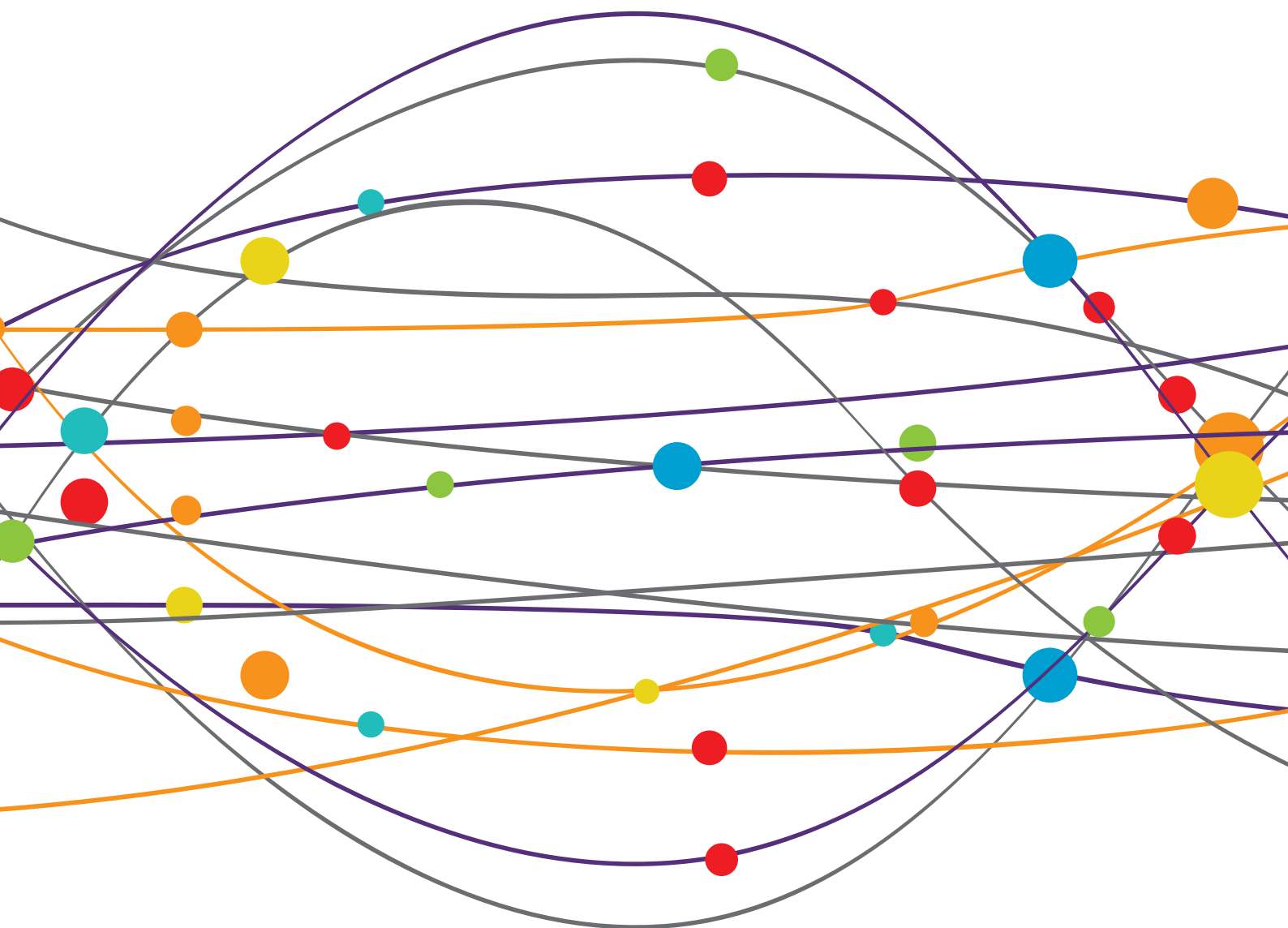


DEVELOPING SUCCESSFUL NEUROPROTECTIVE TREATMENTS FOR TBI: TRANSLATIONAL APPROACHES, NOVEL DIRECTIONS, OPPORTUNITIES AND CHALLENGES

EDITED BY: Stefania Mondello, Anwarul Hasan and Deborah Shear
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DEVELOPING SUCCESSFUL NEUROPROTECTIVE TREATMENTS FOR TBI: TRANSLATIONAL APPROACHES, NOVEL DIRECTIONS, OPPORTUNITIES AND CHALLENGES

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Editorial: Developing Successful Neuroprotective Treatments for TBI: Translational Approaches, Novel Directions, Opportunities and Challenges

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Editorial on the Research Topic

Developing Successful Neuroprotective Treatments for TBI: Translational Approaches, Novel Directions, Opportunities and Challenges

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Traumatic brain injury (TBI) constitutes a critical health problem. More than 50 million new cases occur worldwide each year, accounting for upwards of a million deaths (roughly 2,700 deaths per day) and a global financial burden of \$US400 billion (1, 2). Overall, TBI-related disability is increasing globally, negatively affecting families and society, as well as health-care systems and economies (2). Among survivors, nearly half of those with moderate or severe TBI require years of intensive therapy and face substantial functional impairment and reduced life expectancy (3); however, also mild TBIs and concussions can lead to persistent adverse outcomes.

Despite thousands of preclinical studies in laboratory animals and hundreds of randomized controlled clinical trials (RCTs) testing neuroprotection approaches with different pathophysiological targets (4, 5), to date, no intervention has demonstrated to be unequivocal effective and improve the long-term functional outcome following TBI (1, 6).

This book incorporates reviews and research articles from leaders in the field describing promising therapeutic avenues, which span the spectra of TBI severity and have undergone experimental and clinical investigation. What emerges is a panoramic view of a field actively exploring alternative strategies and novel directions, such as preclinical-drug screening multimodel multispecies consortia (Kochanek et al.) (7, 8), capable to tackle the challenge of heterogeneity in TBI.

Recognizing the critical neuronal loss and wide variability existing in pathophysiologic mechanisms triggered by TBI, the current focus is on the regenerative potential and pleiotropic neuroprotective properties of stem cell therapy for TBI (9, 10). Accordingly, diverse authors have provided perspectives with respect to the application of mesenchymal stromal cells (MSCs) (Carbonara et al.) and the anti-inflammatory role of neural stem cell transplantation (Kassi et al.), and also generated new compelling data (Nasser et al.).

Sophisticated experiments reinterpreting previous unsuccessful therapeutic interventions (11, 12), including erythropoietin (Robinson et al.), hypothermia (Szczygielski et al.), and selective brain

cooling (Leung et al.) have also been conducted providing authors with the opportunity to identify potential reasons for clinical failure. In this analysis, a complex array of factors, comprising a lack of precise diagnose and endophenotype characterization, inadequate measures of outcomes, and tools to inform tailored and individualized treatment emerges as main barriers to advance clinical care in TBI (13). Complementing this conceptual framework, a strong emphasis has also been placed by several contributors on the need for a mechanistic approach to guide drug development. This strategy can better inform target selection and streamline the hard translational decision process. As a consequence, investigations on mitochondrial dysfunction (Pandya et al.), microglial activation (Madathil et al.), cerebral microcirculation impairment (Bellapart et al.), and gut dysbiosis (Rice et al.) following TBI are presented, highlighting their directly involvement in disease pathogenesis and outcome, and role as therapeutic target candidates.

Finally, the volume includes experimental and human studies which add further evidence for the use of neuroimaging (Bajaj et al.) and biofluid markers (Bhatti et al. and DeDominicis et al.) as surrogate endpoints of clinical benefit and treatment response after therapeutic intervention in TBI, supporting their incorporation in future clinical trials (14). Imaging and biofluid markers can, in fact, be instrumental in identifying and characterizing pathophysiologic mechanisms leading to more accurate and finer-grained disease classification which, in turn,

may be used to enrich or stratify patient groups, to demonstrate target engagement, and/or as proof of treatment efficacy (14–16). These factors can play a transformative role in designing effective clinical trials, increasing treatment effectiveness as well as reducing healthcare costs (16–18).

Overall, this volume is intended to provide novel perspectives and insights into the critical research area of TBI treatment, foster knowledge and innovation in the arena of drug development, and stimulates new frameworks and testable hypotheses that can help inform and refine the next generation of TBI clinical trials.

We thank our colleagues for devoting their time, efforts, and clinical and scientific experience. This book would not have been possible without their important contributions. We also thank the editorial team for their dedicated support and assistance. Last, and most important, we are indebted to all patients with TBI and their families for their invaluable contributions. They represent our greatest source of inspiration and all our endeavors are directed toward improving their outcome and quality of life.

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SM wrote the original draft, assembled and incorporated comments from the co-authors, and crafted the final draft. All of the other co-authors contributed to manuscript review and revision.

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Blue-Light Therapy following Mild Traumatic Brain Injury: Effects on White Matter Water Diffusion in the Brain

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Mild traumatic brain injury (mTBI) is a common and often inconspicuous wound that is frequently associated with chronic low-grade symptoms and cognitive dysfunction. Previous evidence suggests that daily blue wavelength light therapy may be effective at reducing fatigue and improving sleep in patients recovering from mTBI. However, the effects of light therapy on recovering brain structure remain unexplored. In this study, we analyzed white matter diffusion properties, including generalized fractional anisotropy, and the quantity of water diffusion in isotropic (i.e., isotropic diffusion) and anisotropic fashion (i.e., quantitative anisotropy, QA) for fibers crossing 11 brain areas known to be significantly affected following mTBI. Specifically, we investigated how 6 weeks of daily morning blue light exposure therapy (compared to an amber-light placebo condition) impacted changes in white matter diffusion in individuals with mTBI. We observed a significant impact of the blue light treatment (relative to the placebo) on the amount of water diffusion (QA) for multiple brain areas, including the corpus callosum, anterior corona radiata, and thalamus. Moreover, many of these changes were associated with improvements in sleep latency and delayed memory. These findings suggest that blue wavelength light exposure may serve as one of the potential non-pharmacological treatments for facilitating structural and functional recovery following mTBI; they also support the use of QA as a reliable neuro-biomarker for mTBI therapies.

Keywords: concussion, diffusion tensor imaging, fractional isotropy, isotropic diffusion, neuropsychological function, quantitative anisotropy, sleep, structural recovery

INTRODUCTION

Mild traumatic brain injury (mTBI) is a common and often unobtrusive wound that occurs when kinetic energy is transferred to the brain through some form of traumatic event, such as a fall, blow to the head, or blast wave. While there are typically no exceptionally conspicuous physical or neuroimaging signs of mTBI, the mechanical trauma to the brain leads to a mild temporary disruption of consciousness or other alteration of ongoing cognition. Also commonly known as “concussion,” mTBI can further lead to persistent alterations in neuropsychological functions, including changes in mood (e.g., depression), poor attention and concentration, and memory problems (1, 2). Importantly, sleep deprivation is also known to produce many of these same symptoms (3, 4).

It is therefore possible that sleep disturbances following mTBI may cause, or at least exacerbate, ongoing post-concussion symptoms. However, the nature of these complaints and their contribution to the experience of daytime sleepiness is not well understood (5). An objective measure of daytime sleepiness is the multiple sleep latency test (MSLT), which is used to determine the time it takes an individual to fall asleep (sleep onset latency) when given the opportunity to take a nap. Following a head trauma, symptoms are believed to result from neuronal damage in the form of diffuse axonal injury (6, 7), leading to the release of specific proteins that in turn promote maladaptive functional and structural changes within the brain (8). Identifying neuro-markers of these changes remains an important challenge in ongoing attempts to understand and treat mTBI and post-concussive symptoms.

A very limited number of treatment options for mTBI have been proposed and experimentally validated. Available treatments include cognitive behavior therapy (9), neuropsychological rehabilitation (10), educational intervention (11), and pharmacological intervention (12). Although the effects are small, some intervention studies report reliable reductions in post-concussion symptoms, including sleep problems, following successful treatment (13). Considering a range of post-concussion symptoms can also occur as the result of sleep loss, it is likely that improving sleep quality in particular would also lead to improvements of other post-concussion symptoms, such as attention, concentration, memory, and mood disturbances. While improving sleep makes sense, this is often easier said than done. A natural and potentially powerful method for regulating the sleep–wake cycle is through targeted exposure to bright light in the morning hours. Exposure to short wavelength light (~430–475 nm; blue wavelength light) has been demonstrated as an alternative to pharmacological treatment methods that focus on improving alertness, concentration, daytime sleepiness, as well as sleep quality (14, 15). Intrinsically photosensitive retinal ganglion cells are particularly responsive to light within the short wavelengths. These cells transmit signals to hypothalamic nuclei, which in turn regulate the production of melatonin (16, 17). Morning exposure to blue wavelength light leads to a suppression of melatonin production, which contributes to a phase delay and stabilization of the circadian rhythm (18), increases daytime alertness and vigilance, and earlier onset of evening sleep (19, 20). Interestingly, a recent clinical trial showed that 4 weeks of 45 min of morning blue-light therapy (BLT) in comparison to longer wavelength placebo light was effective at reducing self-rated fatigue and daytime sleepiness among individuals recovering from TBI (21). However, the extent to which these behavioral changes correspond to structural changes within the brain has not been explored.

When considering the potential influences of BLT on mTBI, it is important to consider that mTBI is associated with microscopic changes in brain structure, particularly within the white matter axonal tracts. Abnormalities in fractional anisotropy (FA) in the brain following an mTBI have been studied extensively using diffusion tensor imaging (DTI), a method that allows high-resolution imaging of the directional movement of water molecules along axonal fiber tracts (i.e., how fast water molecules move along fiber tracts). Abnormalities in FA in individuals with an mTBI are reported in areas such as uncinate fasciculus (UF)

(22), superior longitudinal fasciculus (SLF) (23), anterior corona radiata (ACR) (22), corpus callosum (CC) (24), and thalamus (25). Alterations in FA within (a) UF are reported to be associated with changes in Mini-Mental State Examination (MMSE) scores (cognitive function) and specifically, memory performance (22, 26); (b) SLF and CC are reported to be associated with executive function (attention and memory) (27); (c) ACR changes are correlated with changes in attention (22); and (d) anterior thalamic nucleus changes are also linked to changes in executive function, memory, and attention (25). In addition, studies have found that individuals with mTBI show alterations in white matter within the frontal lobe (frontal cortex/dorsolateral prefrontal cortex, DLPFC), and that these alterations are correlated with lower executive control and related cognitive functions (28). Also, compared to healthy controls (HCs), there are multiple studies that have reported abnormally high FA values in individuals with mTBI within several areas, including the genu and splenium of CC, ACR (bilaterally), IUF, and internal capsule (IC; bilaterally) (29, 30). Recently, new diffusion measures—quantitative anisotropy (QA), isotropic diffusion (ISO), and generalized fractional anisotropy (GFA)—were introduced to the field of DTI for the analysis of diffusion properties of white matter (31). QA and ISO represent *how much* water diffuses (i.e., density) in a specific/restricted direction and in an isotropic fashion (i.e., total isotropic component), respectively. In contrast, GFA, which is calculated from an orientation distribution function, is a measure of *how fast* water diffuses (i.e., diffusivity) in an anisotropic fashion, i.e., it represents degree to which diffusion is anisotropic (31, 32). Highly significant correlations between FA and GFA were reported in the past (33). In addition, the difference between QA and GFA pertains to the fact that QA is a measure of water diffusion along each fiber orientation, whereas GFA/FA is defined for each voxel. Compared to GFA/FA, QA is also reported to have lower susceptibility to partial volume effects of crossing-fibers, free-water diffusion in ventricles, and non-diffusive particles (31). Moreover, normalization of QA helps to stabilize the spin-density measurement across subjects. In this study, we investigated multiple diffusion measures (i.e., diffusivity as well as density measures) simultaneously to better characterize the white matter properties; therefore, in conjunction with GFA, we also estimated normalized QA (NQA) and ISO measures. To the best of our knowledge, no study to date has used these metrics simultaneously to examine the effect of light exposure treatment on the brain following mTBI.

In individuals with mTBI, how changes in post-concussion symptoms following an exposure to BLT may correspond to structural changes within the brain has not yet been explored. Recent evidence suggests that sleep is important for clearing the neurotoxins that build up throughout the day (34) and increases the production of oligodendrocyte progenitor cells that contribute to myelin formation (35), which could conceivably facilitate repair of axonal damage. Based on this, we hypothesized that 6 weeks of daily morning BLT, compared to a placebo condition with an amber-light therapy (ALT) device, would improve sleep and, consequently, lead to changes in white matter water diffusion, improvements in cognitive abilities such as attention and memory, and daytime sleepiness. To this end, we investigated

whether individuals in the BLT and ALT groups showed significant changes in diffusion (i.e., GFA, NQA, and ISO), cognitive, and sleep measures. Furthermore, we examined the correlations between changes in diffusion measures from pre- to post-treatment and changes in neuropsychological performance and sleep onset latency.

MATERIALS AND METHODS

Participants

Twenty-eight individuals meeting criteria for mTBI (mean age = 21.48 ± 3.76 years, 15F) underwent neuroimaging using a Siemens Tim Trio 3T scanner (Erlangen, Germany) at the McLean Hospital Imaging Center. The majority of the individuals (19 out of 28) sustained an mTBI while engaged in a physical activity (e.g., soccer, rugby, hiking, and karate); whereas 9 individuals sustained an mTBI during either a vehicular or household accident. All of the mTBI individuals had a documented mTBI within the preceding 12 months, but not sooner than 4 weeks before their screening. An mTBI was defined based on the criteria established by the VA/DoD practice guidelines (36) as a traumatically induced event (e.g., head impact, blast wave) that was associated with an alteration in mental status (e.g., confusion, disorientation, retrograde, or anterograde amnesia), consciousness (i.e., loss of consciousness less than 30 min; alteration of consciousness up to 24 h), post-traumatic amnesia up to 24 h, and a Glasgow Coma Scale from 13 to 15. All participants were right-handed and primary English speakers. All study participants were required to have some level of self-reported sleep problem, e.g., if they were sleepier during the day, having difficulty in sleeping at night and staying alert during the day, etc. Therefore, all participants were screened using a set of sleep questionnaires where they indicated self-reported sleep problems and endorsed that the sleep problems either emerged or worsened following the injury. Participants with any history of neurological, mood, or psychotic disorder with an onset before the mTBI, or who suffered a loss of consciousness exceeding 30 min following an injury were excluded. Participants were thoroughly briefed on the potential risks and benefits of the study and all completed written informed consent before enrollment. Participants were financially compensated for their time. The experimental protocol was approved by the Institutional Review Board of McLean Hospital, Partners Health Care, and the U.S. Army Human Research Protections Office. All procedures performed in this study were conducted in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Protocol

All the participants underwent DTI, neuropsychological testing, and MSLT sessions on two occasions; separated by 6 weeks of daily morning light therapy with either blue light or a sham placebo amber light. Participants were instructed to rest and relax during scanning. All data were collected within a period of 3 years. All eligible participants completed daily sleep diaries and questionnaires and were fitted with a wrist actigraph for sleep

monitoring throughout the period of the study. Participants were asked to use a commercially available light therapy device (GoLite Blu®, Philips Electronics) for 6 weeks (i.e., 30 min everyday within 2 h of awakening, but before 11:00 a.m.). Half of the individuals ($N = 14$, mean age = 21.75 ± 4.43 years, 8F) were randomly assigned to BLT and half ($N = 14$, mean age = 21.21 ± 3.09 years, 7F) were assigned to ALT. ALT and BLT groups did not differ significantly in age [$F(1,26) = 0.14$, $p > 0.05$, one-way ANOVA], gender [$\chi^2(1) = 0.14$, $p > 0.05$, Pearson's Chi-square], and body-mass index [$F(1,25) = 2.77$, $p > 0.05$, one-way ANOVA]. However, two important covariates were included in our analyses: (1) "light compliance" was calculated as the percentage of the total number of days that the participant acknowledged actually using the light *via* self-report divided by the total number of days in the study (i.e., number of days between baseline and post-treatment assessments), and (2) "time since injury," which was calculated as the number of days between the index mTBI and the baseline assessment.

DTI Data Acquisition and Image Processing

Diffusion-weighted imaging (DWI) data were acquired along 72 directions with a b -value = $1,000 \text{ s/mm}^2$, voxel size = $1.75 \text{ mm} \times 1.75 \text{ mm} \times 3.5 \text{ mm}$, flip angle = 90° , repetition time (TR) = 6,340 ms, echo time (TE) = 99, slices thickness = 3.5 mm, and number of slices = 40 encompassing the whole brain. A set of eight images with no diffusion weighting (b_0 images) was also acquired. Using dcm2nii toolbox [part of MRICron (37)], we converted DWI data from DICOM into NIFTI format. A b -value and b -vector file was generated during this step. Next, we performed standard eddy current correction using FMRIB Software Library v6.0 processing software package¹ on DWI data for head motion correction.

Neuropsychological Assessments and Sleep Measures

The Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) (38), which included scales assessing delayed memory (DM), immediate memory (IM), attention (ATT), visuospatial/constructional (VC), and language (LAN) abilities was administered pre- and post-light exposure.

The RBANS IM is a measure of initial encoding and learning of simple and complex verbal information and RBANS DM is a measure of delayed recall of visual and verbal stimuli and recognition of verbal stimuli. The RBANS ATT is a measure of speed and accuracy of information processing. The RBANS VC is a measure of visuospatial perception, and RBANS LAN is a measure of ability to express language. Lower RBANS IM and DM scores represent difficulty in the recognition and recall of long-term memories and verbal learning, respectively. Lower RBANS ATT scores represent difficulty in the basic attention processes. Lower RBANS VC and LAN scores represent difficulty with using visuospatial information and language (expressive and receptive), respectively. The use of the RBANS has been shown to

¹<http://www.fmrib.ox.ac.uk/fsl>.

be clinically valid and reliable screening tool to assess cognitive deficits following traumatic brain injury (39).

The MSLT has been shown to better reflect the degree of daytime sleepiness when compared to self-report, and with high test-retest reliability (40–42). During each assessment session, participants underwent a modified MSLT protocol, using a standard electrode montage for polysomnographic (PSG) recording (ALICE LE®, Phillips Respironics). Signals were recorded from EEG (C3A2, C4A2, O1A1, and O2A2), electrooculogram, submental electromyogram, and electrocardiogram. On three occasions throughout the testing session (11:50 a.m., 1:50 p.m., 3:50 p.m.), participants were given a 20-min opportunity to take a nap in a sound attenuated bedroom. Increased sleep propensity and/or abnormal daytime sleepiness is inferred from decreased sleep onset latency during these MSLT trials. PSG recordings were monitored for the duration and then scored by certified sleep technicians using 30-s epochs and Somnologica software. Sleep onset latency was classified as the first epoch in which >50% was identified as any stage of sleep. Sleep onset latency was quantified for each trial, as well as the average onset latency across the three MSLT administrations.

Data Analysis

For each participant, we estimated water diffusion parameters such as mean GFA, mean NQA, and mean ISO, using the Q-space diffeomorphic reconstruction (QSDR) approach (43) implemented in DSI Studio.² QSDR is a model-free approach, which calculates the density distribution of water diffusion using a high-resolution standard brain atlas constructed from 90-diffusion spectrum imaging datasets in the ICBM-152 space. Tractography was performed using 25,000 sub-voxel seeds in each region of interest for each participant. A turning angle threshold of 60°, QA threshold of 0.10, and length constrained between 30 and 200 mm was used to estimate diffusion parameters. To ensure consistency across subjects, we normalized the QA measure by scaling the subject-wise maximum QA value to 1. Normalization of QA assumes that all the subjects have identical compactness of white matter. In order to avoid any bias among participants, an identical set of tracking parameters was used for each participant before and after the light therapy. For each participant, GFA, NQA, and ISO were estimated for all the possible tracts crossing 11 brain areas, namely the DLPFC, the genu, body and splenium of the CC, the left and the right uncinate fasciculus (IUF and rUF), the left and the right superior longitudinal fasciculus (ISLF and rSLF), the left and the right anterior corona radiata (lACR and rACR), and the thalamus. DLPFC is attributed anatomically to Brodmann areas (BAs) 9 and 46 (44). To define DLPFC in this study, we integrated BAs 9 and 46 whereas we used the ICBM-DTI-81 white matter labels atlas (45) and the JHU white matter tractography atlas (45) (implemented in DSI Studio) to define all other regions of interest. Diffusion parameters (GFA, NQA, and ISO) from tracts crossing the 11 specified seed regions were used in the analyses. In order to estimate the diffusion measures across all the possible

tracts crossing each of the 11 brain areas, no waypoint regions of interest were included in the analysis.

Metrics of GFA, NQA, and ISO were compared using mixed analysis of covariance (ANCOVA), with light group (BLT/ALT) as a between groups variable and session (pre- versus post-treatment) as a within-subjects variable, while “time since injury” and “light compliance” were included as nuisance covariates. To test the association between the changes in white matter integrity with changes in neuropsychological performance and sleep latency measures, change metrics for each variable were evaluated with partial correlations, controlling for time since injury and light compliance. For this analysis, we used residualized change scores derived by regressing post-treatment scores on pre-treatment scores and determining the residual value for each participant. This provides a metric of post-treatment status controlling for pre-treatment status (i.e., residualized change). We report false discovery rate (FDR) corrected *p*-values for the partial correlations.

RESULTS

In order to estimate different diffusion parameters, we first performed whole-brain tractography, followed by limiting the white matter tracts to those passing through 11 predefined seed regions, namely—R01: the DLPFC, R02: genu, R03: body, R04: splenium of the CC, R05: the lUF, R06: the rUF, R07: the ISLF, R08: the rSLF, R09: the left anterior corona radiata (ACR), R10: right anterior corona radiata (ACR), and R11: the thalamus. Selection of these 11 regions was purely based on previous literature showing abnormalities water diffusion in these regions following mTBI (22–28). In **Figure 1**, we show fiber tracts crossing through each region for a representative participant. Here, fibers are colored coded to represent their direction, where “red” indicates fibers along the X-axis (i.e., left–right), “green” indicates fibers along the Y-axis (i.e., anterior–posterior), and “blue” indicates fibers along the Z-axis (i.e., inferior–superior).

Effect of Light Therapy on Diffusion Properties of the Brain following an mTBI

A detailed comparison of diffusion parameters, GFA, NQA, and ISO, was performed on fiber pathways crossing through the 11 specified seed regions (R01 to R11). All the results were corrected for multiple comparisons using Bonferroni’s method. In **Figure S1** in Supplementary Material, for each area, we showed subject and fiber averaged GFA, NQA, and ISO measures before and following 6 weeks of either ALT or BLT, where error bars represent the SEM. The presented data in **Figure S1** in Supplementary Material are raw data, which are uncorrected for confounds.

GFA

There was a significant time (pre- and post-treatment) $\times$ group (ALT/BLT) interaction [$F(1,24) = 6.151$, $p = 0.021$] such that following BLT, but not ALT, individuals showed a significant decrease in GFA for only the fibers crossing the splenium of the CC. Furthermore, within-subject pairwise comparison showed a significant decrease in GFA following BLT [$F(1,24) = 5.619$,

²<http://dsi-studio.labsolver.org>.

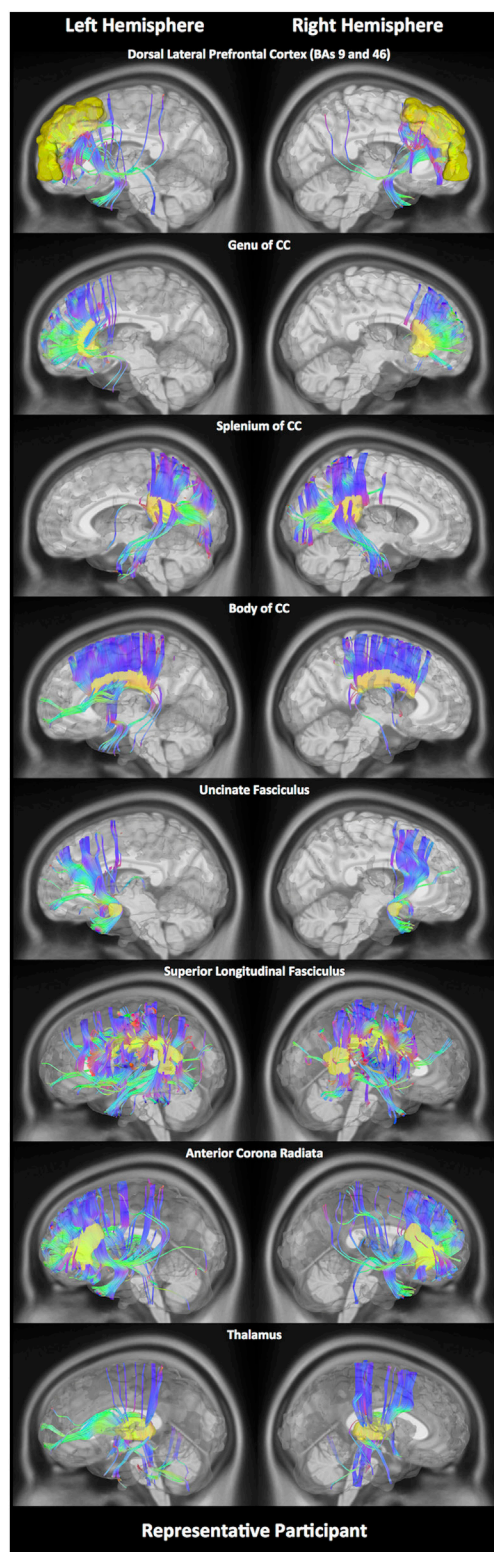


FIGURE 1 | White matter fiber tracking. Here, for a representative participant, we illustrate white matter fiber tracts for each of the 11 regions. Tracts shown in red indicate a fiber direction from left to right or vice versa. Blue indicates a fiber direction from anterior to posterior or vice versa. Green indicates a fiber direction from superior to inferior or vice versa.

$p = 0.026$], but not ALT [$F(1,24) = 1.511$, $p = 0.231$]. ANCOVA results for GFA of the fibers crossing the splenium of the CC are summarized in **Figure 2** and **Table 1**.

NQA

There was a significant time (pre- and post-treatment) $\times$ group (ALT/BLT) interaction such that following BLT, but not ALT, individuals showed a significant decrease in NQA for the fibers crossing three brain areas, i.e., body of CC [$F(1,24) = 4.932$, $p = 0.036$], the left ACR [$F(1,24) = 9.460$, $p = 0.005$], and thalamus [$F(1,24) = 5.688$, $p = 0.025$]. Furthermore, pairwise comparison showed that following BLT, there was significant decrease in NQA for the fibers crossing these three areas, i.e., body of CC [$F(1,24) = 5.984$, $p = 0.022$], the left ACR [$F(1,24) = 12.347$, $p = 0.002$], and thalamus [$F(1,24) = 8.226$, $p = 0.008$], but not following ALT. ANCOVA results for NQA of the fibers crossing these three areas are summarized in **Figure 3** and **Table 2**.

ISO

There were no significant changes in ISO for fibers crossing any of the 11 areas from pre- to post-treatment for either group.

Effect of Light Therapy on Neuropsychological Function and Sleep Onset Latency

Contrary to our expectations, we did not find significant time (pre- versus post-treatment) $\times$ group (ALT/BLT) interaction for neuropsychological function and sleep onset latency. However, because we found significant differences in GFA (for 1 out of 11 brain areas) as well as in NQA (for 3 out of 11 brain areas) following BLT, we then examined whether individual differences in white matter within these 4 brain regions were related to individual differences in our behavioral measures of neuropsychological function (attention and memory) and daytime sleep onset latency during the MSLT trials. Specifically, partial regression analyses were performed (corrected for “time since injury” and “light compliance”) between diffusion measures (GFA and NQA) and neuropsychological function measures (i.e., RBANS scores) as well as sleep onset latency.

Neuropsychological Function

Following BLT or ALT, we did not find significant association between residualized changes in any neuropsychological measures or MSLT scores and residualized changes in GFA for fibers crossing the splenium of the CC. But significant negative partial correlations were observed between residualized changes in RBANS DM scores and residualized changes in NQA for fibers crossing two brain areas: the body of the CC ($r = -0.76$, $p = 0.00$; FDR corrected $p = 0.02$) (**Figure 4A**) and the thalamus ($r = -0.64$, $p = 0.02$; FDR corrected $p = 0.02$) (**Figure 4B**), i.e., greater changes in NQA were associated with better DM performance following BLT. After multiple comparisons correction, we did not find significant association between residualized changes in any neuropsychological measure and residualized changes in NQA for fibers crossing any of the regions of interest following ALT (**Figures 4C,D**).

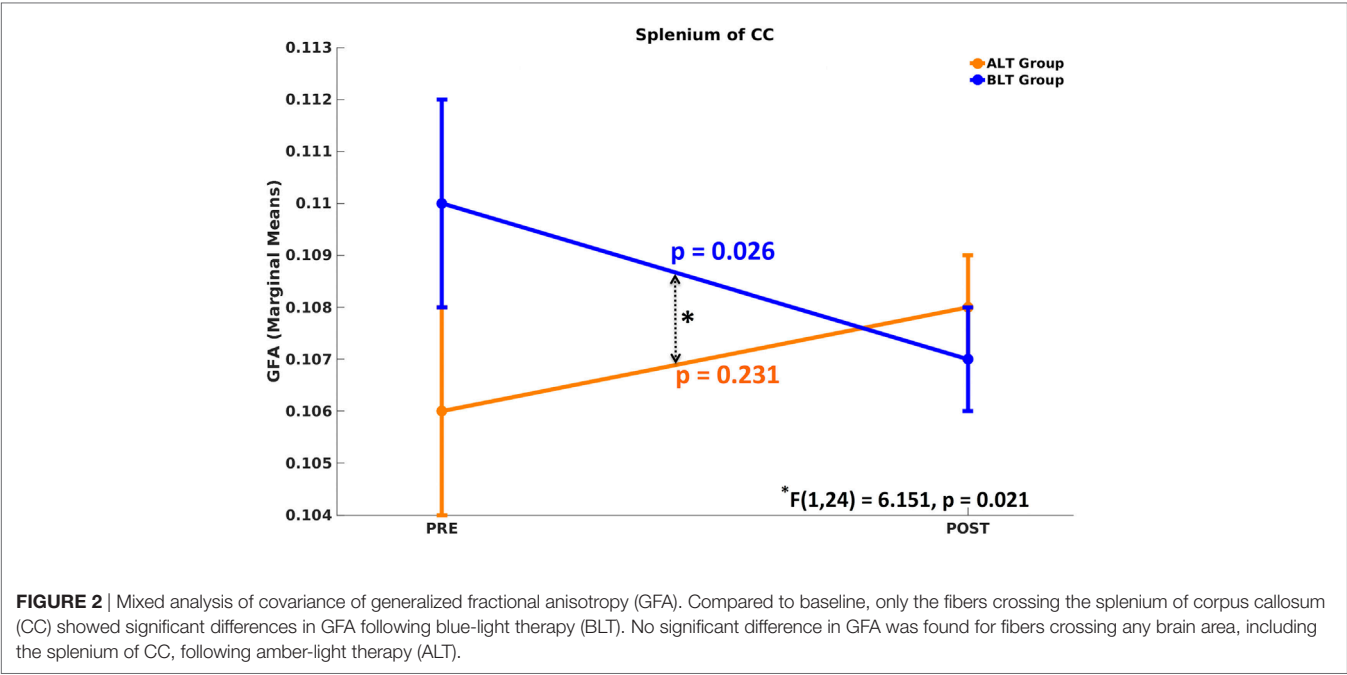


TABLE 1 | Summary of analysis of variance (repeated measures ANOVA) for GFA.

Within-subjects effects					
Interaction					
Source	Brain areas	Type III sum of squares	Mean square	<i>F</i> (1, 24)	Significance (GFA)
Time (pre and post) × group (ALT/BLT) (sphericity assumed)	Splenium of CC	0.000	0.000	6.151	0.021*
Pairwise comparisons (pre versus post)					
Effect of treatment	Brain areas	Groups	<i>F</i> (1, 24)	Significance (GFA)	
Pre versus post	Splenium of CC	ALT	1.511	0.231	
		BLT	5.619	0.026**	

GFA, generalized fractional anisotropy; BLT, blue-light therapy; ALT, amber-light therapy; CC, corpus callosum.

*Interaction is significant at $p < 0.05$.

**Mean difference between post- and pre-treatment is significant at $p < 0.05$.

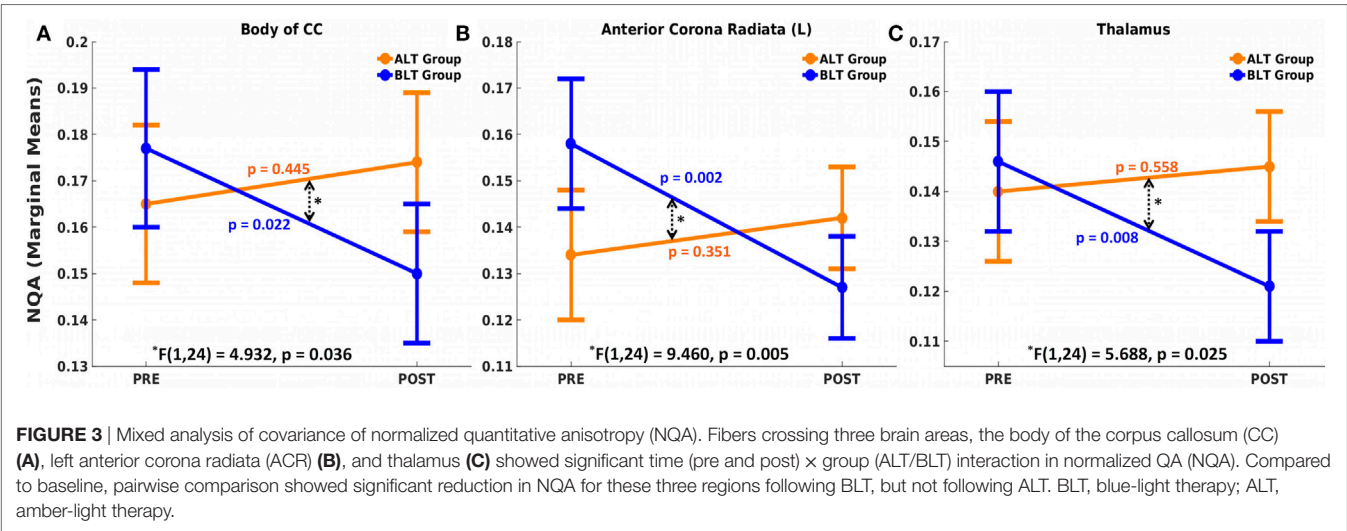


TABLE 2 | Summary of analysis of variance (repeated measures ANOVA) for normalized quantitative anisotropy (NQA).**Within-subjects effects (ANCOVA)**

Interaction					
Source	Brain areas	Type III sum of squares	Mean square	F(1, 24)	Significance ^a (NQA)
Time (pre and post) × group (ALT/BLT) (sphericity assumed)	Body of CC	0.004	0.004	4.932	0.036*
	Left anterior corona radiata	0.005	0.005	9.460	0.005*
	Thalamus	0.003	0.003	5.688	0.025*
Pairwise comparisons (pre versus post)					
Effect of treatment	Brain areas	Groups	F(1, 24)	Significance ^a (NQA)	
Pre versus post	Body of CC	ALT	0.604	0.445	
		BLT	5.984	0.022**	
	Left anterior corona radiata	ALT	0.903	0.351	
		BLT	12.347	0.002**	
	Thalamus	ALT	0.352	0.558	
		BLT	8.226	0.008**	

NQA, normalized quantitative anisotropy; BLT, blue-light therapy; ALT, amber-light therapy; CC, corpus callosum; ANCOVA, analysis of covariance.

^aAdjustment for multiple comparisons using Bonferroni's method.

*Interaction is significant at $p < 0.05$.

**Mean difference between post- and pre-treatment is significant at $p < 0.05$.

Daytime Sleep Onset Latency

Significant negative partial correlations were observed between residualized changes in sleep onset latency during the first MSLT administration and residualized changes in NQA for fibers crossing ACR (L) ($r = -0.72$, $p = 0.01$; FDR corrected $p = 0.01$) (**Figure 4E**), i.e., greater changes in NQA were associated with delayed sleep onset latency during the day following BLT. However, after multiple comparisons correction, we did not find significant association between residualized changes in sleep onset latency during any of the MSLT administrations and residualized changes in NQA for fibers crossing any of the regions of interest following ALT (**Figure 4F**). The findings above are summarized in **Table 3**.

DISCUSSION

In this study, we analyzed several white matter water diffusion properties including GFA, NQA, and ISO, for fibers crossing several brain areas in individuals with a recent mTBI. From a group of individuals with mTBI, half were randomly assigned to a placebo condition of ALT and the other half to an active condition of BLT. Consistent with our hypotheses, we observed significant changes in some of these white matter properties (i.e., GFA and NQA) for multiple brain areas following BLT. Contrary to our hypotheses, we did not observe significant changes in cognitive abilities such as attention and memory, or in the daytime sleep onset latency measures. However, an analysis of cognitive abilities and daytime sleep onset latency measures in relation to white matter properties revealed a significant relationship between increased DM scores and decreased normalized quantitative anisotropy, as well as an association between increased daytime sleep onset latency and decreased normalized quantitative anisotropy after BLT. These

findings suggest that BLT may provide an effective method for facilitating recovery from mTBI.

Previous DTI studies of mTBI have tended to focus on FA as a measure of the diffusion properties of white matter tracts. However, these studies have yielded somewhat inconsistent results, as some report abnormally high (30) and others report abnormally low FA (24) values following an mTBI (i.e., as compared to HCs). Such inconsistencies may be due to several factors, including type, severity and location of injury, time since injury, and variability across subject samples (30). In this study, we examined NQA and ISO, in addition to FA, in order to fully characterize potential treatment effects. In doing so, we found a significant effect of BLT on white matter water diffusion properties (i.e., both GFA and NQA) for several brain areas, which were associated with significant correlations between diffusion measures, behavioral measures of neuropsychological function, as well as daytime sleep onset latency. In contrast, none of the 11 brain areas showed significant change in GFA, NQA, or ISO following the placebo ALT. More specifically, we found that, following BLT (but not ALT), there was significant decrease in GFA for fibers passing through the splenium of the CC, and a significant decrease in NQA for fibers passing through the body of CC, left ACR, and thalamus. These changes in NQA for fiber pathways going through the body of the CC and thalamus were also significantly negatively correlated with changes in RBANS DM scores, suggesting that decreases in NQA were associated with improvements in DM performance from pre- to post-treatment. In addition, changes in NQA for fiber pathways going through the left ACR were significantly negatively correlated with changes in MSLT scores, suggesting that decreases in NQA were associated with improvements in sleep onset latency during the day from pre- to post-treatment. The role of the CC during recovery following BLT might be due to the fact that CC

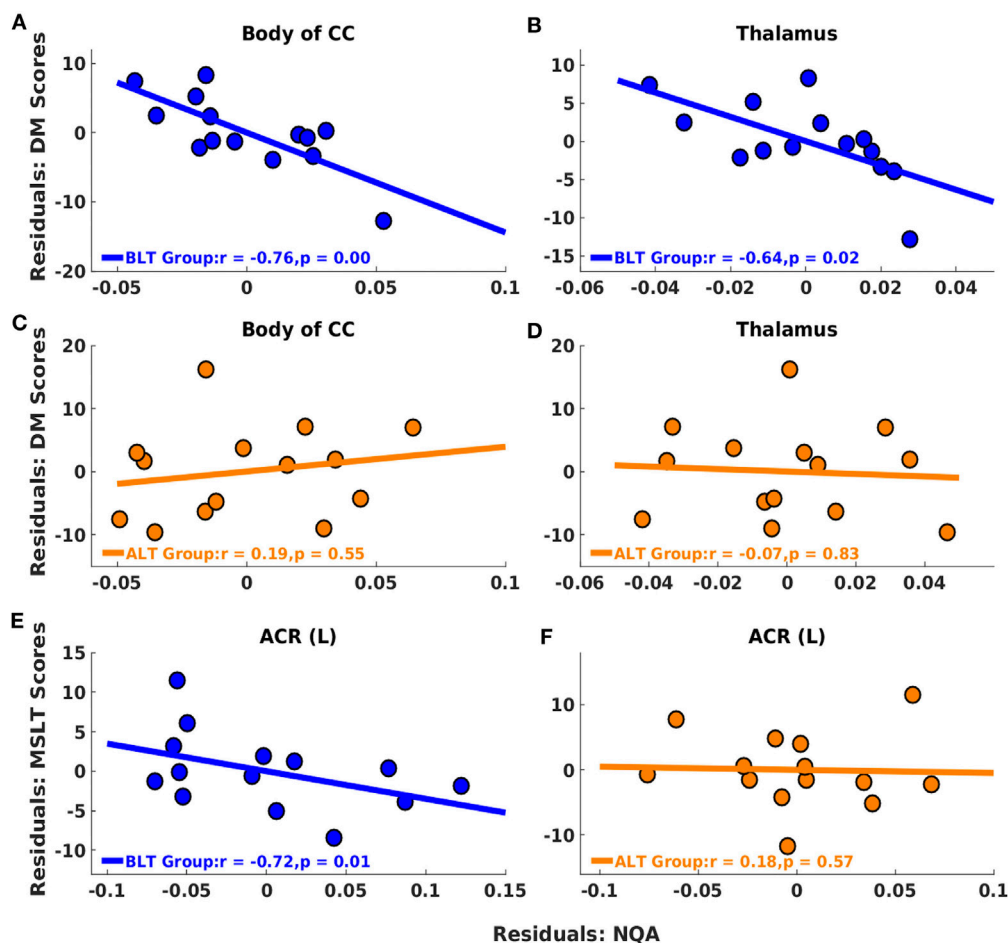


FIGURE 4 | Associations between residualized changes in white matter diffusion measures, neuropsychological measures, and multiple sleep latency test (MSLT) scores following BLT and ALT. For BLT and ALT groups, correlations found between residualized changes in NQA and neuropsychological function measures (DM) [BLT: (A,B), ALT: (C,D)], and between residualized changes in NQA and MSLT scores [BLT: (E), ALT: (F)] are reported. Significant negative correlations between residualized changes in Repeatable Battery for the Assessment of Neuropsychological Status DM scores and NQA measures were found for fibers crossing the body of the corpus callosum (CC) (A) and thalamus (B) for BLT group. No significant correlations were found for ALT group, including for fibers crossing the body of the CC (C) and thalamus (D). Significant negative correlations between residualized changes in sleep onset latency during the first MSLT administration and NQA measures were also found for fibers crossing the left anterior corona radiata (ACR) (E) for BLT group but not for ALT group (F). BLT, blue-light therapy; ALT, amber-light therapy; DM, delayed memory.

tracts facilitate communication of somatosensory information between parietal and occipital lobes (46) as well as communication between the two cortical hemispheres more generally (47). These tracts are known for their vital role in regulating several advanced brain skills such as memory, learning, and abstract thinking. Damage to these tracts could lead to loss of inter-hemispheric connections, causing multiple neuropsychological impairments (47). Moreover, the CC is also a common site affected following a brain injury. In the previous work, axons of the CC were reported to exhibit multiple stages of degeneration following a traumatic brain injury (48). In addition, generation of myelin sheaths within the CC could be responsible for its greater responsiveness to BLT. Furthermore, the connections between the anterior thalamus and hippocampal gyrus are believed to operate in parallel but with different organization and any

damage to such network or any of the participating areas could contribute to impaired memory and discrimination skills (49, 50). The improvements in memory scores as a result of changes in NQA could be due to the effects of blue light exposure on sleep quality, which plausibly results in decreased daytime sleepiness and increases in alertness. Future work assessing PSG or actigraphic changes in sleep duration and quality will be necessary to test these hypotheses directly. In a diffusion kurtosis imaging study, several brain areas including the CC, thalamus, and IC showed correlations between changes in mean kurtosis or radial kurtosis between 1 and 6 months post mTBI and improvements in cognition between the 1- and 6-month visits (51). In that study, no significant differences in other diffusion parameters (such as FA and mean diffusivity) were observed between mTBI patients and age-matched controls. The findings reported in our

TABLE 3 | Summary of correlations between residualized changes in neuropsychological function measures (DM) and GFA, and between residualized changes in MSLT scores and normalized quantitative anisotropy (NQA) measures.

#	ROIs	Partial correlations (<i>r</i> , <i>p</i>) between residualized changes in									
		GFA and					NQA and				
		DM	MSLT 1	MSLT 2	MSLT 3	Mean MSLT	DM	MSLT 1	MSLT 2	MSLT 3	Mean MSLT
BLT group											
1	Splenium of CC	0.35, 0.27	−0.33, 0.29	−0.17, 0.60	−0.48, 0.12	−0.37, 0.23			–		
2	Body of CC			–			−0.76, 0.00**	−0.18, 0.58	−0.43, 0.16	−0.17, 0.60	−0.37, 0.24
3	ACR (L)			–			−0.37, 0.23	−0.72, 0.01**	−0.45, 0.14	−0.45, 0.15	−0.58, 0.05
4	Thalamus			–			−0.64, 0.02**	−0.45, 0.14	−0.48, 0.11	−0.39, 0.21	−0.54, 0.07
ALT group											
1	Splenium of CC	−0.02, 0.96	−0.56, 0.06	−0.61, 0.04*	−0.38, 0.22	−0.61, 0.04*			–		
2	Body of CC			–			0.19, 0.55	−0.52, 0.08	−0.52, 0.08	−0.51, 0.09	0.60, 0.04*
3	ACR (L)			–			0.43, 0.16	0.18, 0.57	0.04, 0.91	−0.04, 0.92	0.07, 0.83
4	Thalamus			–			−0.07, 0.83	0.13, 0.70	−0.19, 0.56	0.42, 0.17	0.17, 0.60

ROIs, regions of interest; GFA, generalized fractional anisotropy; NQA, normalized quantitative anisotropy; DM, delayed memory; MSLT, multiple sleep latency test; BLT, blue-light therapy; CC, corpus callosum; ACR (L), anterior corona radiata (left); ALT, amber-light therapy; FDR, false discovery rate.

**p* < 0.05 (uncorrected for multiple comparisons).

***p* < 0.05 (FDR corrected for multiple comparisons).

study are also consistent with previous findings demonstrating microstructural white matter changes in the ACR for patients suffering from narcolepsy, a disorder characterized by rapid sleep onset latency during the daytime (52).

We observed a significant reduction in diffusion measures (GFA and NQA) following BLT. One of the potential explanations for changes in GFA and NQA measures could be attributed to the way axons are packed. Previously, changes in FA are reported to be dependent on axonal packing. It was reported that light axonal packing leaves more intercellular water as compared to dense packing causing less restriction to water molecules, which further results into lower FA values whereas higher degree of myelination results into higher FA values due to tight axonal packing (53). In addition, in a separate study, we recently demonstrated that acute exposure to 30 min of blue light subsequently led to increased functional brain responses within the prefrontal cortex and improved cognitive performance during a working memory task (54). Blue light exposure in the morning may therefore facilitate brain function later during the day, possibly when individuals are at work. If individuals are exposed to 30 min of morning blue light every day for 6 weeks and are able to sustain regular attentional focus, this may plausibly also be reflected in better white matter integrity and improved performance on neuropsychological tasks and decreased daytime sleepiness. Another possible reason for changes in diffusion measures (GFA and NQA) following BLT could be that before BLT, GFA, and NQA were higher and BLT helped to restore these diffusion levels back to normal. In fact, increased water diffusion after an mTBI has been associated with the stretching and deformations of axons following mTBI, which leads to an increase in intra- but decrease in extra-cellular water causing an increase in diffusion along the axons (55, 56). Modeling studies have shown that the inter-hemispheric fibers, especially of the CC, could be more sensitive to mechanical strain following brain deformation after a concussion (57). Diffusion of water molecules through strained axons could further be responsible for higher GFA or

NQA. Abnormal disruption of water due to axonal swelling, compression of axons, and expansion of tissues may also lead to abnormal changes in water diffusion (30, 58). Myelin also plays a significant role in axon susceptibility following an mTBI. For instance, compared to myelinated axons, unmyelinated fibers within white matter are more adversely affected following traumatic axonal injury (59). BLT may improve myelination and help in regenerating new structural fibers, which could cause the observed improvements in neurobehavioral scores and possibly the observed changes in GFA and NQA values. However, the potential mechanism behind increased myelination or regeneration of structural fibers following light therapy is not completely understood. It may involve clearance of neurotoxins (34) and increases in oligodendrocyte precursor cells (35) due to shifts in circadian rhythms and improved sleep (18, 60, 61). Previously, it was reported that mean water diffusivity values were reduced within several brain regions including CC, corona radiata, and thalamic radiation in patients with obstructive sleep apnea compared to HCs (62). In a study of patients with bipolar disorder, reduced water diffusivity within the same regions identified here (CC, corona radiata, and thalamic radiation) indicated that sleep quantity could be associated with integrity of myelin sheaths (63). Therefore, BLT may enrich or stimulate the production of myelin-enriched brain debris, which may further stimulate microglial/macrophage activation in white matter tracts (64), especially within the CC, corona radiata, and thalamic radiation, which are associated with various sleep problems. Adaptive alterations in water diffusivity following BLT may also act to strengthen brain function. The association between sleep and variation in diffused water quantity could also be responsible for circadian changes in diffusion measures (65, 66), which may further lead to improvements in brain structure and function following BLT. Furthermore, it is known from other studies that acute exposure to blue light also has a positive impact on brain function and cognitive performance and it makes people faster at responding during working memory tasks without

loss of accuracy (54). Separate from the effects of blue light on melatonin suppression, it is possible that blue light may have more direct cognitive alerting effects *via* direct stimulation of the locus coeruleus, which in turn releases norepinephrine throughout the cerebral cortex (54, 67, 68). While speculative, it is conceivable that the effects could be even more robust during periods of insufficient sleep that are extremely common following a traumatic brain injury (69). This is an important area for further research.

Finally, it is noteworthy that NQA appeared to yield a larger number of significant findings than GFA or ISO. One possibility is that NQA is a more sensitive measure to detect microstructural changes of white matter integrity following an mTBI. By contrast, we predict that GFA could be a more sensitive measure to determine white matter differences between controls and mTBI patients. This is also consistent with the previous literature, which has suggested that density measures like NQA are more sensitive to individual physiological differences, whereas diffusivity measures like GFA are more sensitive to pathological conditions (70). NQA is also generally considered to be a more robust measure for deterministic tractography, due to its lower susceptibility to partial volume effects (31). It has also been found that NQA has the capability to filter out noisy fiber tracts, which further results in a higher spatial resolution in NQA-aided tractography. By contrast, voxel-based indices, such as GFA, are not capable of filtering out the noisy fibers since the same magnitude of anisotropy is shared by all the fiber orientations within a voxel (31). These considerations support the idea that NQA-aided tractography may be a better approach than GFA-based tractography for examining abnormal white matter content following an injury and injury-related therapies. It should be noted that the deterministic tractography methods implemented in DSI Studio has achieved the highest “valid connection” examined by an open competition among 96 methods submitted from 20 different research groups around the world.³

This study had several limitations. First, we acknowledge the fact that there is no way to assert the accuracy of tractography. Thus, further research will be needed to provide convergent validity to these findings. Second, our data sample was focused on participants with mTBI and did not include healthy controls. The goal was to compare the active versus a placebo condition on the recovery of a patient population, but future work would benefit from a sample of healthy individuals to determine the extent to which the outcomes represent full normalization of brain structure. Third, our mTBI sample also included individuals with different injury mechanisms. Mild injuries of this type are extremely heterogeneous and may vary significantly among samples. Finally, our data sample was relatively small. Low statistical power due to smaller sample size could account for the non-significant findings observed in many neuropsychological function and sleep onset latency measures following BLT.

³http://www.tractometer.org/ismrm_2015_challenge/results.

In summary, these findings provide preliminary evidence that BLT can affect recovery of brain structure and function following mTBI. Following BLT, normalized values of water diffusion were associated with increases in memory and sleep latency scores. While more research is warranted, these preliminary findings raise the possibility that BLT might be useful as a means of facilitating brain and cognitive recovery among individuals with mTBI. Finally, our results also support the use of NQA as a sensitive measure to analyze the effect of treatment following a brain injury.

ETHICS STATEMENT

Participants were thoroughly briefed on the potential risks and benefits of the study and all completed written informed consent before enrollment. The experimental protocol was approved by the Institutional Review Board of McLean Hospital, Partners Health Care, and the U.S. Army Human Research Protections Office (HRPO). All procedures performed in this study were conducted in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

AUTHOR CONTRIBUTIONS

SB conducted the neuroimaging analyses and wrote the initial draft of the manuscript and organized the revisions. JV, RS, and ND each contributed to the writing of revisions of the manuscript and helped with data analysis. WK designed the study, oversaw data collection and analysis, and contributed to writing revisions of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/article/10.3389/fneur.2017.00616/full#supplementary-material>.

FIGURE S1 | Subject-averaged generalized fractional anisotropy (GFA), NQA, and isotropic diffusion (ISO) measure. Here, we plot the subject-averaged magnitude of raw diffusion measures before and after either amber-light therapy (ALT) (A–C) or blue-light therapy (BLT) (D–F) for GFA (A,D), NQA (B,E), and ISO (C,F). Error bars represent the SEM.

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Elevated Serum α -Synuclein Autoantibodies in Patients with Parkinson's Disease Relative to Alzheimer's Disease and Controls

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Early diagnosis of neurodegenerative diseases is of paramount importance for successful treatment. Lack of sensitive and early biomarkers for diagnosis of diseases like Parkinson's disease (PD) is a handicapping problem for all movement disorders specialists. Using serum autoimmune antibodies (AIAs) against neural proteins is a new promising strategy to diagnose brain disorders through non-invasive and cost-effective method. In the present study, we measured the level of AIAs against α -synuclein (α -syn), which is an important protein involved in the pathogenesis of PD. In our study patients with PD (46 patients), Alzheimer's disease (AD) (27 patients) and healthy controls (20 patients) were evaluated according to their sera α -syn AIAs levels. Interestingly, α -syn AIAs were significantly elevated in PD group compared to AD and healthy controls, which advocates their use for diagnosis of PD.

Keywords: autoantibodies, biomarkers, α synuclein, Parkinson's disease, Alzheimer's disease

INTRODUCTION

Dementia of Alzheimer's type and Parkinson's disease (PD) are considered the first and the second most common neurodegenerative disorders worldwide within adults. The prevalence of Alzheimer's disease (AD) is 1–2% at the age of 65 years, which doubles every 5 years to >35% at the age of 85 years, while the PD prevalence is 1% over 65 years and reaches 4% over 80 years (1, 2).

Parkinson's disease is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) and the accumulation of insoluble cytoplasmic protein inclusions (Lewy bodies), which is composed of α -synuclein (α -syn) (1, 3). Thus, α -syn aggregation is a central component of the pathogenesis of PD, which interferes with different cellular functions including lysosomal and mitochondrial functions, autophagy, vesicular homeostasis, and microtubule transport, and induces neuroinflammatory process (4). Additionally, α -syn aggregation has a role in the pathogenesis of AD through its interaction with tau protein and amyloid and has been used as potential biomarker by previous studies (5–7). Accordingly, some studies investigated the α -syn autoantibodies (AIAs) as putative biomarker compared patients with PD, to patients with AD along with controls (8).

Furthermore, several animals and patients' studies confirmed the connection between neuroinflammation and neurodegenerative disorders through activation of microglia and astrocytes. Moreover, humoral immunity has an integrative role in PD pathogenesis, which might prevent further progression (9). Subsequently, the inflammatory and immune mediators have been investigated as potential biomarkers (3, 10).

Discovering biomarkers for confirming diagnosis and/or determining progression of these neurodegenerative disorders was the target of several recent trials. A working group of National Institute of Health defined the biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (11). Ideal biomarker should be linked to neurodegeneration mechanisms, specific, reproducible, non-invasive, easy to use, and inexpensive (12).

In neurodegenerative disorders, naturally occurring AIAs are produced by the immune system against released disease-associated proteins or their fragments into circulation from regions of ongoing pathological changes and cell death. Thereafter, AIAs bind to the disease-associated debris in blood and could gain access to the brain to bind to their related antigens (13).

Consequently, several studies explored the levels of α -syn or its AIAs in patients' cerebrospinal fluid (CSF) (5, 14, 15) and/or plasma (16), with contradictory results (17). Recently, several studies reported association of α -syn and cognitive impairment in PD patients (16). Despite the plasma studies showed more inconsistency, the non-invasive nature, lower costs, and the advances of methodological methods maintained the ongoing exploration of the plasma biomarkers (3, 10).

Exploring biochemical biomarkers is of remarkable importance in chronic neurodegenerative disorders such as PD and AD. They could improve diagnostic accuracy in early stages, distinguish both diseases with overlapping symptoms, develop disease modifying treatments, and explore novel molecular neuropathological processes (5).

Thus, the aim of this study was to investigate the value of the naturally occurring serum autoantibodies of α -syn protein as potential biomarker for diagnosis of PD in comparison to AD and healthy controls.

MATERIALS AND METHODS

A total of 93 individuals were enrolled in the study between June 2016 and June 2017 after approval through a written formal consent and after receiving the approval of the scientific ethical committee of Faculty of Medicine—Ain Shams University.

The study was composed of three groups, 27 clinically confirmed cases of dementia of Alzheimer's type, 46 clinically confirmed cases of PD, and 20 healthy controls. All participants were subjected to complete medical history, brain imaging, and basic laboratory investigations to guard against any other possible cause serving their manifestations. PD patients, diagnosed according to the British Parkinson's Disease Society Brain Bank criteria, were included (18) by a consultant neurologist from Ain Shams University, Movement Disorders Clinic, Cairo, Egypt. Patients with PD were assessed using the Unified Parkinson's

Disease Rating Scale (UPDRS), Hoehn and Yahr scale (H&Y), and Schwab and England scales (S&E) in "medication Off" and "On" states. Exclusion criteria of parkinsonian group included the presence of dementia, atypical or secondary parkinsonism, and familial parkinsonism.

Patients with AD were recruited from outpatients clinic of department of Neurology and institute of psychiatry, Ain Shams University, diagnosis according to the NINCDS-ADRDA and DSM-IV criteria for dementia (19) and assessed using the Arabic version of the Montreal Cognitive Assessment (MoCA) test (20, 21).

Following confirmation of diagnosis, blood samples were withdrawn, centrifuged immediately, sera were obtained from collected blood samples, and stored in -20 freezers prior to their collection in Biobank. All collected sera were stored eventually in -80 freezer of the Medical Experimental Research Center (MERC) of Mansoura University.

Autoantibodies Estimation

IgG anti α -syn antibodies have been tested using commercially available enzyme-linked immunosorbent assay (ELISA) kit purchased from MyBioSource Inc. (San Diego, CA, USA; Cat. No. MBS 2086950). Testing steps have been carried out according to the manufacturer's provided protocols. The titers were estimated on the base of calibration curve of autoantibody standards and expressed in nanograms per milliliter (ng/ml). The sensitivity in the assay was 0.1 ng/ml.

Data Analysis

The collected data were revised, coded, tabulated, and introduced to a PC using Statistical package for Social Science (SPSS 20). Data were presented and suitable analysis was done according to the type of data obtained for each parameter as descriptive study of the three different groups, followed by comparative, correlation and regression studies.

Analysis was performed to identify the ability of autoantibodies to separate cases (PD and AD) from controls. Moreover, further analysis was made to find a differentiation threshold between different neurodegenerative diseases, e.g., PD from AD (in our case). Analyses included: correlation value for each marker, histograms for levels of each autoantibody in different categories (controls, PD, and AD) and finally distribution level analysis. Correlations were calculated for serum levels of α -syn autoantibodies (AIAs) with clinical data of studied groups using Pearson's (for parametric data) and Spearman's (for non-parametric data) coefficients tests.

RESULTS

Group characteristics of study populations are shown in **Table 1**. The mean age of parkinsonian subgroup was 56.26 ± 12.26 years (range 29–81 years), and of Alzheimer's subgroup was 70.07 ± 8.31 years (range 54–81 years), while the mean age of the control group was 53.95 ± 10.65 years (range 33–72 years). There was no statistical significant difference between both the control and parkinsonian subgroup ($p = 0.431$), while there is high statistical significance between the control group and Alzheimer's

TABLE 1 | Demographic and clinical data of the three studied groups.

	Parkinson subgroup	Alzheimer's subgroup	Control group
	No. = 46	No. = 27	No. = 20
Gender; males (%) / females (%)	23 (50.0%) / 23 (50.0%)	15 (75.0%) / 5 (25.0%)	12 (44.4%) / 15 (55.6%)
Age; mean $\pm$ SD (range)	56.26 $\pm$ 12.26 29–81	70.07 $\pm$ 8.31 54–81	53.95 $\pm$ 10.65 33–72
Duration of illness; mean $\pm$ SD (range)	5.20 $\pm$ 3.36 (0.5–15)	4.046 $\pm$ 2.54 (1–9)	
Age of onset; mean $\pm$ SD (range)	51.07 $\pm$ 12.18 (25–79)		
H&Y—Off; median (range)	3 (1–5)		
H&Y—On; median (range)	1 (1–2)		
S&E—Off; mean $\pm$ SD (range)	54.75 $\pm$ 25.22 (10–90)		
S&E—On; mean $\pm$ SD (range)	85.00 $\pm$ 17.05 (40–100)		
Unified Parkinson's Disease Rating Scale (UPDRS) I Off; mean $\pm$ SD (range)	4.58 $\pm$ 2.93 (0–11)		
UPDRS III Off; mean $\pm$ SD (range)	43.00 $\pm$ 19.91 (8–77)		
Montreal Cognitive Assessment; mean $\pm$ SD (range)		16.63 $\pm$ 5.41 (7–25)	
Serum α -syn autoantibodies (ng/ml); median (range)	4.23 (3.3–5.63)	2.9 (1.2–3.3)	0.46 (0.07–0.93)

subgroups ($p < 0.001$) and the parkinsonian and Alzheimer's subgroups ($p < 0.001$).

The mean duration of illness in parkinsonian patients was 5.20 ± 3.36 years and in Alzheimer's patients was 4.46 ± 2.54 years. The median of H&Y—Off was 3 (1.5–3.5), 75% of patients was in stage 1–3, and the mean of S&E—Off was $54.75 (\pm 25.22)$. The mean of MOCA test for Alzheimer's patients was 16.63 ± 5.41 (11–22; **Table 1**).

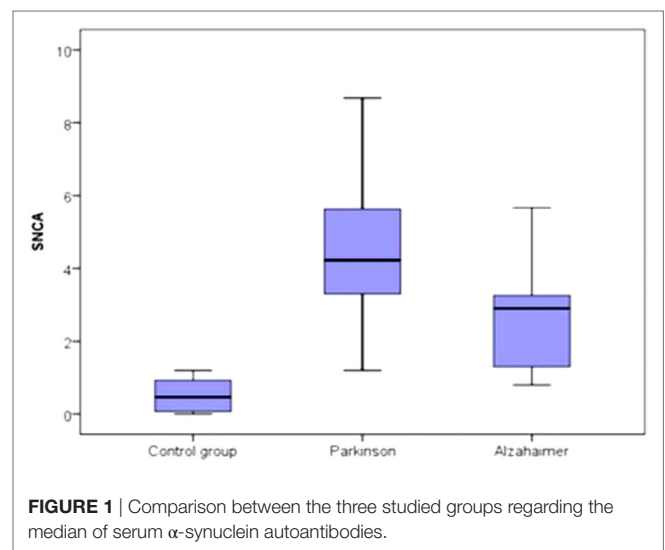
α -Syn AIAs, α -syn Autoantibodies

The median of serum α -syn AIAs was highest in patients with PD [4.23 ng/ml (3.3–5.63)], and elevated in Alzheimer's patients [2.9 ng/ml (1.2–3.3)] less than PD patients, while lowest in controls [0.46 ng/ml (0.07–0.93)]. On comparing these levels of the three studied groups, there was high statistical significant difference ($p < 0.001$) (**Figure 1**).

Within PD group, patients elder than 60 years (18 patients) had significantly higher serum α -syn antibodies compared to patients younger than 60 years ($p = 0.037$). Furthermore, PD cohort was divided to two subgroups according to disease staging, <3 (17 patients) and ≥ 3 (23 patients). Patients with milder stages had non-significantly higher serum α -syn AIAs (4.94 ± 2.61) compared to more advanced stage (4.29 ± 1.80), while both were significantly higher than controls ($p < 0.001$). There was no significant difference between PD patients of duration <5 years (25 patients, 4.62 ± 2.08) and ≥ 5 years (21 patients, 4.84 ± 2.34) ($p = 0.739$). There were no significant differences between males and females within each subgroup.

In the Parkinson's subgroup, there was significant correlation between the serum level of α -syn AIAs and age of patients ($r = 0.390$, $p = 0.007$) and age of onset ($r = 0.383$, $p = 0.009$) (**Figure 2**), while there were no significant correlations with disease stage or UPDRS cognition and motor sub-scores. There were no significant correlations in the Alzheimer's subgroup.

On further analysis of the serum level of α -syn AIA as a predictor biomarker in Alzheimer's and PD using Roc curve. In the Alzheimer's subgroup, the cutoff point of α -syn AIA was >1.2 , its AUC was 0.955, its sensitivity reached 74.07, and its specificity was 100.00. In the Parkinson's subgroup, its cutoff point was >1.2 ,



its AUC was 0.999, its sensitivity reached 97.83, and its specificity was 100.00 (**Figure 3**).

DISCUSSION

The inconsistency and infrequency of studies that explored anti α -syn AIAs in serum of PD patients warranted further studies of this non-invasive and inexpensive biochemical biomarker (3). The current study investigated the serum anti α -syn AIAs as potential biomarker in patients with PD compared with AD and healthy controls. Remarkably, this study found significantly elevated serum anti α -syn AIAs in PD patients more than AD patients and healthy controls, with high sensitivity and specificity of cut off level >1.2 as predictor biomarker (97.83 and 100%, respectively). Additionally, the serum anti α -syn AIAs was significantly elevated in AD patients compared to healthy controls.

Similar findings were reported by prior studies. Horvath and his colleagues also found that the titers of anti α -syn AIAs in

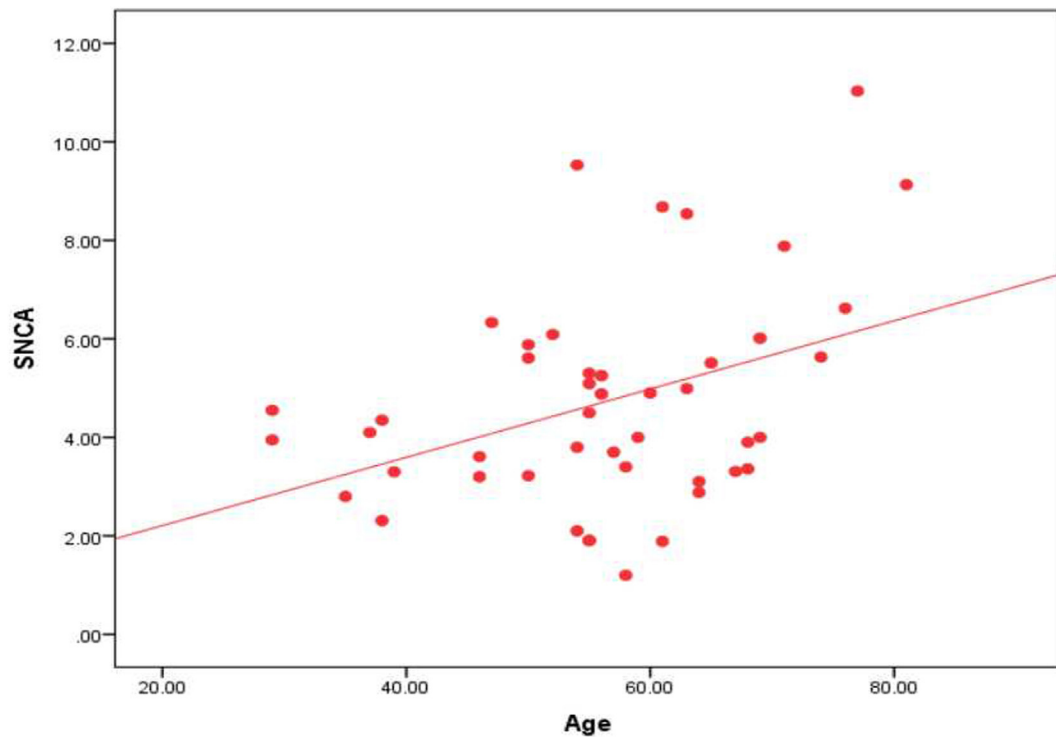


FIGURE 2 | Correlation between the level of α -syn autoantibodies and age in the Parkinson's subgroup.

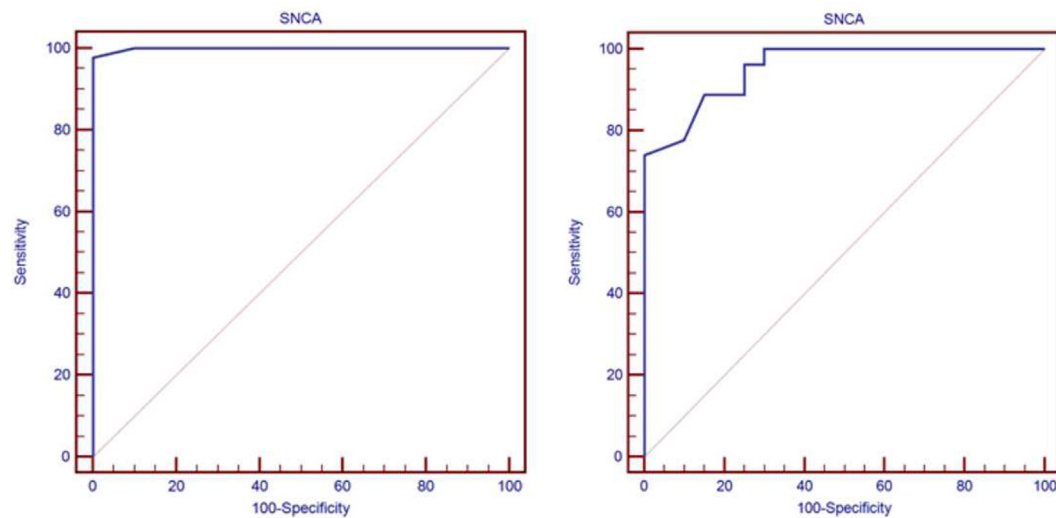


FIGURE 3 | Roc curve of predicting Parkinson's and Alzheimer's groups.

blood samples of 60 recently diagnosed parkinsonian patients was higher compared to age-matched controls, which was correlated also to CSF α -syn AIAs (22). Likewise, other prior studies also demonstrated higher serum α -syn AIAs versus controls (23). However, they reported return of their titer to controls level with longer duration (24).

In contrast to the current study, other studies reported comparable (25–27) or lower (8, 28, 29) serum titer of α -syn AIAs compared to controls. Maetzler et al. reported comparable serum α -syn AIAs in 93 PD patients compared to controls; however, their study was of elder age, longer duration, and non-age-matched controls (25). Besong-Agbo et al. reported lower level of serum

α -syn Abs in PD patients than patients with AD and controls (8). They attributed their findings to advanced stage of their PD cohort, methodological differences, and low avidity of naturally occurring Abs (8). Recently, Brudek and his colleagues have investigated the apparent affinity of anti- α -syn AIAs in plasma samples from 46 PD patients and 41 controls using ELISA and found that the occurrence of high affinity anti- α -syn AIAs in plasma from PD patients is reduced compared to healthy controls (28).

Variability of findings of previous studies could be attributed to methodological and patients' characteristics variability (3, 8, 27). Avidity of natural autoantibodies was characterized as methodological causes of variability (8). Patients' variability included different age's range, duration, severity, and genetic inheritance (22, 25). Noteworthy, the current study is characterized by younger age and shorter duration compared to other studies.

Several prior studies demonstrated higher serum α -syn AIAs in patients with shorter duration (9, 23, 24). In contrast to these studies and in accordance to another studies (8, 25), this study did not show correlation with disease duration. However, we detected non-significant decline of serum α -syn AIAs with more advanced stages, in accordance to the recent study by Horvath et al. (22). In this study, the serum α -syn AIAs was correlated to age of PD patients. Other studies did not detect association with age (8, 22, 25). Furthermore, we could not detect correlation between and disease stage (H&Y scores), similar to other studies (8, 25, 27).

Recently, despite using different approaches, El-Agnaf group revealed significant elevation of α -syn in PD patients' CSF (27) with parallel decline in their plasma level (28). The elevated levels of protein in CSF with their decline in plasma—in accordance to our findings—suggest a possible role for AIAs in clearance of α -syn from sera of PD patients compared to controls.

On the other hand, this study detected elevated α -syn AIAs in serum of patients with AD, compared to controls, in concur with previous studies (7, 9, 30). However, it was lower than PD cases. Moreover, recently, serum α -syn was higher in PD, and correlated with cognitive decline, rather than motor severity (16). Although previous reports suggested possible interaction between tau, B-amyloid, and α -syn levels (30, 31), further studies are needed to confirm a possible contribution of α -syn in AD. The present findings support our previous studies on identification of AIAs against different cytoskeletal proteins in brain insult (32, 33).

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CONCLUSION

The present study showed that serum level of AIAs against α -syn could help as biomarker for PD as they could identify PD patients compared to healthy controls and patients with other neurodegenerative disease (AD). Moreover, our work showed that α -syn AIAs level were higher in AD patients compared to healthy controls, which suggest possible role for α -syn in AD that need to be studied in future research. Further studies are warranted to reproduce these findings, investigating larger number of patients, differentiating types of PD, distinguishing younger onset and classic types, and examining different disease severity and durations.

ETHICS STATEMENT

The study was conducted after approval through a written formal consent and after receiving the approval of the scientific ethical committee of Faculty of Medicine—Ain Shams University and IRB of Faculty of Medicine, Mansoura University.

AUTHOR CONTRIBUTIONS

AS: concept, study design, data generation, data analysis, and writing manuscript. MS: hypothesis, concept, study design, data generation, data analysis, and writing manuscript. MM: data generation. TR: data analysis and critical review. HE: data generation, data analysis, and drafting manuscript. WM: study design and data analysis. ME-B: data generation, data analysis, and critical review. MA-D: hypothesis, concept, study design, and critical review.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Systematic Review of Human and Animal Studies Examining the Efficacy and Safety of N-Acetylcysteine (NAC) and N-Acetylcysteine Amide (NACA) in Traumatic Brain Injury: Impact on Neurofunctional Outcome and Biomarkers of Oxidative Stress and Inflammation

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Background: No new therapies for traumatic brain injury (TBI) have been officially translated into current practice. At the tissue and cellular level, both inflammatory and oxidative processes may be exacerbated post-injury and contribute to further brain damage. N-acetylcysteine (NAC) has the potential to downregulate both processes. This review focuses on the potential neuroprotective utility of NAC and N-acetylcysteine amide (NACA) post-TBI.

Methods: Medline, Embase, Cochrane Library, and ClinicalTrials.gov were searched up to July 2017. Studies that examined clinical and laboratory effects of NAC and NACA post-TBI in human and animal studies were included. Risk of bias was assessed in human and animal studies according to the design of each study (randomized or not). The primary outcome assessed was the effect of NAC/NACA treatment on functional outcome, while secondary outcomes included the impact on biomarkers of inflammation and oxidation. Due to the clinical and methodological heterogeneity observed across studies, no meta-analyses were conducted.

Results: Our analyses revealed only three human trials, including two randomized controlled trials (RCTs) and 20 animal studies conducted using standardized animal models of brain injury. The two RCTs reported improvement in the functional outcome post-NAC/NACA administration. Overall, the evidence from animal studies is more robust and demonstrated substantial improvement of cognition and psychomotor performance following NAC/NACA use. Animal studies also reported significantly more cortical sparing,

reduced apoptosis, and lower levels of biomarkers of inflammation and oxidative stress. No safety concerns were reported in any of the studies included in this analysis.

Conclusion: Evidence from the animal literature demonstrates a robust association for the prophylactic application of NAC and NACA post-TBI with improved neurofunctional outcomes and downregulation of inflammatory and oxidative stress markers at the tissue level. While a growing body of scientific literature suggests putative beneficial effects of NAC/NACA treatment for TBI, the lack of well-designed and controlled clinical investigations, evaluating therapeutic outcomes, prognostic biomarkers, and safety profiles, limits definitive interpretation and recommendations for its application in humans at this time.

Keywords: *N*-acetylcysteine, *N*-acetylcysteine amide, traumatic brain injury, neurofunctional outcome, animal models, oxidative stress, inflammation modulation

INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of death and disability in the United States and globally (1, 2). In the USA, TBI results in more than 250,000 hospitalizations and 2.5 million hospital visits (3), and the costs of immediate TBI care is estimated to be up to 100 billion US\$ (4). Moreover, the burden caused by the consequent degree of disability that TBI patients suffer is estimated to be \$2.5–\$6.5 million (5). These disabilities include, but are not limited to, severe motor and cognitive impairments and mental health problems, such as addiction and mood disorders (6).

At the brain tissue level, the damage from the primary insult is mostly irreparable (7, 8). Additionally, the initial tissue damage may be worsened by a complex secondary injury process following the primary insult (9). These processes consist of a cascade of metabolic, cellular, and molecular events related to extensive tissue destruction and repair (10). These mechanisms are represented by the imbalance of glucose demand and supply, disruption of calcium homeostasis, increased formation of free radicals, lipid peroxidation, mitochondrial dysfunction, and local release of catecholamines (11–13). It has been shown that these processes, or lack thereof, result in further damage to the already critically injured brain tissue (9, 14). Local consequences of this intricate process include vasoconstriction and formation of microthrombi in the microvasculature, with further ischemia and edema (15); initiation and exacerbation of peripheral and central inflammatory process with release of pro- and anti-inflammatory mediators (16); a subsequent rise of intracranial pressure (ICP) with unfavorable neurological outcome or death (17).

Part of this process, involving disruption of the capacity of mitochondria to scavenge free radicals or reactive oxygen species (ROS), is of particular interest in this review (18). The level of glutathione, a naturally available antioxidant within the mitochondria, decreases rapidly after brain tissue injury (19), which leads to accumulation of cytotoxic ROS. *N*-acetyl L-cysteine (NAC), a thiol containing L-amino acid, replenishes glutathione synthesis (20), and thereby may ameliorate secondary brain injury (20) as it counters the deleterious effects oxidative stress, promotes redox-regulated cell signaling, and dampens excessive immuno-inflammatory responses (21). NAC has been an FDA approved drug since 1985 (22) and has been used for

management of acetaminophen toxicity (23). Additionally, a few clinical trials have evaluated NAC targeting neurological diseases, including autism (24), major depression and other psychiatry conditions (9, 25, 26), neonatal asphyxia (27), and neurodegenerative disease (28). Furthermore, recent studies have shown that NAC can reduce levels of oxidative-stress biomarkers following surgical trauma, such as in abdominal aortic aneurysm repair and surgical repair of atrial fibrillation (29, 30).

N-Acetylcysteine is relatively safe to administer, has mild side effects such as nausea, vomiting, rash, and fever, and rarely results in anaphylaxis (23). However, a limitation of using NAC is that it has a low blood–brain barrier (BBB) permeability (20). More recently, an amide derivate of NAC known as *N*-acetylcysteine amide (NACA) was developed with a higher BBB permeability than NAC resulting in increased central nervous system bioavailability (31). However, this new derivate has never been used in studies conducted in humans (32). Both NAC and NACA have not been approved for use in TBI by the FDA or Health Canada.

Given the lack of therapies shown to improve outcome following TBI, we sought to survey the current literature on the underlying the biological and clinical effects of NAC and/or NACA, with respect to their ability to improve neurofunctional outcome, *via* modulation of oxidative stress pathways, inflammatory responses, and cell death signaling in both humans and animals sustaining brain trauma.

METHODS

This systematic review was reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (33).

Search Methods

MEDLINE (1946–Nov 2017), EMBASE (1947–Nov 2017), Cochrane Controlled Trials Register, and Cochrane Database of Systematic Reviews (from inception to July 2017) were searched. The search was not restricted by date and language. Search terms were defined *a priori* and by reviewing the MeSH (Medical Subject Headings) terms of articles identified in preliminary literature searches. The search strategy was based on the initial Medline search strategy and was modified as necessary for the other databases. We used a sensitive search strategy combining

MeSH headings and the keywords “acetyl-cysteine” or “acetyl-cysteine” or “cysteine hydrochloride” or “cystine L-cysteine” or “NAC” or “*N*-acetyl-B-cysteine” or “*N*-acetyl-L-cysteine” or “*N*-acetylcysteine” AND “brain injury” or “trauma.”

Eligibility Criteria and Study Selection

We included experimental studies in humans or animals that measured the neurofunctional outcome (primary outcome) post NAC or NACA use in patients with TBI or in animal models of brain injury. To be included, studies should have performed standardized neurocognitive or behavioral tests to measure neurofunctional outcome. We also included studies that measured levels of biomarkers of oxidative stress, inflammation or cell death (secondary outcomes). Studies were required to have at least one comparator group without NAC administration or placebo, including before and after intervention comparison. We included human studies involving adult or pediatric patients. We excluded studies involving isolated spinal cord injuries, case reports, case series, and conference proceedings. Two of the review authors (JB and UA) not blinded to journal, institution or authors, independently screened the abstracts of identified studies, and determined the eligibility of each study. Each author screened the titles and abstracts of every record retrieved to determine which of the studies would be assessed further. If it was clear from the title and abstract that the article was irrelevant, the article was rejected. Full texts of the studies with questionable eligibility or considered eligible, were retrieved in this phase for evaluation. The reference lists of the retrieved articles were also searched for additional citations. In case of disagreement, consensus was reached by discussion with the senior author (LTDL).

Interventions

In both human and animal studies, we included all regimens of NAC and NACA used (different loading and maintenance doses, intervals, duration and routes). Information about which placebo and its regimen was also retrieved. A summary of the characteristics of included studies is available in **Tables 1** and **2**.

Outcome Measures

The primary outcome in this review was the neurofunctional status of the participants after administration of NAC or NACA, compared with a control group, during the follow-up period established in each study. Several tests for assessment of different levels of neurocognition have been previously validated in the human and animal literature. For example, the use of novel object recognition in Morris Water Maze Task (57) for assessment of neurocognition, and Y-maze (40), for assessment of psychomotor skills, both used in animals. Other tests were used in humans, such as the MicroCog®—Assessment of Cognitive Functioning (MACF) (58), Controlled Oral Word Association test with animal naming (59), Romberg test (balance) (60), and the dynamic gait index (61). In addition, assessment of post-traumatic symptoms such as hearing loss, headache, dizziness, memory loss, and sleep disturbances were also conducted (35). The secondary outcomes were tissue biomarkers of inflammation, such as pro-inflammatory cytokines [e.g., interleukin (IL)-1 β (62),

TABLE 1 | Characteristics of human studies.

Reference	Population, mean/median age	Sample size, % male	Injury type	Control	NAC(A) dose	Via	First dose	Other doses	Length of follow-up	Outcome(s) measured	Findings
Amen et al. (34)	Retired NFL players, age: NR	N = 30, 100%	Repeated mild TBI	Self-matched	Diet with NAC	Oral	NR	Diet supplement, dose NR	2–12 months	Microcognitive test, SPECT image analysis	1 Improvement of general cognition 2 Improvements in brain perfusion
Hoffer et al. (35)	Military personnel, median age: 22 years	N = 81, 99%	Mild post blast injury	Placebo	4 g loading dose	Oral	Up to 72 h	4 g/day for 4 days followed by 3 g/day for 3 days	7 days	Hearing loss, headache, confusion, memory, sleep and balance problems, COWA, animal naming	1 Improvement in cognition and balance 2 Significant resolution of symptoms
Clark et al. (36)	Children, mean age: 9 years	N = 14, 60%	Severe, GCS $\leq$ 8	Placebo	NAC 140 mg/kg/dose and probenecid 25 mg/kg/dose	NG tube	NR	NAC 70 mg/kg/dose, 17 doses over 3 days and probenecid 10 mg/kg 11 doses over 3 days	14 days	Adverse events and antioxidant reserve	1 No adverse events in the NAC group

COWA, controlled oral word association test; GCS, Glasgow coma scale; NAC, *N*-acetyl cysteine; NR, not reported; SPECT, single-photon emission computed tomography.

TABLE 2 | Characteristics of 20 animal studies (21 experiments).

Reference	Animal model	N	Injury type	Control group(s)	Intervention	Via	Initiation of intervention	Other doses	Follow-up
Abdel-Baki et al. (37)	Sprague–Dawley rats, 250–300 g	NR	CCI, moderate	1. Sham injury + NS 2. Injury and NS	NAC 150 mg/kg	IP	1 h	Once daily on days 1 and 2	1 week
Chen et al. (38)	Wistar rats, 250–300 g	51	Weight drop, moderate	1. Sham injury + NS 2. Injury and saline	NAC 150 mg/kg	IP	15 min	Once daily on days 1, 2, and 3	3 days
Du et al. (39)	Long Evans pigmented rats, 360–400 g	74	Blast exposure, 14psi, mild	Normal control	NAC 300 mg/kg HPN-07 98.5%	IP	1 h	Twice daily on days 1 and 2	7, 14, and 21 days
Eakin et al. (40)	1. Sprague–Dawley rats, 350–400 g	26	FPI, 1.8–1.9 atm, mild	1. Sham 2. Injury	NAC 50 mg/kg	IP	30 min	Once daily on days 1, 2 and 3	14 days
Eakin et al. (40)	ICR mice, 30–40 g	32	Weight drop, ~30 g, mild	1. Sham vehicle (DMSO) 2. Sham + drug 3. Injury	NAC 100 mg/kg Topiramate 30 mg/kg	IP	1 h	NR	7 days 30 days
Ellis et al. (41)	Cats	17	FPI, mild	Injury	1. Pre-TBI 326 mg/kg 2. Post-TBI 163 mg/kg	IP	30 min	NR	80 min
Ewert et al. (42)	Long Evans pigmented rats, 360–400 g	48	Blast exposure, ~ 14psi, mild	Injury	NAC 300 mg/kg HPN-07 300 g/kg	IP	1 h	Twice daily for 2 days	3 and 24 h 7 days and 21 days
Gunther et al. (43)	Sprague–Dawley rats, 250–400 g	24	Penetrating ballistic like, moderate	1. Injury 2. Sham surgery	NACA 300 mg/kg	IP	2 min	24 h survivors 300 mg/kg	2 and 24 h
Haber et al. (44)	Sprague–Dawley rats, 250–300 g	NR	CCI, moderate	1. Sham + NS 2. Injury + NS 3. Minocycline 45 mg/kg	1. NAC 150 mg/kg 2. NAC 150 mg/kg + minocycline 45 mg/kg	IP	1 h	Once daily on days 1 and 2	31 days
Hicdonmez et al. (45)	Sprague–Dawley rats, 280–320 g	36	Weight drop ~0.5J, moderate	(1) No injury (2) Injury	NAC 150 mg/kg	IP	15 min	NR	2 and 12 h
Kawoos et al. (46)	Sprague–Dawley rats, 300–350 g	88	Blast overpressure, mild	Placebo (6 groups based on repetitive BOP)	NACA 500 mg/kg in each group, for 6 different groups	IP	2 h in 6 groups and 15 min prior TBI in 1 group	1 group: NACA at 2 + 4 h post TBI	7 days
Naziroglu et al. (47)	Sprague–Dawley rats, 330 ± 20g	36	Weight drop contusion, moderate	1. No injury 2. TBI 3. TBI + Se	NAC 150 mg/kg	Oral	1 h	Once at 24 h, 48 h, and 72 h	3 days
Pandya et al. (48)	Sprague–Dawley rats, 300–350 g	51	CCI, moderate	TBI + vehicle	1. NAC 150 mg 2. NACA 150 mg/kg and 18.5 mg/kg/h	IP	5–30 min	18.5 mg/kg/h NAC, NACA or vehicle	25 h to 15 days
Senol et al. (20)	Sprague–Dawley rats, 300–340 g	36	Weight drop, moderate	1. No injury 2. TBI 3. TBI + Se	NAC 150 mg/kg	Oral	1 h	Once at 24 h, 48 h, and 72 h	4 weeks
Silva et al. (49)	Wistar rats, 270–300 g	NR	FPI, moderate	Injury + NS	NAC 100 mg/kg	Oral	Immediately	Once daily for 5 weeks	5 weeks
Thomale et al. (50)	Sprague–Dawley rats, 300–350 g	48	CCI, moderate	1. Injury + NS	NAC 163 mg/kg	IP	Immediately	2 and 4 h	24 h
Thomale et al. (51)	Sprague–Dawley rats, 300–350 g	62	CCI, moderate	1. Injury + NS	NAC 163 mg/kg	IP	15 min	2 and 4 h	24 h
Xiong et al. (52)	Sprague–Dawley rats, 200–350 g	NR	CCI, moderate	1. Sham 2. Injury 3. Vehicle	NAC 163 mg/kg	IP	4 groups: 1. 5 min before 2. 30 min after 3. 1 h after 4. 2 h after	2 groups post TBI: 1. 5 m and 15 m 1. 5 m and 30 m	12 hours 14 days

(Continued)

TABLE 2 | Continued

Reference	Animal model	N	Injury type	Control group(s)	Intervention	Via	Initiation of intervention	Other doses	Follow-up
Xiong et al. (53)	Sprague–Dawley rats, 200–350 g	NR	CCI, moderate	1. Sham 2. Injury	NAC 163 mg/kg	IP	30 min	NR	1, 4 and 12 h 1 and 14 days
Yiand Hazell (54)	Sprague–Dawley rats, 200–350 g	64	FPI, moderate	1. Sham + NS 2. Injury + NS	NAC 163 mg/kg	IP	5 min	6 and 24 h	6 and 24 h
Yi et al. (55)	Sprague–Dawley rats, 200–350 g	66	FPI, moderate	1. Sham + NS 2. Injury + NS	NAC 163 mg/kg	IP	5 min	NR	6 and 24 h 3 and 7 days

atm, atmospheric pressure; BOP, blast overpressure; CCI, controlled cortical impact; DMSO, dimethyl sulfoxide; FPI, fluid percussion injury; HPN, 2,4-disulfonyl α -phenyl tertiary butyl nitron; ICR, imprinting control region; IP, intra-peritoneal; NACA, *n*-acetylcysteine amide; NAC, *n*-acetylcysteine; NR, not reported; NS, normal saline; PSI, pounds per square inch; Se, selenium; TBI, traumatic brain injury.

tumor necrosis factor alpha (63)], neural injury [e.g., glial fibrillary acidic protein (GFAP) (64)], neurodegeneration [e.g., amyloid- β (64)], apoptosis (e.g., deoxy-nucleotide transferase dUTP nick and labeling) (38), and oxidative stress [e.g., cytosolic free Ca^{++} , cytosolic ROS (65)].

Risk of Bias Assessment

Risk of bias was assessed in duplicate for each study included. Any disagreement was resolved through discussion and consensus. Each included study was classified as a randomized controlled trial (RCT) or a non-randomized study. We assessed risk of bias in each human study incorporated describing the risks (low-risk, high-risk, and unclear risk) for selection bias, performance bias, detection bias, attrition bias, reporting bias, and other bias. For animal studies, we used the tool proposed by Krauth et al. (66) which includes randomization, allocation concealment, blinding, sample size, ethical compliance, statistical methods, outcome assessment, and follow-up.

Analysis

Clinical and methodological heterogeneity across the studies were assessed by examining study design, details on subjects, baseline data, interventions and outcomes, to determine whether the studies were sufficiently similar or not. Large heterogeneity, and the absence of common outcome measures reported, precluded meta-analyses. Therefore, all studies were analyzed qualitatively with a descriptive systematic approach.

RESULTS

The database search identified 251 potential studies for inclusion. After completion of the screening process, 23 studies were included in the qualitative analysis (20, 34–55). Three studies were conducted in humans (34–36) and 20 in animals (20, 37–45, 47–55). Other studies were excluded because they did not meet inclusion criteria, i.e., commentaries, conference abstracts, case reports and case series, or studies including spinal cord injuries. **Figure 1** demonstrates the flow of the screening process. Studies excluded during the review process are reported in Supplementary SI in Supplementary Material.

Clinical and Methodological Characteristics

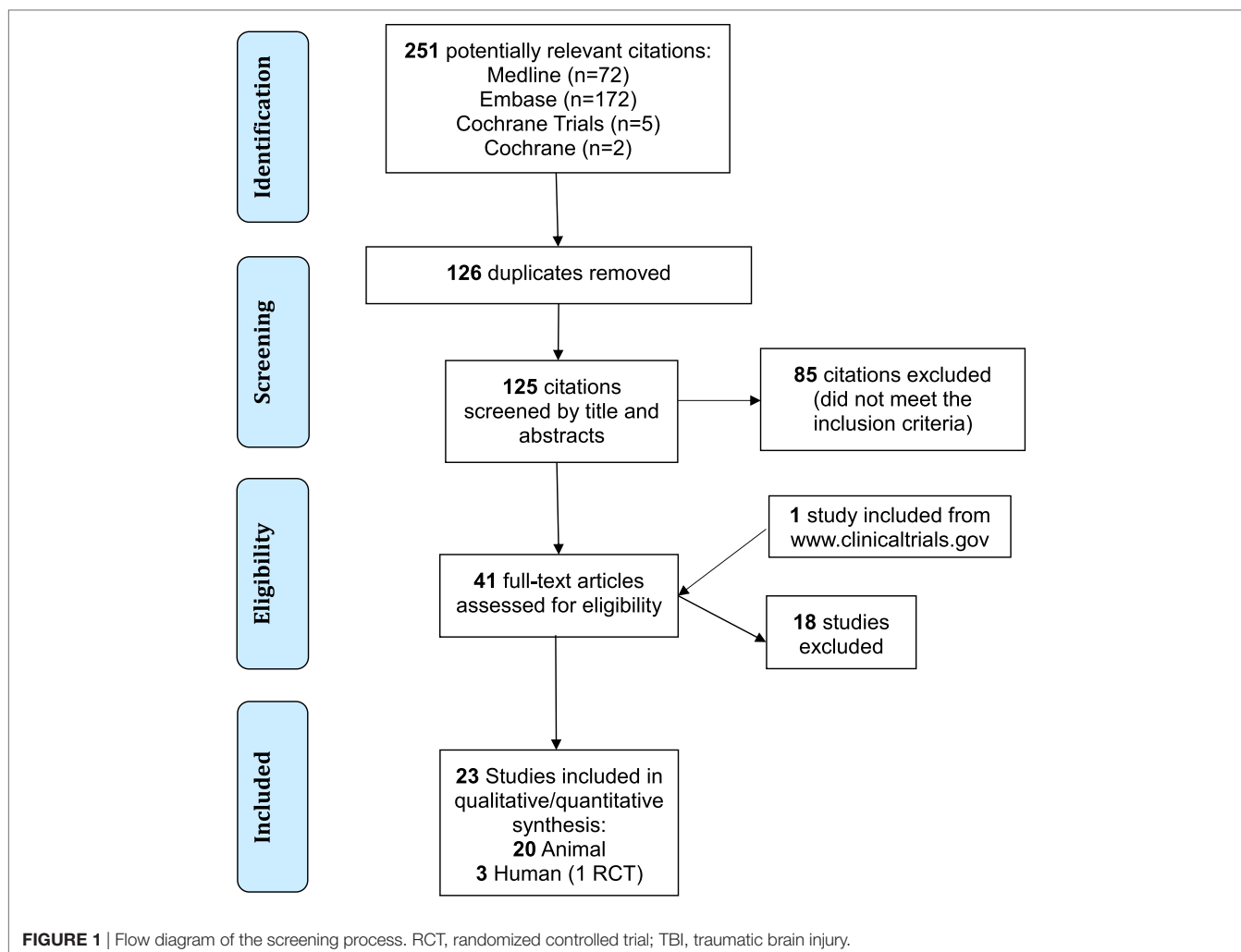
The three studies conducted in humans were represented by two RCT (35, 36) and one observational cohort study (34) (Tables 1 and 2). The study with the largest sample size enrolled 81 active duty military personnel or veterans with blast-related mild TBI (35), whereas the other RCT recruited 14 pediatric patients with severe TBI (36). The observational study (34) was conducted in 30 retired professional football players who sustained repeated head impacts over extended periods of time with evidence of brain damage (mTBI/concussion) and cognitive impairment.

The 20 animal studies (20, 37–55) included 21 experiments with over 700 animals in total. Nineteen studies included rats ($n = 700$), however, five studies did not report sample size (37, 44, 49, 52, 53). One study examined mice ($n = 32$) (40), and one included cats ($n = 17$) (41). Sprague–Dawley rats were included in 15 studies ($n = 491$) (20, 37, 40, 43–48, 50–55). Studies used different brain injury models, such as controlled cortical impact in seven experiments (37, 44, 48, 50–53), weight drop in five experiments (20, 38, 40, 45, 47), fluid percussion injury in five experiments (40, 41, 49, 54, 55), blast exposure in three experiments (39, 42, 46), and ballistic-like TBI in one experiment (43). A moderate injury was inflicted on animals in 15 of these experiments (20, 37, 38, 43–45, 47–55) and mild injury in 6 experiments (39–42, 46).

Interventions

Human studies used different regimens of NAC. For example, in the placebo-controlled RCT conducted involving military members (35), a loading dose of 4 g was administered orally within 72 h of mild TBI followed by 4 g/day for 4 days, and 3 g/day for 3 days. In the pediatric placebo-controlled trial (36), NAC was administered with probenecid with loading dosages of 140 and 25 mg/kg, respectively. A total of 17 maintenance doses of 70 mg/kg of NAC were administered over three days along with 11 maintenance doses of 10 mg/kg of probenecid. In the non-randomized trial (34), NAC was administered as one of the active agents of dietary supplements with no clarification of regimen.

In the animal studies, the loading doses of NAC ranged from 100–326 mg/kg with median dose of 163 mg/kg used in seven



experiments (41, 50–55). In some experiments, other agents such as selenium (20, 47), 2,4-disulfonyl α -phenyl tertiary butyl nitron (HPN-07) (39), topiramate (40), and minocycline (44) were used in combination with NAC. NACA, the BBB permeable derivative of NAC, was provided in three experiments (43, 46, 48). The route of administration in 17 studies (18 experiments) was intraperitoneal (37–46, 48, 50–55) and the drug was delivered *via* injection immediately after or up to 2 h post-injury (median 1 h). Subsequent doses were given in 16 studies, usually up to 48–72 h of the injury (20, 37–40, 42–44, 46–52, 54).

Risk of Bias

Only the RCT conducted on military personnel following blast (35) had an appropriate design and sample size calculation required to detect differences between treatment groups (Tables 4 and 5). The pediatric RCT (36), though well controlled, had a small sample size of 14 patients and no sample size calculation was reported. Finally, the study conducted in retired professional athletes (34) was non-randomized, self-matched, and unblinded. This study reported limited information concerning attrition, the NAC regimen used, leading to a high risk of experimental bias.

Ten animal studies had a random sequence of allocation (20, 38–40, 42, 43, 45, 47, 48, 51) and only 1 of them had allocation concealment and blinding (48). Other limitations of some studies included lack of reporting animal inclusion criteria, sample size calculation, and reporting of outcomes. For detailed information on each domain, please see Table 5.

Outcomes

Neurofunctional Status—Human Studies

The RCT conducted on military personnel (35) showed significant improvements in TBI symptoms, such as imbalance and headache, both assessed on day 7 in the treatment group compared to the placebo group (odds ratio 3.6, $p = 0.006$). The authors demonstrated that the proportion of symptom improvements was about 86% in those who were treated earlier with NAC (i.e., within 24 h post injury) as compared to 46% in those who received the drug between 24 and 72 hours. Significant improvements were from baseline values in NAC treated patients for trail making tasks A [$F_{(1,74)} = 6.64$, $p < 0.05$] and B [$F_{(1,74)} = 4.87$, $p < 0.05$]. Similar significant improvements were not seen in the placebo group. No significant differences

TABLE 3 | Outcome measures and summary of findings in 20 animal studies (21 experiments).

Reference	Outcome(s) measured	Summary of findings in <i>N</i> -acetylcysteine treated animals compared to controls
Abdel-Baki et al. (37)	Variants of place avoidance task (56)	1 NAC + minocycline: improvement of active place avoidance ($F_{4,25} = 34.68$; $p = 0.01$).
Chen et al. (38)	NH-kB; IL-1 β ; TNF- α ; IL-6; ICAM-1 micro-vessels; brain water; BBB; cell apoptosis	1 NF-kB, UL-1 β , IL-6, TNF- α , ICAM-1: upregulated following TBI and suppressed with NAC 2 NAC: $\downarrow$ Brain edema, barrier permeability, and apoptosis
Du et al. (39)	4-HNE; c-fos; amyloid beta (A4); APP; GFAP; NF-68; caspase 3	1 Blast exposure: upregulated 4-HNE, c-fos, GFAP, APP, and HNF 68 2 NAC: reduced the levels of all biomarkers in specific brain areas
Eakin et al. (40)	MWM: hidden platform, probe trial, visible platform	1 Treatment group: Improved performance in MWM tasks, probe trial, and visible platform tasks ($p < 0.05$)
Eakin et al. (40)	Novel object recognition; Y maze paradigm	1 Treatment group: significantly improved performance for both tasks ($p < 0.05$)
Ellis et al. (41)	Mean arterial pressure	1 Cats receiving NAC demonstrated vasoconstriction (one of mechanism involved in oxidative stress)
Ewert et al., (42)	ABR; DPOE level shifts; hair cell loss on cochlear histology	1 ABR threshold was 10 dB less at 24 h. Difference in thresholds was around 20 dB at 7 and 21 days between the treated and control group 2 DPOE levels shift recovered around 7d in treated group 3 Hair cell loss was significantly less in treated group
Gunther et al. (43)	Fluoro-Jade B; TUNEL; MnSOD; Ox-42; iNOS; 3-NT; NFkB; Caspase 3; Cytochrome C; bcl-2; Cy3; Alexa 488; Biotinyl	1 Significant lower levels of necrotic cell death and apoptosis 2 Levels of antioxidant enzyme MnSOD were significantly higher
Haber et al. (44)	Four variants of place avoidance tasks (56). Brain sections of injured area	1 NAC + minocycline: performed better on conflict active place avoidance task and limited memory deficits 2 NAC + minocycline: associated with decreased CD68 expression and increased microglial activation.
Hicdonmez et al. (45)	MDA; SOD activity; GPx activity; catalase activity Number of neurons (/mm ²); caspase 3 activity	1 Treatment group: significant increased activity of SOD and GPx at 12 h 2 Treatment group: significant GPx activity at 2 h 3 Morphology of neurons was well protected in the NAC group
Kawoos et al. (46)	Intracranial pressure monitoring	1 Single NACA dose reduced ICP after single BOP induced injury 2 Single NACA dose was not effective in reducing ICP after multiple BOP 3 Two NACA doses were effective in reducing ICP after multiple BOP 4 A pre-injury NACA dose was most effective in reducing ICP after multiple BOP
Naziroglu et al. (47)	Intracellular Ca ²⁺ ; apoptosis; caspase activity, and ROS in hippocampal cells	1 NAC group: $\downarrow$ Intracellular free Ca ²⁺ , apoptosis, caspase 3 and 9 activity, and ROS levels 2 Se group: some effect, but NAC group had a much greater effect
Pandya et al. (48)	Exp 1—tissue sparing; Exp 2—oxidative stress; Exp 3—mitochondrial bioenergetics and glutathione content; Exp 1–3 cognitive behavioral assessment	1 NACA: better than NAC for tissue sparing and cognitive behavior. 2 NACA: $\downarrow$ oxidative stress, improved mitochondrial bioenergetics, and maintenance of GSH
Senol et al. (20)	Lipid peroxidation; GSH; GSH peroxidase; protein assay; brain cortex β -Carotene, Vitamins A, C, and E	1 NAC group: significantly higher levels of GSH, Vitamin A, Vitamin E, and total oxidant status
Silva et al., (49)	Drug induced seizures; Na + K + ATPase activity; TBAR; protein carbonyl	1 NAC group: more protection from drug induced seizures and Na + , K + ATPase inhibition
Thomale et al. (50)	Brain edema, ABG; ICP; contusion volume	No differences were observed
Thomale et al. (51)	MAP; ABG; CBF; ICP; water content; contusion volume	No differences were observed
Xiong et al. (52)	Mitochondrial activity; Ca + 2 content and transport; GSH	1 NAC: significant impact on mitochondrial bioenergetics and Ca uptake 2 NAC: restored GSH levels during the 14-day observation period
Xiong et al. (53)	Apoptosis-related proteins; shortened bcl-2 and Bax; cytochrome c	1 NAC: diminished levels of shortened bcl-2 and Bax
Yi and Hazell (54)	HO-1 activity in specific brain regions; volume of injury	1 NAC: lower levels of HO-1 in cerebral cortex, thalamus, and hippocampus 2 NAC: associated with significantly decreased volume of injury
Yi et al. (55)	Complexin I and II; neuronal cell loss	1 NAC: reversed increased in Complexin levels in the injured cortex 2 NAC: significantly less neuronal cell loss

HNE, 4-hydroxy-2-nonenal; SOD, superoxide dismutase; ABG, arterial blood gas; ABR, auditory brain stem; BB, blood-brain barrier; BOP, blast overpressure; CBF, cerebral blood flow; c-fos, genetic biomarker; COWA, controlled oral word association test; DPOE, distortion product optoacoustic emissions; GPx, glutathione peroxidase; HO-1, hemoxygenase -1; ICP, intracranial pressure; ICAM, intercellular adhesion Molecule-1; IL-1 β , interleukin 1-beta; IL-6, interleukin 6; MPO, myeloperoxidase; MDA, malondialdehyde; NAC, *N*-acetylcysteine; NACA, *N*-acetylcysteine amide; NH-kB, nuclear factor kappa B; ROS, reactive oxygen species; Se, selenium; SOD, superoxide dismutase; TBAR, thiobarbituric acid reactive substances; TBI, traumatic brain injury; TNF- α , tumor necrosis factor alpha.

were observed in hearing loss and memory problems between the NAC treated and the placebo group on day 7. The study on retired professional football players (34) showed significant improvements compared to their baseline measures in overall cognitive functioning (mean = 43 vs. 32, $p < 0.001$), cognitive proficiency (mean score = 35 vs. 25, $p < 0.001$), processing speed (mean score = 39 vs. 33, $p < 0.001$), processing accuracy (mean score = 49 vs. 41, $p = 0.01$), attention (mean score = 49 vs. 41, $p = 0.01$), reasoning (mean score = 42 vs. 33, $p < 0.01$), and memory (mean score = 43 vs. 34, $p = 0.02$). Improvements in cognitive functioning were associated with significant improvements in the brain perfusion ($p < 0.001$) in specific brain regions in the prefrontal, orbital, parietal, and occipital cortices. A recent phase I RCT conducted in 14 pediatric patients (36) reported no difference in the Glasgow outcome scale recorded upon hospital discharge or at three months follow-up.

Neurofunctional Status—Animal Studies

As compared to controls, animals treated with NAC showed significant improvements in specific neurocognitive and psychomotor tasks (Table 3). These tasks included active place avoidance (spatial memory) (37), novel object recognition (memory) (40), Y-maze (spatial memory) (40), probe trial (learning) (40), and visible platform tasks (visual acuity and motor ability) (40). In a study conducted in rats with moderate TBI (44) where NAC use was associated with minocycline, authors concluded that there was a possible synergy between the two drugs, leading to improvement of long-term memory and set-shifting, compared to controls. In another study where NACA was used (48), improvements in the acquisition phase of the Morris Water Maze task (spatial learning and memory) were demonstrated, compared to controls. Furthermore, NAC protected against hair cell loss that caused subsequent hearing impairment in another study (42). The effect of NAC on seizure disorder following TBI was addressed in a study with Wistar rats (49) and reported a reduced risk for pentylenetetrazol-induced seizures, compared to controls.

Biological Markers—Human Studies

In the most recent phase I RCT conducted in 14 pediatric patients (36), which evaluated the use of NAC and probenecid (pro-NAC), it was reported increased levels of both drugs in the cerebrospinal fluid of patients in the intervention group. The authors also measured levels of serum neuro-injury biomarkers, such as the Neuron Specific Enolase (NSE) and the Glial Fibrillary Acidic Protein (GFAP); however, levels of these biomarkers were not different between the intervention and control groups (NSE $F[1,45] = 0.60$, $p = 0.441$ and GFAP $F[1,45] = 0.29$, $p = 0.596$, respectively).

Biological Markers—Animal Studies

Recent animal experiments have focused on assessing the effects of NAC administration on levels of several oxidative stress biomarkers and glutathione (48) (Table 3). For example, several studies indicate better mitochondrial respiration and a higher glutathione content in animals with brain injury treated with NAC, compared to controls (48, 52, 53). Treatment with NAC

was also associated with lower levels of IL-1 β —a potent pyrogenic cytokine protein, nuclear factor (NF)- κ B—prominent transcription factor that regulates inflammation and cellular survival, TNF- α —prototypical pro-inflammatory cytokine, IL-6—a key inflammatory and immunoregulatory cytokine, intercellular adhesion molecule-1—early adhesion protein that promotes leukocyte transmigration, 4-hydroxy-2-nonenal (4-HNE)—a marker of oxidative stress, c-fos—an immediate gene expressed in cell proliferation, regulation, and survival, GFAP—an astrocyte injury marker, the beta-amyloid precursor protein (β -APP)—a marker of chronic axonal damage, and neurofilament light—also a key marker of axonal injury (38, 39). Other studies have demonstrated improvements in makers known to modulate the oxidative stress, such as in apoptosis-related proteins [B-cell lymphoma-2 (bcl-2) protein and bcl-2-associated X protein (Bax)] (53), in a protein involved in the oxidative stress cascade [hemeoxygenase-1 (HO-1)], and in membrane proteins involved in neurotransmission (Complexin I and II) (55). Combined administration of NAC and Selenium was associated with reductions in cytosolic-free Ca^{++} , apoptosis, cytosolic ROS, capsase-3, and capsase-9 (proteases responsible for the disassembly of the cell into apoptotic bodies), lipid peroxidation, total oxidant status, plasma IL-1 β , and plasma IL-4 activities in rats inflicted with moderate TBI, as compared to untreated controls (20, 47).

N-Acetylcysteine amide has been used in three animal studies (43, 46, 48); two of which, examined biomarkers (43, 48) and one that reported its effect on ICP levels (46). Significant reductions in Fluoro-Jade (a marker of neuronal degeneration) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, a marker of apoptosis) were documented in Sprague–Dawley rats subjected to moderate TBI (43). The authors also demonstrated an increase in Manganese superoxide dismutase (MnSOD, an antioxidant enzyme) relative to controls (43). In another study (48), the use of NACA was associated with improvement of mitochondrial bioenergetics, glutathione content, cortical sparing, and reduced HNE levels.

Other Secondary Outcomes Reported

Six studies (38, 41, 46, 50, 51, 54) assessed other secondary outcomes, such as the size of brain contusion, vasoconstriction or dilatation, ICP, edema, or imaging (Table 3). For example, in a study conducted in rats, use of NAC showed a non-significant decrease of 19% in contusion volume compared to untreated brains, as morphometrically measured using slice staining and imaging (50). The same investigators noted in another study with a larger sample size, that NAC treatment has no significant impact on ICP or water content (51). Similarly, the administration of NAC had no effects on cerebrovascular responsiveness as measured by intra-arterial pressure in brain vessels (41). At the cellular level, brain injury models where NAC was used for treatment, showed decreased brain edema, BBB permeability, and apoptotic cell death compared to untreated brains (38). Lastly, in a recent study, NACA significantly reduced the ICP in rats sustaining single and multiple injuries (two-way repeated measure ANOVA $p < 0.05$) (46). This study found that pre-injury and repeated doses of NACA were dose-dependently effective in reducing ICP after TBI (two-way repeated measure ANOVA $p < 0.05$).

TABLE 4 | Risk of bias in human trials assessing the role of *N*-acetylcysteine for traumatic brain injuries.

	Selection bias		Performance bias	Detection bias	Attrition bias	Reporting bias	Other bias
	Random sequence generation	Allocation concealment					
Amen et al. (34)							
Ranking	High-risk	High-risk	High-risk	Unclear	Unclear	High-risk	High-risk
Explanation	Not randomized	No allocation concealment	No blinding	No information provided	No information provided	Selective results presented	Other drugs used, no dosage, compliance not reported
Hoffer et al. (35)							
Ranking	Low-risk	Low-risk	Low-risk	Low-risk	Low-risk	Low-risk	High-risk
Explanation	Randomized	Allocation concealment done	Blinding of participants and assessors	Blinding of outcome assessor	All subjects followed to endpoint	Trial protocol and study reported	Generalizable? (conducted in the military setting)
Clark et al. (36)							
Ranking	Low-risk	Unclear	Low-risk	Low-risk	Low-risk	Low-risk	High-risk
Explanation	Randomized	No information provided	Blinding of participants and assessors	Blinding of outcome assessor	All subjects were followed to endpoint	Reported prior publication in clinicaltrials.gov	Small sample size

Adverse Events

No drug-attributable safety events were reported in any of the TBI studies cited. This conclusion is consistent with previous literature reports on the use of NAC for other medical conditions and as a potential performance enhancing ergogenic aid (67–69).

Combined Therapies

The association of NAC with other drugs has demonstrated a potential synergistic effect in four animal studies included in this review (39, 40, 42, 44). Probenecid, well known to augment the systemic exposure of antimicrobial agents *via* inhibition of drug elimination through membrane transporters, can also prevent intracellular depletion of GSH (70). The study in children with severe TBI (36) has demonstrated that the co-administration of probenecid increases NAC concentration in both the brain and plasma, thus offering additive mechanisms for therapeutic synergy. In an animal model of blast injury (42) that produced auditory damage as primary sequela, a combination of an antioxidant (2,4-disulfonyl *a*-phenyl tertiary butyl nitron [HPN-07]) and NAC could both enhance temporary auditory recovery and prevent permanent cellular damage when administered early post-blast exposure. The association of minocycline and NAC in a model of mild TBI (44) lead to a regulation of inflammation at tissue level (i.e., modulation of microglia), which may be an additional site of drug synergy between minocycline and NAC since microglial cell activation is known to be redox-regulated. Lastly, another experimental study where topiramate was administered in association with NAC (40) demonstrated the synergistic effectiveness of this combination as an adjunct for headache in a subgroup of animals. NAC alone resulted in a significant behavioral recovery after injury not affected by the use of topiramate.

DISCUSSION

Main Findings

To our knowledge, this systematic review is the first to summarize and appraise the current evidence on the use of NAC in brain injury in human and in animal trials. The primary outcome of neurofunctional status, and the secondary outcomes of effect on markers of inflammation and oxidative stress at cellular and tissue levels and safety, were assessed to a limited extend in the included studies. Overall, NAC improved the neurofunctional status in humans and animals, reduced levels of mediators of oxidative stress and the inflammatory response at tissue and cellular levels, cell death, and had no safety concerns. The effect of NAC at tissue and cellular levels reducing inflammation and the oxidative stress has a potential to decrease secondary brain injury, which can affect positively the neurofunctional outcomes in brain injury patients.

Under physiological conditions, endogenous antioxidant systems maintain the redox homeostasis within the mitochondria to avoid accumulation of cytotoxic free radicals or ROS, which are formed as result of regular cellular respiration and metabolism. Brain injury causes dysregulation of this homeostatic process, with imbalance between ROS production and the cell's antioxidant capacity, resulting in exacerbation of oxidative processes (71). The oxidative stress occurs within minutes of the primary mechanical impact (72) and is an important contributor to the pathophysiology of acute brain injury. Excitotoxicity provokes an excessive calcium uptake, reduces the membrane potential of mitochondria, increases production of ROS from the membrane enzyme complexes I and III, and subsequently reduces ATP production (18, 73). ROS initiate tissue damage by contributing to metabolic failure, to the breakdown of macromolecules, and to the oxidization of proteins, lipids and nucleic acids. Additionally,

TABLE 5 | Risk of bias in animal studies.

Reference	Random allocation	Allocation concealment	Blinding	Inclusion/exclusion criteria	Sample size calculation	Compliance with animal requirements	Conflicts of interests disclosed	Statistical model explained	Animals without comorbidity	Test animal details	Every animal accounted	Dose-response model	Optimal time window used	Total
Abdel-Baki et al. (37)	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	7/13
Chen et al. (38)	Yes	No	No	No	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	8/13
Du et al. (39)	Yes	No	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	9/13
Eakin et al. (40)	Yes	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	9/13
Ellis et al. (41)	No	No	No	No	No	Yes	No	Yes	No	No	Yes	Yes	Yes	5/13
Ewert et al. (42)	Yes	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	9/13
Gunther et al. (43)	Yes	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	9/13
Haber et al. (44)	No	No	No	No	No	Yes	No	Yes	Yes	Yes	No	Yes	Yes	6/13
Hicdonmez et al. (45)	Yes	No	No	No	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	8/13
Kawoos et al. (46)	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	7/13
Naziroglu et al. (47)	Yes	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	9/13
Pandya et al. (48)	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	10/13
Senol et al. (20)	Yes	No	No	No	No	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	8/13
Silva et al. (49)	No	No	No	No	No	Yes	No	No	No	No	No	Yes	No	2/13
Thomale et al. (50)	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	8/13
Thomale et al. (51)	Yes	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	9/13
Xiong et al. (52)	No	No	No	No	No	Yes	No	Yes	Yes	Yes	No	Yes	Yes	6/13
Xiong et al. (53)	No	No	No	No	No	Yes	No	Yes	Yes	Yes	No	Yes	Yes	6/13
Yi and Hazell (54)	No	No	No	No	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	7/13
Yi et al. (55)	No	No	No	No	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	7/13
	10/20	1/20	1/20	1/20	0/20	20/20	11/20	20/20	18/20	15/20	14/20	19/20	18/20	

ROS enhance other secondary injury processes, including the excitotoxicity itself, inflammation, hyperadrenergic activation (13), mitochondrial dysfunction, which ultimately will lead to irreversible cell damage and death (72).

The recent interest in therapies targeting the damaged brain tissue, such as NAC, NACA, and the use of beta-blockers (74) are due to a better understanding of the pathophysiology of brain damage at tissue, cellular and molecular levels. Studies in animals and humans are focusing on identifying biomarkers of tissue damage, impaired metabolism, inflammation, oxidative stress, and cell death. Several biomarkers are currently associated with outcome in TBI and have been used as prognostic indicators. As an example, the hyperadrenergic response and catecholamine surge occurring in the early post-injury period following TBI was independently associated with unfavorable outcome and peripheral inflammatory cytokine/chemokine dysregulation in patients with moderate to severe isolated TBI (13, 16). Similarly, studies investigating the safety and efficacy of beta blockers in patients with acute TBI are currently ongoing (74).

Despite considerable investment by both the pharmaceutical industry and the US National Institutes of Health, to date, there are no pharmacotherapies that will definitively improve unfavorable outcomes post-brain injury. Human literature lacks properly designed phase 2 and 3 clinical studies evaluating new drugs, including NAC and NACA, as demonstrated in this review. There is a lack of studies that properly assess the effects of these promising compounds on the neurofunctional outcome, including cognition. Only one RCT (35) with an adequately powered sample size calculation was obtained. This study reported beneficial effects of NAC on patients with mild TBI-related balance disturbances and headache only. Conversely, a larger number of pre-clinical studies employing standardized brain injury animal models reported more convincing evidence of the utility of NAC/NACA. These models, previously validated in the animal literature (75) have demonstrated improvements in behavioral tasks related to memory, cognition, and auditory complications. Moreover, assessment of biomarkers of oxidative stress, inflammation (pro-/anti-inflammatory cytokines), and cell death (apoptosis-related proteins) was conducted mostly in the animal studies. They have demonstrated substantial modulation and downregulation of these pathways after the application of NAC. Evidence on other outcomes, such as the effect of NAC on brain perfusion, ICP, size of contusion, and brain reactivity remains modest to date.

***N*-Acetylcysteine Amide**

N-acetylcysteine amide is related to NAC with exception of a minor change in the chemical structure, an amide side chain substitution, that gives the compound a neutral charge and improves hydrophobicity and lipophilicity (76). As result of this minor modification, the efficacy of NACA as compared to NAC is significantly enhanced. The new physiochemical and pharmacological properties lead to an easier penetration into the BBB, mitochondria, and other cellular constituents. NACA seems to be a more attractive drug, with possibly stronger therapeutic properties for modulation of inflammation and oxidative stress post TBI due to its superior BBB permeability. It could be even

more protective in TBI by simultaneously and effectively reducing the concomitant trauma-induced systemic inflammatory processes by reducing pulmonary injury (46). For example, in a study (77) where rats were administered with NACA and were exposed to a blast injury, there was a significant reduction of the infiltration of neutrophils into the lung and immunomodulation. NACA facilitated lung recovery from the inflammatory damage, which can be important in cases of severe ALI/ARDS and development of systemic inflammation that could further damage affect the already injured brain. Limited research on NACA has demonstrated that significant levels are detectable in the brain after oral and intraperitoneal administration, compared to NAC (21). If NACA is formulated as a co-crystal with an excipient, it may have a prolonged plasmatic half-life compared to NAC (78). Oxidative stress measures were reported in a model of intracellular oxidation in human red blood cells (79). In this experiment, NACA reduced oxidative activity five times more effectively than NAC and restored 91% of endogenous GSH compared to 15% with NAC, which may suggest an easier penetration of NACA through cellular membranes. The rationale for use of NACA in brain injury therefore seems robust. However, additional pre-clinical and clinical evidence is still required to better establish both mechanism of action and therapeutic efficacy.

Strengths and Weaknesses of This Review

To our knowledge, no systematic review has been conducted in human and animal studies addressing the effects of NAC and NACA on neurofunctional outcome, tissue biomarkers of inflammation and oxidative stress, and safety in brain injury. Major limitations of this review are related to the weaknesses of many of the studies included. For example, it was not feasible to conduct a meta-analysis due to clinical and methodological heterogeneity across the studies, which subsequently precluded analysis of potential publications bias. Furthermore, in reviewing current evidence, the main limiting factor observed was that pre-clinical research on NAC and NACA in TBI is substantially more robust compared to clinical research. We identified several studies conducted in animals and only a few in humans. Pre-clinical work was mostly performed using previously validated animal models of brain injury; however, regimens of NAC and NACA administration, including dose (single vs. multiple), route (oral vs. intravenous), and time post injury still warrant better standardization. In addition, the effect of both drugs on other important biomarkers of brain and BBB damage predictors of outcome in TBI, such as the protein S100 beta (80, 81), need to be evaluated in pre-clinical and clinical studies. The use of NAC or NACA concomitantly with other drugs, such as the ones cited in this review, need more robust investigation to confirm whether synergistic or complimentary effects may occur. Moreover, the human literature presented in this review is restricted to small RCTs, not adequately powered to detect clinical differences, such as favorable or unfavorable neurofunctional outcome, mortality, and drug safety. Studies in humans also may lack generalizability, as they were conducted only in military personnel with specific blast injuries and in cases of pediatric neurotrauma.

Unanswered Questions and Future Research

Although studies conducted in animal models of brain injury can provide insight and guidance to NAC and NACA use in TBI in patients, assessment and management of patients with TBI during clinical practice is considerably different from addressing TBI in these established animal models of brain injury (82). These differences may challenge the subsequent long-term outcome evaluation of NAC/NACA effects on TBI. Ultimately, findings from animal models must be translated and validated in the clinical setting, and this is importantly demonstrated in this review. Such knowledge translation strategies require major efforts and collaboration between clinicians and scientists, and can be challenging to achieve. However, those efforts are justified considering that TBI is a global leading cause of death and disability, have a substantial economic burden due the expenses in immediate and late care, and affects individuals regardless of age, sex, or race worldwide. Phase 3 and 4 trials are warranted for a better understanding of the efficacy and safety of NAC and NACA in TBI patients. Some questions still need to be answered in phase 1 and 2 trials. However, we believe that with the previous knowledge of efficacy and safety of NAC use in other clinical settings, a RCT is warranted. Future studies should include a double-blind, placebo controlled, parallel trial, adequately powered to detect differences in laboratory and, more importantly, meaningful clinical endpoints, including neurofunctional outcome, mortality, and adverse events. We hope that this review will enable clinicians to better appreciate the current state of NAC/NACA use in TBI and prompt investigators to conduct well designed studies in future.

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CONCLUSION

In summary, our systematic review demonstrated moderate quality evidence of efficacy and safety of the use of NAC and NACA in pre-clinical studies. However, these studies still have important questions to be addressed and are substantially heterogeneous, which precludes a more robust interpretation. We found very limited clinical research addressing this subject in brain injury patients. Overall, the literature reported improvement of some aspects of neurofunctional outcome in human and animals, with decreased oxidative stress and inflammation at cellular and molecular levels, and no safety concerns. The promising effects of these drugs on the outcome of TBI warrant further animal research and translation to the clinical setting.

AUTHOR CONTRIBUTIONS

LL was the method expert responsible for study design, planning of data collection, data analysis, draft, and revision of the manuscript. JB and UA participated in the study design, data collection and analysis, and drafting of the manuscript. BN and SR were the content experts and participated in the study design, data analysis and revision of the manuscript. HT and AN participated in data analysis and revision of the manuscript. The final manuscript was approved by all authors.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/articles/10.3389/fneur.2017.00744/full#supplementary-material>.

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Prehospital Intubation and Outcome in Traumatic Brain Injury—Assessing Intervention Efficacy in a Modern Trauma Cohort

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Background: Prehospital intubation in traumatic brain injury (TBI) focuses on limiting the effects of secondary insults such as hypoxia, but no indisputable evidence has been presented that it is beneficial for outcome. The aim of this study was to explore the characteristics of patients who undergo prehospital intubation and, in turn, if these parameters affect outcome.

Material and methods: Patients ≥ 15 years admitted to the Department of Neurosurgery, Stockholm, Sweden with TBI from 2008 through 2014 were included. Data were extracted from prehospital and hospital charts, including prospectively collected Glasgow Outcome Score (GOS) after 12 months. Univariate and multivariable logistic regression models were employed to examine parameters independently correlated to prehospital intubation and outcome.

Results: A total of 458 patients were included ($n = 178$ unconscious, among them, $n = 61$ intubated). Multivariable analyses indicated that high energy trauma, prehospital hypotension, pupil unresponsiveness, mode of transportation, and distance to the hospital were independently correlated with intubation, and among them, only pupil responsiveness was independently associated with outcome. Prehospital intubation did not add independent information in a step-up model versus GOS ($p = 0.154$). Prehospital reports revealed that hypoxia was not the primary cause of prehospital intubation, and that the procedure did not improve oxygen saturation during transport, while an increasing distance from the hospital increased the intubation frequency.

Conclusion: In this modern trauma cohort, prehospital intubation was not independently associated with outcome; however, hypoxia was not a common reason for prehospital intubation. Prospective trials to assess efficacy of prehospital airway intubation will be difficult due to logistical and ethical considerations.

Keywords: traumatic brain injury, advanced airway management, prehospital trauma care, human, emergency medical services

INTRODUCTION

Traumatic brain injury (TBI) constitutes a major public health issue every year for approximately 10 million people globally (1). Prehospital TBI management focuses on prevention of secondary insults, such as prehospital hypoxia (blood oxygen saturation <90%) and hypotension [systolic blood pressure (SBP) <90 mmHg], which have been shown to lead to intracranial lesion deterioration as well as unfavorable long-term outcome (2–6). Current regional guidelines state that a compromised airway should be secured in TBI patients, especially when a long prehospital transport time is expected, or when hypoxia cannot be corrected by other means (7). Consequently, endotracheal intubation is recommended for TBI patients with a prehospital Glasgow Coma Scale (GCS) ≤ 8 (unconscious), as is suggested by the Brain Trauma Foundation (7, 8). Unconscious patients may lose protective airway reflexes which may lead to aspiration (9), as well as to obstruction of a collapsed epiglottis, tongue, and soft palate, conditions leading to hypoxia (10). By providing immediate care at the trauma scene, ensuring appropriate airway management, oxygenation, and adequate blood pressure, improvement in outcome has been shown (8, 11, 12). However, due to its complexity, prehospital intubation in TBI patients is a procedure that can itself result in hypoxia (13, 14), hypotension (15), or even hypertension (16, 17), complications especially unfavorable for TBI patients. It has also been established that when performed poorly, the procedure is hazardous and might even worsen outcome (18–21). Moreover, two other factors shown to influence outcome in trauma is the prehospital duration (“the golden hour”) (22) and the distance to the hospital (23), of course both closely related. Although a large number of studies on prehospital intubation have been conducted, there are only a few on the relationship between advanced prehospital airway management and the distance to hospital. Generally, those studies that have addressed the correlation between prehospital time duration and intubation have not uniquely focused on TBI patients (24–27).

In 2008, the Scandinavian guidelines for prehospital management of severe TBI were published to guide and standardize prehospital care (7) and were also implemented regionally. These guidelines stressed the need for standardized prehospital treatment for patient suffering from suspected TBI. Today, there is no clear consensus on whether prehospital intubation improves outcome, supported by a meta-analysis (28). Some main reasons for this are the lack of good prospective trials and that retrospective trials have difficulties adjusting for the treatment and selection bias. While this study does not constitute a

prospective trial, it aims to provide detailed information from a modern prehospital trauma care system containing detailed information from hospital charts and prospectively gathered outcome data.

In contrast to similar studies, we wished to primarily analyze the characteristics of patients who underwent prehospital intubation, and in turn, which of these factors that independently affected long-term functional outcome. As a secondary aim, we analyzed different aspects of the prehospital management logistics, focusing on the role of prehospital intubation.

MATERIALS AND METHODS

Ethics and Study Design

The study received ethical approval from the Regional Ethical Review Board in Stockholm reference numbers 2007/1113-31, 2010/1979-32, 2013/1718-32, 2014/691-32, and 2015/1675-31/1. This is an observational cohort study of TBI patients.

Study Population

Included patients were; adult and late adolescent trauma patients (≥ 15 years of age) with prehospital trauma charts, a computer tomography verified TBI (ICD-10 S06.2-S06.9) treated at the only neurosurgical unit (at Karolinska University Hospital, Stockholm, Sweden) in the region during the period January first 2008 to December 31st 2014 in Stockholm, Sweden (following prehospital guideline implementation). Patients were excluded if declared dead on scene, admitted to the reporting hospital >6 h after the trauma or in cases when the exact time of trauma was unknown. In addition, we excluded patients transported from another county for specialist care and/or transfers after >24 h to the university hospital after admission to any of the other hospitals.

Prehospital Data Collection

Data were collected from the neuro trauma registry at the Karolinska University Hospital. Prehospital data were retrieved from the electronic prehospital records network (CAK-net) used by all ambulance caregivers. The ambulances are equipped with a global position satellite system (GPS) that delivers a GPS coordinate according to the SWEREF 99 (Swedish reference frame 1999) system (29). The SWEREF 99 has been shown to have a margin of error within 0.5 m of the WGS 84 (World Geodetic System 1984) that the commercially available GPS system uses as reference (29). The electronic prehospital records also provide the exact address on the scene of accident. If the SWEREF 99 coordinates were not available, Google Maps® was used to generate the WGS 84 coordinates using the entered address (used for $n = 161$, 35%). The preferred ambulance route from the scene of accident to the primary hospital was chosen. Travel distances were adjusted for recent infrastructure projects in the Stockholm region during the study period to indicate the correct paths for the ambulances. The first author (Rebecka Rubenson Wahlin) who is an experienced staff member of the Stockholm Emergency Medical Services (EMS) did perform these assessments. For helicopter transport, the linear distance to the hospital was used.

Abbreviations: ACOS, American College of Surgeons; AIS, Abbreviated Injury Scale; CPR, cardiopulmonary resuscitation; ED, Emergency department; EMCC, Emergency Medical Communications Centre; EMS, Emergency medical services; EMT, Emergency medical technicians; GCS, Glasgow Coma Scale; GOS, Glasgow Outcome Score; ICU, intensive care unit; ISS, injury severity score; KSS, Karolinska University Hospital in Solna; LOS, length of stay (days); PHETI, prehospital endotracheal intubation; ROSC, return of spontaneous circulation; RTS, revised trauma score; RR, respiratory rate; SBP, systolic blood pressure; SCC, Stockholm County Council; TBI, traumatic brain injury.

Clinical Variables

Age and gender were included from hospital charts. Mechanism of injury was included from prehospital records. Multitrauma, defined as an injury to any other major organ system except the head and spine, were noted (30). The energy of the trauma, as defined by advanced trauma and life-support guidelines (31), were defined as “low energy” or “high energy,” if available. Prehospital hypoxia was defined as a peripheral oxygen saturation <90%, and a prehospital hypotension if the SBP <90 mmHg, at any time during the prehospital duration. If serum ethanol was positive at admittance to the hospital, it was noted as it has been shown to be associated with a favorable outcome (32). GCS was noted, and “unconscious” patients were defined as a GCS $\leq$ 8 at the scene of accident (33). If one, or two, pupil(s) presented without light reflex, it was defined as “pupil unresponsiveness.” To assess the neuro-radiological damage, we assessed the admission CT scans according to Marshall (34) classification, Rotterdam CT-score (35), and Stockholm CT-scores (36). We chose to use the Stockholm CT-scores in the analysis as they are presented as continuous variables where higher levels and have been shown to best correlate to outcome (36). Moreover, head abbreviated injury scale (AIS) > 3, as defined as at least a “severe” TBI, were noted together with injury severity score (ISS) and new injury severity score (NISS) (37). S100B, a protein of brain tissue fate and a potent biomarker of brain injury (38), were assessed at admission and at 12–48 h after injury as later samples have been shown to be less influenced by extracranial trauma (39, 40). Intensive care unit stay was defined as the length of stay in days. Survival status was noted, as well as 12 months Glasgow Outcome Score (GOS) (33) assessed by clinic visits and questionnaires regarding health-related quality of life.

The prehospital variables were collected and defined in accordance with The Utstein Trauma Template (41) and Utstein-style template for prehospital airway management (42) to increase the possibility to compare data with other prehospital studies; time from alarm until hospital arrival, highest level of prehospital care provided, prehospital airway management, type of prehospital airway management, and type of transportation, time from alarm until arrival at scene were all extracted from the prehospital records as well as SBP, respiratory rate, heart rate and GCS on scene, indication for airway intervention, attempts of airway intervention, intubation success, device used in success, and post intervention ventilation.

The time periods were defined as follows; time on scene and the time of departure from scene until hospital arrival were defined in minutes and seconds, the distance from scene of accident to hospital were defined in kilometers.

The saturation from pulse oximetry devices were acquired from the scene and at arrival at the hospital, this “delta-saturation” (oxygen saturation at the emergency department—oxygen saturation at the scene) was reported.

Prehospital Conditions

The Stockholm County Council (SCC) includes 26 municipalities covering 6,519 square kilometers, an archipelago of approximately 30,000 islands, and is responsible for the EMS of 2.1 million inhabitants (43). The SCC responsibility includes both

the EMS and the seven emergency hospitals, of which, solely one is a level-1 trauma center according to the American College of Surgeons’ criteria (44). The EMS are provided by one SCC owned company and by two private companies contracted by the SCC. One Emergency Medical Communications Centre operates in the area.

During the study period (2008–2014), there were 55–61 ground ambulances, and three rapid-response vehicles during daytime (07:00–20:00) (43). A rapid-response vehicle was physician-manned and the two others by nurse anesthetists, as well as emergency medical technicians (EMTs). All ground-based ambulances were manned by two people, an EMT and one registered nurse. During nighttime, there is no physician on call, and about 38 ambulances operate in the area (45). In addition, there is also a nurse anesthetist manned helicopter (one additional helicopter during summer time) and one mobile intensive care unit operating in the area.

As per the new guidelines that were implemented in 2008, registered nurses may administer drugs and handle the laryngeal mask after personal delegation (46). Nurse anesthetists with more than 1 year of clinical experience are also allowed to perform prehospital endotracheal intubation (PHETI) without drugs (46). Nurse anesthetists with more than 3 years of experience may perform drug-assisted rapid sequence induction after personal delegation.

Statistical Analysis

For descriptive purposes, continuous data are presented as medians with interquartile ranges (except the normally distributed variable age as mean and SD). Mann–Whitney *U*-test and Chi-square test were used to compare continuous and categorical parameters, respectively. A univariate regression analysis was used to correlate factors to prehospital intubation (“lrm” function in R, “rms”-package) (47). For outcome prediction, a similar univariate proportional odds regression was used toward GOS levels. We know from previous studies using the same database that the proportional odds of GOS levels results in similar results as dichotomizing it into GOS levels 1–3 versus 4–5 (38, 40). In the two univariate models, un-imputed data were used. Nagelkerke’s pseudo- R^2 was used to illustrate the pseudo explained variance, where “0” does not provide any variance while “1” fully explains the model. Multivariable models, utilizing Multiple Imputation (MI) (“mice”-package in R), including all parameters significant in the univariate analyses, were performed to determine factors independently correlated to intubation and functional outcome. Only parameters significant in univariate analyses were included in the multivariate models and the models were bias-adjusted for multiple parameters. Dependant variables were GOS or prehospital intubation. To examine how prehospital intubation affected outcome in the multivariate model, a step-up procedure where used. Conditional density plots and box plots were used to illustrate continuous versus categorical variables and box plots comparing continuous variables (delta-saturation).

The statistical program R was used, utilizing the interface R-studio Version 0.99.902 (47). The statistical significance level was set to $p < 0.05$.

Missing Data

Some data were missing from the hospital charts and were imputed in order to optimize multivariate analyses, thus being able to utilize all patients. MIs (“mice” package in R) were performed, retaining seven imputed dataset, which were used to look for parameters independently correlated to functional outcome and prehospital intubation. The current method is recommended in this type of multivariate analyses, as is advocated by the statistical literature as well as the IMPACT research group (48, 49).

RESULTS

Patient Demographics

During the period January first 2008 to December 31st 2014, 738 TBI patients were considered for inclusion and, out of these, 122 patients were excluded due to missing prehospital records, 75 patients due to uncertain trauma time or admittance more than 6 h after trauma, and 83 patients as they had been referred from other counties (i.e., secondary transports). In total, 458 patients fulfilled inclusion criteria. Demographics for all patients, as well as missing data for each parameter, are presented (Table S1 in Supplementary Material). Out of these 458 patients, 178 were unconscious at the scene of accident and thus represented patients in potential need of prehospital airway management according to the implemented guidelines. Among the 178 unconscious patients, 61 were intubated (a total of 66 were intubated, but in five cases, this was because the patient was conscious, but uncooperative or combative at the scene).

The unconscious group was more severely injured (according to all classifications), with higher in-hospital mortality and worse long-term functional outcome compared to the conscious patients (Table 1). In the unconscious cohort, the intubated patients were almost 10 years younger (38.8 versus 48.9 years), more often victims of high-energy trauma (however, this parameter must be interpreted with caution due to the amount of missing data) and were more often transported by helicopter (52% compared to 16% for non-intubated patients) (Table 2). The intubated group also had a longer distance from scene of accident to the hospital (in median almost 10 km to the hospital) and were longer at-scene as compared to the non-intubated patients. The intubated patients remained in median 12 min longer at the scene of accident (Table 2).

Parameters Correlated to Prehospital Intubation

The parameters that were independently associated with prehospital intubation among the unconscious patients were mode of transportation (by helicopter), amount of energy involved in the trauma, time from alarm to hospital arrival, pupil responsiveness, prehospital hypotension, and distance from trauma scene to the hospital (Table 3). A multiregression toward prehospital intubation using significant variables in the univariate regression exhibited an adjusted pseudo- R^2 of 0.393 (Table 3). Notably, prehospital hypoxia was not significantly correlated to prehospital intubation in univariate analysis for the unconscious patients ($p = 0.547$).

TABLE 1 | Patient characteristics and outcome data between conscious and unconscious patients.

	Conscious (<i>n</i> = 280)	Unconscious (<i>n</i> = 178)	<i>p</i> -Value
Prehospital data			
Age, years (SD)	48.9 (19.6)	45.3 (19.2)	0.055
Gender, male (%)	<i>n</i> = 204 (73%)	<i>n</i> = 131 (74%)	0.948
Multitrauma, <i>n</i> (%)	<i>n</i> = 52 (19%)	<i>n</i> = 79 (44%)	<0.001
Positive blood ethanol, <i>n</i> (%)	<i>n</i> = 100 (36%) (24 missing, 9%)	<i>n</i> = 75 (42%) (7 missing, 4%)	0.375
Hypoxia at SoA	<i>n</i> = 9 (3%) (20 missing, 7%)	<i>n</i> = 32 (18%) (17 missing, 10%)	<0.001
Hypotension at SoA	<i>n</i> = 6 (2%) (23 missing, 8%)	<i>n</i> = 9 (5%) (34 missing, 19%)	0.088
Trauma energy, high <i>n</i> (%)	<i>n</i> = 34 (12%) (172 missing, 61%)	<i>n</i> = 68 (38%) (78 missing, 44%)	<0.001
Hospital data			
Pupil unresponsiveness, <i>n</i> (%)	<i>n</i> = 19 (7%) (6 missing, 2%)	<i>n</i> = 61 (34%) (6 missing, 3%)	<0.001
Stockholm CT Score	1.9 (1–2.5)	3 (2–3.5)	<0.001
Head-AIS > 3	<i>n</i> = 191 (68%)	<i>n</i> = 157 (88%)	<0.001
ISS, median IQR	21.5 (13–26)	26 (22–38)	<0.001
NISS, median IQR	29 (24–41)	48 (34–57)	<0.001
S100B admission, median $\mu\text{g/L}$	0.99 (0.36–2.35) (68% missing)	2.9 (1.4–7.35) (34% missing)	<0.001
S100B peak at 12–48 h, median $\mu\text{g/L}$	0.22 (0.13–0.42) (38% missing)	0.36 (0.20–0.74) (8% missing)	<0.001
Hospital length of stay (LOS), median days (IQR)	9 (5–19)	20 (9–34)	<0.001
ICU LOS, median days (IQR)	1.7 (0–7)	10.6 (3–19)	<0.001
Outcome data			
In-hospital mortality	<i>n</i> = 19 (7%)	<i>n</i> = 31 (17%)	0.001
Long-term GOS 1–3 (unfavorable), <i>n</i> (%)	<i>n</i> = 81 (29%)	<i>n</i> = 96 (54%)	<0.001

Table illustrating the demographic data between conscious and unconscious patients. Missing data are mentioned for each parameter, if present. Difference between groups are compared using chi-square or Mann–Whitney test, where applicable. SoA, scene of accident; CT, computerized tomography; AIS, Abbreviated Injury Scale; ISS, Injury Severity Score; NISS, New Injury Severity Score; ICU, intensive care unit; GOS, Glasgow Outcome Score; IQR, interquartile range.

Predictably, if all 458 patients were included in the model (Table S2 in Supplementary Material), the parameter “Unconscious” had the strongest association toward prehospital intubation (pseudo- R^2 0.361). Apart from that, the combined patient cohort presented similar results (Table S2 in Supplementary Material).

Parameters Correlated to Long-Term Functional Outcome

The parameters that independently correlated to functional outcome in the multivariate proportional odds analysis of unconscious patients were: levels of the biomarker S100B 12–48 h after trauma, Stockholm CT-score, NISS, age, and pupil responsiveness (Table 4). This model exhibited an adjusted pseudo explained variance in relation to long-term GOS of 0.502 (we defined this as our “base” model). Prehospital intubation did not significantly correlate to outcome in univariate analysis ($p = 0.296$), and did not add any significant independent information to the base model ($p = 0.154$) (Table 4). In an exploratory approach, we

TABLE 2 | Patient characteristics and outcome data, intubated and non-intubated groups among unconscious patients.

Parameters	Not intubated (<i>n</i> = 117)	Intubated (<i>n</i> = 61)	<i>p</i> -Value
Prehospital data			
Age, years (SD)	48.9 (18.7)	38.8 (18.7)	0.001
Gender, male (%)	<i>n</i> = 86 (74%)	<i>n</i> = 43 (73%)	0.618
Multitrauma, <i>n</i> (%)	<i>n</i> = 42 (36%)	<i>n</i> = 37 (61%)	0.003
Positive blood ethanol, <i>n</i> (%)	<i>n</i> = 54 (46%) (missing 6%)	<i>n</i> = 21 (34%)	0.091
Hypoxia at SoA	<i>n</i> = 19 (16%) (missing 12%)	<i>n</i> = 13 (22%)	0.689
Hypotension at SoA	<i>n</i> = 2 (20% missing)	<i>n</i> = 7 (11%)	0.015
Trauma energy, high, <i>n</i> (%)	<i>n</i> = 26 (22%) (missing 58%)	<i>n</i> = 42 (69%) (missing 16%)	0.003
Hospital data			
Pupil unresponsiveness, <i>n</i> (%)	<i>n</i> = 30 (27%) (missing 4%)	<i>n</i> = 31 (51%) (missing 1%)	0.002
Stockholm CT Score	2.9 (2.0–3.5)	3.0 (2.0–3.5)	0.877
Head-AIS > 3	<i>n</i> = 106 (91%)	<i>n</i> = 53 (87%)	0.641
ISS, median IQR	26 (21–34)	29 (25–42)	0.005
NISS, median IQR	43 (34–57)	50 (34–57)	0.237
S100B admission, median µg/L	2.7 (1.3–4.9) (missing 41%)	4.6 (1.7–11) (missing <i>n</i> = 14, 23%)	0.093
S100B peak at 12–48 h, median µg/L	0.38 (0.21–0.80) (missing 9%)	0.33 (0.20–0.69) (missing 8%)	0.456
Hospital length of stay (LOS), median days (IQR)	19 (9–33)	22 (8–35)	0.700
ICU LOS, median days (IQR)	10 (3–18)	13 (4–22)	0.194
Outcome data			
In-hospital mortality	<i>n</i> = 21 (18%)	<i>n</i> = 10 (16%)	0.959
Long-term GOS 1–3 (unfavorable), <i>n</i> (%)	<i>n</i> = 57 (49%)	<i>n</i> = 39 (64%)	0.076
Prehospital transportation data			
Transported with helicopter, <i>n</i> (%)	<i>n</i> = 19 (16%)	<i>n</i> = 32 (52%)	<0.001
Time from alarm until hospital arrival, mm:ss, median (IQR)	36:29 (28:04–47:57)	49:34 (37:33–60:08)	<0.001
Time from alarm until arrival at scene, mm:ss, median (IQR)	09:38 (06:51–14:49)	12:56 (09:00–20:50)	0.013
On-scene time, mm:ss, median (IQR)	14:31 (10:23–21:14)	26:40 (21:01–16:41)	<0.001
Time from scene until hospital arrival, mm:ss, median (IQR)	10:07 (06:29–15:24)	10:35 (06:27–16:59)	0.731
Distance from scene of accident to the hospital, median kilometers (IQR)	9.2 (5.1–18.1)	17.2 (10.8–32.22)	<0.001

Table illustrating the demographic data between intubated and non-intubated patients. Missing data are mentioned for each parameter, if present. Difference between groups are compared using chi-square or Mann–Whitney test, where applicable.

SoA, scene of accident; CT, computerized tomography; AIS, Abbreviated Injury Scale; ISS, Injury Severity Score; NISS, New Injury Severity Score; ICU, intensive care unit; GOS, Glasgow Outcome Score; IQR, interquartile range; mm, minutes; ss, seconds.

analyzed the unconscious patients who had prehospital hypoxia (*n* = 32) to see if intubation specifically improved outcome in this cohort, but could not see any significant association (*p* = 1.0, data not shown).

When assessing the combined patient cohort of 458 patients, similar correlations toward outcome were found, with the obvious addition of “unconscious” patients having a more unfavorable outcome (Table S3 in Supplementary Material). Prehospital hypoxia was an independent predictor of unfavorable outcome in the combined cohort, as well as prehospital intubation (Table S3 in Supplementary Material). Interestingly, neither “distance from the trauma to the hospital” nor the “total prehospital” or “on-scene” times were correlated to the long-term outcome (Table 3; Table S3 in Supplementary Material).

Logistics of Prehospital Airway Management

Of the 178 unconscious patients, 61 patients (41%) were in need of PHETI for different reasons, a majority were intubated due to decreased level of consciousness (40%) or “ineffective ventilation” (18%), only two (3%) were intubated primarily due to hypoxia according to the prehospital charts (Table 5). Out of the patients who were *conscious* at the scene of accident, *n* = 5 were intubated. In none of these cases was the airway compromised,

instead, these patients were sedated due to psychomotor agitation (Table 5). The number of intubation attempts varied, but in 85% of the cases, only one intubation attempt was necessary (Table S1 in Supplementary Material). There were nine failed intubations at the scene of accident. In an exploratory sub-group analysis, long-term GOS were neither related to multiple intubation attempts, nor failed intubation in the unconscious cohort (data not shown).

Moreover, we could not detect any difference in the intubation success rate depending on care provider, EMS physician, or nurse (*p* = 0.423, data not shown).

With increasing distance from the scene of accident, the rate of prehospital intubation escalated and at >10 km almost 50% of all patients were intubated (Figure 1), in line with the introduced guidelines.

The delta-saturation during the prehospital transportation did not improve significantly (*p* = 0.568) in the intubated group (Figure 2). Thus, prehospital intubation did not significantly improve saturation on group level during transport.

In an exploratory approach, we investigated the helicopter transportations more thoroughly. Of all air transports carrying intubated patients, *n* = 18 (60%) were intubated by the EMS personnel arriving by helicopter rather than by the EMS that first arrived on scene.

TABLE 3 | Parameters correlated with prehospital intubation in the unconscious population.

Univariate analysis	For unconscious patients (n = 178)		
Parameter (s)	p-Value	Pseudo-R ²	Correlation coefficient
Age	<0.001	0.085	–
Gender	0.500	NS	NS
Multitrauma	0.002	0.075	+
High/low energy trauma*	<0.001	0.160	+
Positive blood ethanol	0.063	NS	NS
Prehospital hypoxia	0.547	NS	NS
Prehospital hypotension*	0.006	0.070	+
Pupil responsiveness*	<0.001	0.095	+
Stockholm CT Score	0.816	NS	NS
AIS	0.475	NS	NS
ISS	<0.001	0.086	+
NISS	0.141	NS	NS
S100B admission	0.031	0.053	+
S100B 12–48 h	0.549	NS	NS
Mode of transportation (helicopter)*	<0.001	0.181	+
Distance from trauma to hospital	0.003	0.068	+
Time from alarm to hospital arrival	<0.001	0.121	+
Time from alarm until EMS arrival at scene	0.022	0.041	+
EMS on-scene time*	<0.001	0.165	+
Time from scene to hospital arrival	0.328	NS	NS
Multivariable analysis	Adjusted pseudo-R ²		

Parameters significant in a bivariate regression analysis versus prehospital intubation with a un-imputed dataset, p-value for significance, Nagelkerke's pseudo-R² for the explained variance and correlation coefficient if an increase of the parameters was positively or negatively correlated to pre-hospital intubation. In the multivariate proportional odds model, an imputed dataset was used. Due to co-variance of the parameters, time for EMS on scene was the only time duration that was used. NS, not significant; CT, computerized tomography; AIS, Abbreviated Injury Scale; ISS, Injury Severity Score; NISS, New Injury Severity Score; EMS, Emergency medical services.

DISCUSSION

In our modern TBI cohort from a level 1 trauma center, we found a difference between parameters correlated to prehospital intubation and functional outcome. Previously, no study has used a similar approach to analyze prehospital advance airway management in this patient group. Prehospital hypotension, pupil unresponsiveness, high energy trauma, longer distance to hospital, and helicopter transportation were independently associated with an increased intubation frequency, and among them, only pupil unresponsiveness was an independent outcome predictor. The added effect of prehospital intubation did not significantly influence outcome. Moreover, prehospital hypoxia was not associated with an unfavorable outcome in the multivariable analysis and while some patients clearly suffered from this condition, the EMS on scene did not primarily focus on this parameter when deciding on prehospital intubation. The failure to show an independent association between hypoxia and

TABLE 4 | Parameters correlated to functional outcome in the unconscious cohort.

Univariate analysis	For unconscious patients (n = 178)		
Parameter	p-Value	Pseudo-R ²	Correlation coefficient
Age*	<0.001	0.089	–
Gender	0.219	NS	NS
Multitrauma	0.475	NS	NS
High/low energy trauma	0.170	NS	NS
Positive blood ethanol level	<0.001	0.074	+
Prehospital hypoxia	0.016	0.037	–
Prehospital hypotension	0.142	NS	NS
Prehospital intubation	0.296	NS	NS
Pupil responsiveness*	<0.001	0.082	–
Stockholm CT score*	<0.001	0.164	–
AIS	0.026	0.030	–
ISS	<0.001	0.069	–
NISS*	<0.001	0.099	–
S100B admission	<0.001	0.153	–
S100B 12–48 h*	<0.001	0.302	–
Distance from trauma to hospital	0.496	NS	NS
Mode of transportation	0.212	NS	NS
Time from alarm to hospital arrival	0.867	NS	NS
Time for EMS to reach the trauma scene	0.423	NS	NS
Time for EMS on scene	0.692	NS	NS
Time from scene to hospital arrival	0.512	NS	NS
Multivariable analysis	Adjusted pseudo-R ²		

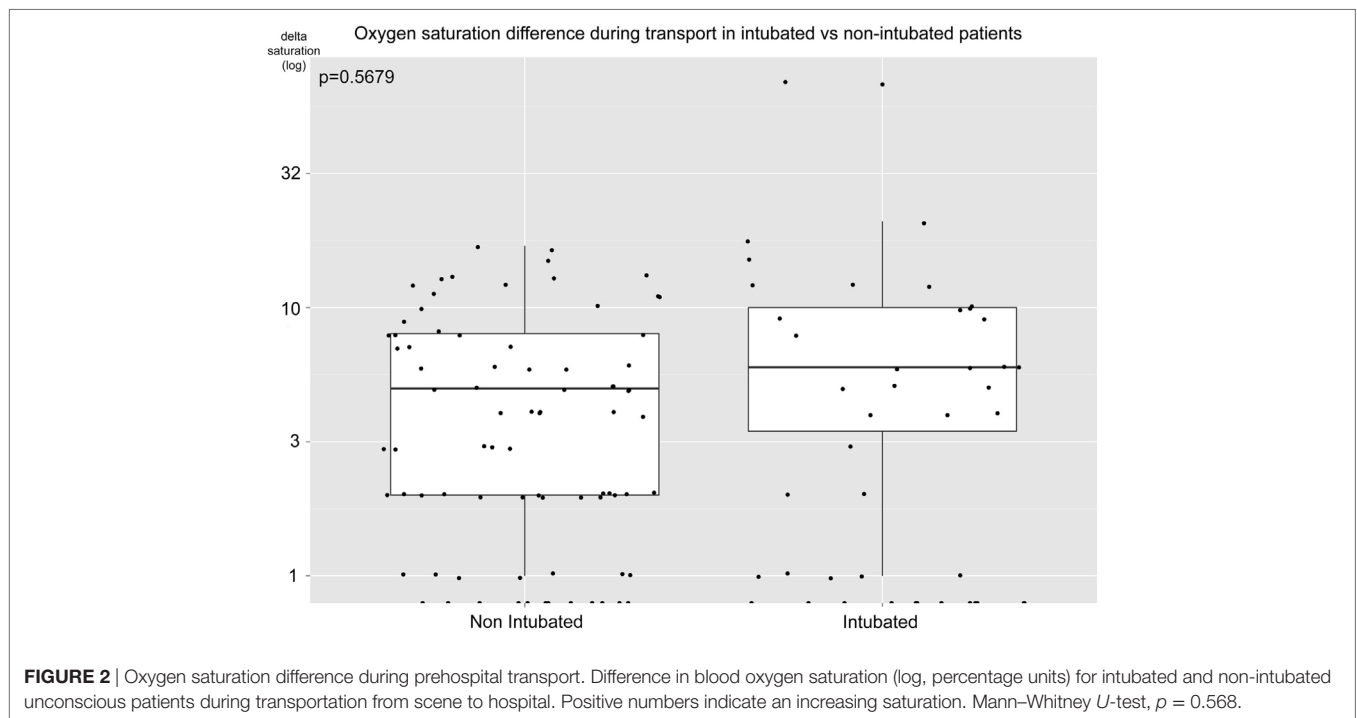
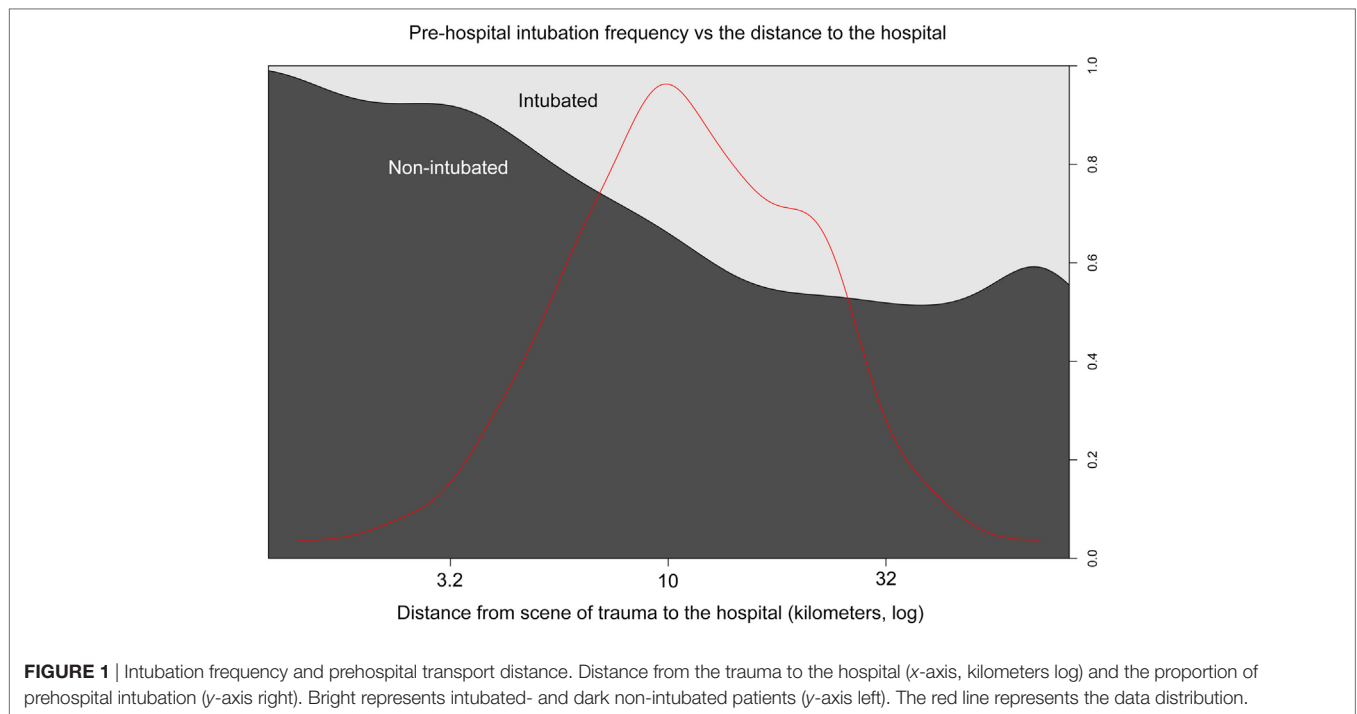
Parameters significant in a proportional odds regression analysis versus outcome (GOS1–5) with an un-imputed dataset, p-value for significance, Nagelkerke's pseudo-R² for the explained variance and correlation coefficient if an increase of the parameters was positively or negatively correlated to an increase in GOS (better outcome). In the multivariate proportional odds model, an imputed dataset was used. Due to co-variance of the parameters, S100B 12–48 h was preferred to admission S100B, and NISS was preferred to AIS and ISS in the model. A step-up model was used to see if prehospital intubation added independent information to the multivariate model. NS, not significant; CT, computerized tomography; AIS, abbreviated injury scale; ISS, injury severity score; NISS, New Injury Severity Score; EMS, Emergency medical services.

TABLE 5 | Reason for prehospital endotracheal intubation.

Reason for endotracheal intubation	n = 66 (% of intubated patients)
1. Decreased level of consciousness	n = 26 (40%)
2. Hypoxemia	n = 2 (3%)
3. Ineffective ventilation	n = 12 (18%)
4. Existing airway obstruction	n = 8 (12%)
5. Impending airway obstruction	n = 9 (14%)
6. Combative or uncooperative	n = 5 (8%)
7. Relief or pain or distress	n = 1 (2%)
8. Cardiopulmonary arrest	n = 3 (5%)

Primary reason for endotracheal intubation, as stated in prehospital trauma charts.

an unfavorable outcome could mean that the correct patients were intubated. This could be seen as the medical professionals making the right decision and treating the patients appropriately



such that the expected effect of hypoxia (negative) is ameliorated. Further, the discrepancy between factors correlated with intubation and outcome, as well as EMS primarily not intubating because of hypoxia, could explain why this study, and the trauma literature, have failed to show a robust association between PHETI and outcome.

Parameters Easily Assessable on the Scene Were Associated With Intubation

As suggested by the implemented guidelines (7), low level of consciousness and long distance to the hospital were factors associated with an increased rate of prehospital intubation, together with prehospital hypotension, pupil unresponsiveness,

high energy trauma, and if a helicopter was used for transport. Thus, the EMS' decision to intubate appear guided by factors involved in the field triage criteria for trauma steering (50). In the prehospital airway management literature, different guidelines apply but to intubate unconscious patients is a general rule (51). Naturally, the guidelines applied in different studies determine which parameters that would be most frequently associated with prehospital intubation. Unfortunately, many studies fail to adequately describe these and may define it as "Standard guidelines for the triage of trauma victims are used" (52). Directly analyzing which parameters that are associated with prehospital intubation has never been performed in a similar fashion in a TBI cohort. Previously, unconsciousness, respiratory insufficiency, and cardiac arrest have been described as predictors of on scene intubation in a mixed prehospital patient cohort (53), thus similar, but not identical, to our TBI cohort. Analogously to our findings, groups have seen that air transportation results in an increasing frequency of intubation (52). In our region, helicopters are often used for long distance transports from rural areas, where predominantly high-energy, motor vehicle accidents occur. We saw a marked increase in intubation frequency using helicopter transportation. In theory, the EMS in the helicopter should not be more prone to prehospital intubation than any other EMS. After thorough investigation of these cases, we believe that the addition of another EMS individual at the scene assisting in the procedure is the reason why intubation was more frequently performed in the helicopter sub-group, and not due to more severe injuries. The association between intubation and longer on-scene time is presumably not related to the severity of injury, but the extra time on-scene necessary to perform the intubation. It could also be an effect of the high frequency of helicopter use in the intubated cohort as the helicopter was often recruited after the first EMS crew had arrived on scene, thereby delaying arrival. In aggregate, it seems like the EMS intubated according to the implemented guidelines and based on parameters easily accessible on the scene.

Surrogate Markers of Brain Injury Severity Were Associated With Outcome

Chesnut and co-workers highlighted the importance of prehospital hypoxia (and hypotension) and its role as an unfavorable outcome predictor using the Traumatic Coma Databank (2), something that has also been shown by the IMPACT study group (3) as well as other groups (54). This has resulted in airway management being a cornerstone in prehospital care of unconscious TBI patients, so as to ensure sufficient oxygen delivery to the injured brain (55, 56).

At the scene, as seen in this study, it is extremely difficult for the EMS to assess the extent and severity of the intracranial lesion and determine which patients have the most extensive, brain injury and thus who would probably be most suited for sedation and endotracheal intubation in order to prevent secondary injury development. Unexpectedly, prehospital hypotension and hypoxia were not independently associated with unfavorable outcome in our study, even if this could indicate that these conditions are properly managed. Moreover, many of the historical cohorts

[used by Chesnut and IMPACT (2, 48)] did not report on the time from trauma to EMS arrival, but as these are cohorts from the 1970s to the 1990s, it is presumably longer than what was seen in our study (median 11 min). As the severity of these secondary insult depends on the time that the patient is exposed by them (8, 57), they would influence outcome to a lower extent in our cohort compared to many others.

The use of field triage criteria probably explain why the Stockholm CT score (40) and 12–48 h peak concentration of the brain enriched protein ("biomarker" of tissue fate) S100B (38), the two parameters that most strongly correlated to long-term functional outcome in the study, were not correlated to prehospital intubation. Other parameters correlated to an unfavorable outcome in the unconscious cohort were high age, pupil unresponsiveness [both strong, independent IMPACT predictors of poor outcome in TBI (48)], and increased NISS. These parameters presented similar pseudo- R^2 for outcome prediction as in the IMPACT cohort (48). Age is an important aspect in this study, as increasing age was a predictor for unfavorable outcome, while a *decreasing* age was correlated to prehospital intubation (albeit not independently, presumably because of high co-variance between younger patients and the parameter high-energy trauma). Thus, it is seemingly not the patients who have the highest risk for an unfavorable outcome related to the TBI injury that are intubated on scene, although they could be expected to benefit most from an improved airway management during transport. Why NISS was superior to ISS in outcome prediction may be due to the fact that ISS is more influenced by extracranial trauma than NISS (58). As previously have been pointed out by studies investigating prehospital intubation and its effect on outcome is the fact that intubation is performed on patients with more severe injuries (28). This is something that can be seen in our study as well, as if the whole cohort of conscious patients were taken into account (Table S3 in Supplementary Material), prehospital intubation came out as a negative outcome predictor in the univariate analysis. Altogether, surrogate markers of brain injury severity were strongly associated with outcome in our study, creating a discrepancy to parameters associated with prehospital intubation.

Intubation Frequency Increased with Distance, but Unfavorable Outcome Did Not

The EMS in Stockholm showed an intubation rate of 41% in unconscious patients and about 50% was intubated if the transport exceeded 10 km (**Figure 1**). While some studies recommend endotracheal intubation for longer transports (7, 59), there is no strong evidence suggesting that it improves outcome or ensures oxygen delivery. However, a longer travel distance for EMS personnel has been shown to be unfavorable for outcome in rural settings (60). The authors of that study noted that the mean distance to the scene for patients that died was 9.33 miles (15 km) compared to 7.71 miles (12.5 km) for patients that survived, thus similar distances as in our study (including similar transportation times) (60). Importantly, this study does not mention the

use of helicopters, which could explain the discrepancy seen in our cohort.

Grosman et al. has shown that if the response time is longer than 30 min, there is an increase in unfavorable outcome in trauma cohorts (61). Even though many transfers were from peripheral islands in the Stockholm archipelago (median distance between scene of accident to the nearest hospital was 11.8 km) in our cohort, the median response time was as short as 11 min. Almost all of our patients had a time duration from alarm to arrival at the hospital underneath 1 h, thus falling within the “golden hour,” a cornerstone of many trauma systems when the risk of unfavorable outcome increases (22). This could be why we did not detect any association between transportation times and outcome. Further, recent findings suggest that this time-frame may not be as important as it once was for outcome in TBI patients (24, 62), presumably as some treatment can be provided in the prehospital setting. It is a difficult compromise to decide if either stay on scene and optimize the patient versus to quickly load the patient for transportation (“scoop and run”). The EMS for the intubated cohort spend in average 12 min additional on scene as compared to non-intubated patients (14 versus 26 min); however, this was not associated with any unfavorable outcome. This is supported by a recent meta analyses showing that an extended on-scene time is not associated with an increased risk for unfavorable outcome in trauma patients (27). In summary, we could not show that increased transportation time and distance were associated with increased risk of unfavorable outcome, which could be explained by rapid transports and adequate prehospital treatment in our cohort.

Hypoxia Was Not a Key Reason for PHETI

In contrast to other studies in the field, we had unique data as to why the EMS performed prehospital intubation. This revealed that “decreased level of consciousness” (40%), “ineffective ventilation” (18%), and “impending airway obstruction” (14%) were the most common causes and, only in 3%, was the reason purely hypoxia (Table 5). While there were patients with hypoxia at the scene (18% in the unconscious cohort), this indicates that other priorities were taken instead of the hypoxic threshold of a saturation of 90%. Because of our set-up to compare endotracheal intubation with everything else, a situation arises where supraglottic devices such as oropharyngeal, nasopharyngeal, and laryngeal mask or even bag valve masks could have been used to improve oxygenation (which would presumably be escalated to endotracheal intubation, but only if necessary) versus a cohort that had endotracheal intubation. There is evidence indicating that these methods of non-intubated advanced airway managements are equally good as endotracheal intubation when looking at survival (63, 64) is a safer way to secure the airway (65), and even shows improved outcome in non-trauma cohorts (66). Presumably, as oxygen saturation is such a common treatment goal for EMS at the scene as soon as the pulse oximetry device has been deployed, it cannot be adequately used as an outcome predictor any more. In economics, this is referred to as the Goodhart’s Law (“when a measure becomes a target, it ceases to be a good measure”) (67). This is a similar route as the intracranial pressure (ICP) metric has taken in TBI studies, as with modern therapy intensities, ICP is

such a targeted metric that only mortality can be discriminated in observational studies, for patients with refractory high ICP levels (68). Luckily, there were no patients with refractory low levels of prehospital oxygen saturation following EMS arrival on scene. Moreover, as can be seen in Figure 2, there were no differences in oxygen saturation between intubated and non-intubated patients during transport from scene to the hospital. To our knowledge, delta saturation during transport has not been previously reported in this fashion. Figure 2 clearly shows that while there were two outliers in the intubated group with large improvements in saturation, the average patients improved equally well during transport independent of airway management. This is in line with the theme of this study, where the EMS seems to escalate airway management if necessary to ensure oxygen delivery. A meta-analysis from 2015 revealed that clinical experience of the EMS is a significant predictor of survival in prehospital intubated TBI patients (69). Similar findings have been reported with physicians having a greater chance of a successful prehospital intubation as compared to nurses (70), as well as less prehospital hypoxia during transport (71). However, in our study, we could not find any differences in outcome in patients intubated by nurses as compared to prehospital physicians, which could be a positive result of the training provided to the EMS following the implementation of the Scandinavian guidelines. However, as the incidence of unsuccessful intubation is low, comparison is difficult in our study.

In summary, hypoxia alone was an uncommon reason for PHETI, presumably due to a general escalated airway management difficult to assess in a retrospective setting. Our findings support that the EMS should only spend time on endotracheal intubation on scene if the patient desaturates despite other types of non-invasive airway management techniques.

The Complexity of Analyzing Efficacy of Prehospital Interventions in a Retrospective Cohort

A great number of studies have analyzed the association between outcome and prehospital intubation in retrospective trauma cohorts, where some have shown improvement (52, 72, 73), and others deterioration and an unfavorable outcome (74–76), for the intubated cohort. We believe, as we have shown in this study that it is difficult to determine the benefit of prehospital intubation as the EMS will assess every patient individually and determine, using clinical experience, and “hidden” skills difficult to detect using these types of studies. Moreover, prehospital intubation is likely to be performed more on patients assessed to be sicker, possibly with more severe pre-morbidities. This integrated qualified and on the fly assessment is hard to quantify and may introduce a treatment bias. While we could not detect any general improvement of prehospital intubation for unconscious patients, for the individual patient, prehospital intubation may very well be an escalated therapy that is beneficial, and/or even life-saving.

A main finding and conclusion of this study is that, due to multiple confounders and possible interactions in the logistically complex prehospital situation, the merits or dangers of prehospital intubation are difficult to adequately assess in a retrospective study. As has been previously mentioned by other

groups, well-designed prospective study protocols are warranted to answer this question (77, 78), but even then it will be difficult in the heterogeneous injury as TBI. In aggregate, this study suggests that decisions to intubate or not at the scene are based on judgments that are multi-factorial and hard to quantify for analysis, but are generally correct in the study region.

Limitations

There are limitations to this study. First, the retrospective method is in itself a limitation and, in this case, a retrospective registry-based study on a single, relatively low volume, trauma center. Still, as we captured data over several years, we believe we have achieved a good sample size, which reflects the full population of patients at our trauma center and could be extrapolated to similar regions in Europe and North America. Second, some data were missing from our datasets, which we, according to standards within the field, imputed. While we retain the uncertainty of the non-imputed dataset, conclusions from heavily imputed data (such as energy level of the trauma) should be drawn with caution. Third, we were not able to control for pre-existing medical conditions or comorbidities, which of course might have influence on the results. However, as the cohort has a relatively low median age, particularly in the intubated group, fewer comorbidities are expected. Fourth, while we included different injury scores including NISS, ISS, and AIS, we did not look specifically at subcomponents of these as to highlight if thoracic injuries would be more associated with intubation. We do plan to better stratify these injuries and the importance of them in upcoming studies of our TBI population. Finally, as the trauma database we recruited our patients from is in itself a selected group of TBI patients (consisting primarily of severe and moderate TBI patients), and therefore, findings might not apply to all TBI patients in other regions with different EMS and trauma systems. Yet, this is the full population of the most severely injured TBI patients, a cohort that we think is most important to study, when analyzing prehospital airway management.

Despite these caveats, to our knowledge, this is one of the first studies to incorporate several aspects of the pre-injury management into assessment of endotracheal intubation, such as reason for intubation, saturation differences during transport, intubation frequency over distance, and intubation's potential effect on long-term functional outcome, in both uni- and multivariable models, in a TBI cohort.

CONCLUSION

Parameters associated with prehospital intubation and long-term outcome showed discrepancy in our study. This may indicate that the decisions to intubate or not at the scene, based on judgments that are multi-factorial and hard to quantify for analysis, are

clinically appropriate in the study region. Difficulties with retrospective studies in an area with complicated logistics and hard to document clinical evaluations in the field become evident and can question the validity of findings. With this taken into consideration, our results support that the EMS should only spend time on PHETI if the patient desaturates despite other types of non-invasive airway management techniques. Large multi-center prospective studies with structured protocols in this area will be affected by logistic and ethical considerations.

ETHICS STATEMENT

The study received ethical approval from the Regional Ethical Review Board in Stockholm reference numbers 2007/1113-31, 2010/1979-32, 2013/1718-32, 2014/691-32, and 2015/1675-31/1.

AUTHOR CONTRIBUTIONS

RW planned the study, collected data, and drafted the manuscript. B-MB planned the study and aided in drafting of the manuscript. DN planned the study, aided with statistical analyses, and drafting of the manuscript. MS planned the study and aided in the drafting of the manuscript. AH planned the study and aided in the drafting of the manuscript. ET planned the study, collected data, performed the statistical analyses, and drafted the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <https://www.frontiersin.org/articles/10.3389/fneur.2018.00194/full#supplementary-material>.

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Neuromodulatory Effect of Thymoquinone in Attenuating Glutamate-Mediated Neurotoxicity Targeting the Amyloidogenic and Apoptotic Pathways

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Overexposure of the glutamatergic N-methyl-D-aspartate (NMDA) receptor to the excitatory neurotransmitter L-glutamic acid leads to neuronal cell death by excitotoxicity as a result of increased intracellular Ca^{2+} , mitochondrial dysfunction, and apoptosis. Moreover, it was previously reported that prolonged activation of the NMDA receptor increased beta-amyloid ($\text{A}\beta$) levels in the brain. Thymoquinone (TQ), the active constituent of *Nigella sativa* seeds, has been shown to have potent antioxidant and antiapoptotic effects. The aim of the present study was to explore the neuromodulatory effects of different doses of TQ (2.5 and 10 mg/kg) against apoptotic cell death and $\text{A}\beta$ formation resulting from glutamate administration in rats using vitamin E as a positive control. Behavioral changes were assessed using Y-maze and Morris water maze tests for evaluating spatial memory and cognitive functions. Caspase-3, Lactate dehydrogenase, $\text{A}\beta$ -42, and cytochrome c gene expression were determined. TQ-treated groups showed significant decreases in the levels of all tested biochemical and behavioral parameters compared with the glutamate-treated group. These findings demonstrated that TQ has a promising neuroprotective activity against glutamate-induced neurotoxicity and this effect is mediated through its anti-amyloidogenic, antioxidant, and antiapoptotic activities.

Keywords: amyloid-beta, caspase-3, cytochrome c, excitotoxicity, glutamate, thymoquinone

INTRODUCTION

Glutamate (Glu) is the primary excitatory neurotransmitter in the brain which contributes to many physiological processes, including learning and memory (1). Pathological overstimulation of glutamatergic receptors produces an excessive influx of Ca^{2+} and Na^{+} , leading to glutamate-induced neuronal apoptosis and eliciting a marked excitotoxicity (2). Over-activation of the glutamatergic N-methyl-D-aspartate (NMDA) receptor in the brain provokes excitotoxic neuronal death which plays a crucial role in many pathological conditions, including ischemic stroke, traumatic brain injury, Alzheimer's disease (AD), Parkinson's disease (PD), and epilepsy (3). Overactivation of NMDA receptors by Glu is associated with apoptotic cell death *via* release of cytochrome c (Cyto-c) and induction of the intrinsic apoptotic pathway (4, 5). Cyto-c initiates the caspase-dependent

apoptotic pathway, which causes proteolytic processing of pro-Caspase-9 and subsequently activation of the downstream effectors caspase-3 (Casp-3), -6, and -7 (6). Casp-3 causes degradation of nuclear DNA (7) and formation of peroxynitrite radicals (ONOO^-), provoking depletion of cellular energy (8). Moreover, NMDA overactivation was found to increase the production of amyloid-beta ($\text{A}\beta$) protein *via* different potential mechanisms: (1) Ca^{2+} -dependent shift from non-amyloidogenic to amyloidogenic processing of the amyloid precursor protein (APP) (9–11) and impaired clearance of the $\text{A}\beta$ protein from brain and blood (12, 13) and (2) alteration of proteolysis of APP by Casp-3 leading to an increase in $\text{A}\beta$ -42 peptide levels (14, 15).

Owing to its antioxidant property, thymoquinone (TQ) was proven to protect different organs against pathological conditions caused by oxidative damage. TQ was also reported as a potent neuroprotective agent against neurodegeneration induced by forebrain ischemia by attenuating oxidative stress (16). Its antioxidant activity may be attributable to the effect of its reduced form tert-butylhydroquinone, which acts as a hydrogen donating antioxidant that inhibits lipid peroxidation, or to the scavenging effect of multiple reactive oxygen species by TQ and thymohydroquinone, mimicking superoxide dismutase activity (17). Moreover, TQ can restore the abnormal matrix metalloproteinase and hence decrease reactive oxygen species levels (18). *In vitro*, TQ had been found to counteract the oxidative stress and membrane potential collapse induced by $\text{A}\beta$ -42 aggregation (19). TQ was found to protect against hepatic apoptosis induced by ischemia reperfusion injury (20, 21). It was also proven to be an effective antineoplastic agent by triggering cancer apoptosis and autophagic cell death (22), as well as suppressing tumor angiogenesis (23). TQ also exerted an anti-inflammatory effect against rheumatoid arthritis (24) and bronchial asthma (25). Moreover, TQ demonstrated potential anticonvulsant activity against pettimal epilepsy (26).

Vitamin E (Vit E) is one of the most powerful natural antioxidants and is essential for protecting the body from free radical damage (27). It is considered to be a potent protective agent against atherosclerosis, AD, and cancer (28). Moreover, Vit E was found to protect against apoptotic cell death induced by cinnamaldehyde in PLC/PRF/5 cells (29), ultra-violet B radiations in chicken embryonic fibroblasts (30), and haloperidol in hippocampal cell lines (31).

The aim of the present study was to explore the neuromodulatory effect of different doses of TQ 2.5 and 10 mg/kg against glutamate-induced neuronal damage, by assessing the behavioral changes (spatial memory and cognitive function) and biochemical parameters [Cyto-c, Casp-3, lactate dehydrogenase (LDH), and $\text{A}\beta$ levels] in an animal model using Vit E as a positive control.

MATERIALS AND METHODS

Material

Experimental Animals

Adult male albino rats (weight: 250–300 g; age: ~2 months) were used. The animals were obtained from the animal colony

of the National Institute of Research (Cairo, Egypt). The rats were housed in a temperature-controlled room (23–24°C) with a 12-h light:dark cycle and with free access to food and water. They were allowed to acclimatize to the animal house of the German University in Cairo for at least 1 week before initiating the experiments. Animal procedures were performed following the approval of the Ethics Committee of the German University in Cairo in association with the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985). All efforts were made to minimize animal discomfort and suffering.

Drugs and Chemicals

L-Glutamic acid monosodium hydrate, TQ, and Vit E were purchased from Sigma-Aldrich Co., USA. Saline 0.9% (NaCl), 70% ethanol, and olive oil were purchased from Adwic (El-Nasr Pharmaceutical Chemicals Co., Egypt), and phosphate buffered saline was purchased from Lonza Ltd., Switzerland.

Experimental Design

Two control groups were analyzed in the present study. The first group received 1 ml of 0.9% saline i.p. daily for 14 consecutive days and the second group received 1 ml of olive oil i.p. daily for 14 consecutive days. No statistically significant differences were noted in the results among the two control groups, thus, they were pooled into a single control group referred to as the Negative control group in the experimental design and the description of results.

Seventy-two rats weighting were used in the present study and were allocated to five groups, of 12 rats each.

Group I was the Negative control group, while rats in Group II were injected with Glu (2 g/kg, i.p.) once daily for seven consecutive days (1). Group III was referred to as the VitE/Glu Group in which rats were injected with Vit E (50 mg/kg, i.p.) once daily for seven consecutive days followed by glutamate (2 g/kg, i.p.) for an additional seven consecutive days (32). In Group IV; the TQ2.5/Glu Group, rats received TQ (2.5 mg/kg, i.p.) once daily for seven consecutive days, followed by glutamate (2 g/kg i.p.) for an additional seven consecutive days (33). The same protocol was applied in Group 5 (the TQ10/Glu Group) although the dose of TQ was increased to 10 mg/kg (33).

Methods

Neurobehavioral Tests

On the last day of drug treatment, animals were trained for the Y-maze and day 1 of the Morris water maze (MWM). The following day, animals were tested for the Y-maze test and day 2 of MWM. Then, the third and fourth trials of MWM as well as the probe test were conducted on three successive days. Every apparatus was thoroughly washed with 70% ethanol between each use and after every animal in both the training and testing sessions (34).

Y-Maze

The Y-maze is used to measure spatial working memory in rodents *via* the spontaneous alternation behavior (SAB) calculation (35).

TABLE 1 | Effects of Glu, Vit E/Glu, TQ 2.5/Glu, and TQ 10/Glu on the spatial memory in the Y-maze test.

Groups	–ve control	Glu	Vit E/Glu	TQ 2.5/Glu	TQ 10/Glu
SAB%	75.92 ± 8.200	36.53 ± 7.609*	78.85 ± 6.99 [®]	76.50 ± 4.15 [®]	77.60 ± 4.885 [®]

Effect of Glu, Vit E/Glu, TQ2.5/Glu, and TQ10/Glu on spatial memory. Glu was injected i.p. as a single dose for 7 days. TQ and Vit E were injected as a single dose for 7 days.

Animals were subjected to the Y-maze test and the SAB% for each rat was recorded for 8 min and the mean SAB% for all rats in each group was calculated. Statistical analysis of mean SAB% were carried out by one-way ANOVA followed by Tukey's multiple comparison tests, by graph pad prism 5. Each value represent the mean value of 12 rats ± SEM.

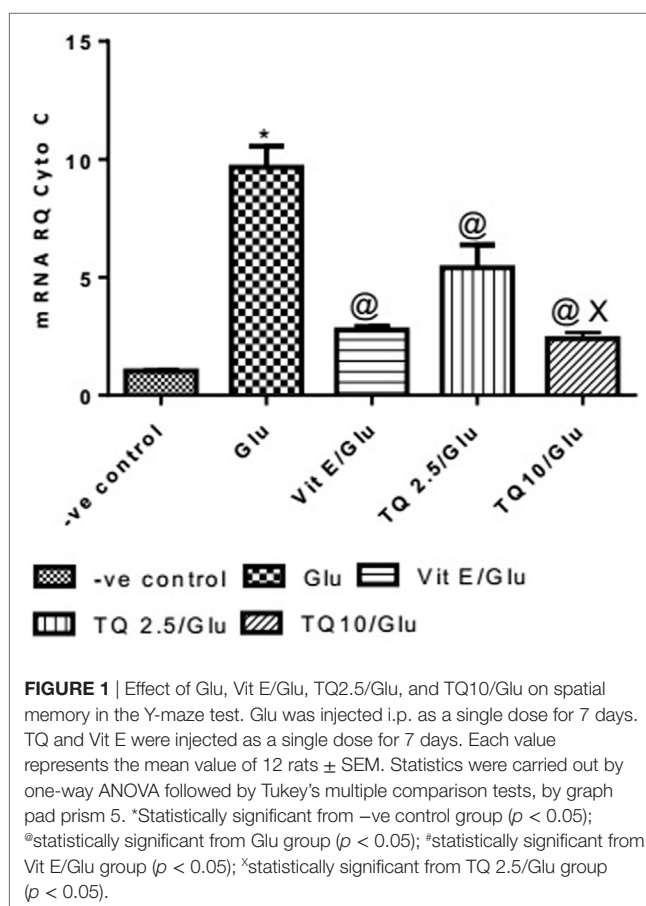
*Statistically significant from –ve control group ($p < 0.05$).

[®]Statistically significant from Glu group ($p < 0.05$).

Spontaneous alternation measures the ability of the animal to alternate its choice of arm entry on subsequent trials based on its memory of previous arm entries performed (35), which depends on the natural exploratory behavior of rats for new environments (36). The maze is a Y-shaped apparatus consisting of three arms, each with the same dimensions, 35-cm long, 25-cm high, and 10-cm wide, and each extending from a central platform, with the angle between each arm equal 120°. The apparatus was placed on the floor of the experimental room. The test was performed for 2 days; the first day was for the purpose of training, during which each rat was positioned in the central platform and allowed to explore the maze freely for 8 min. The same procedure was followed on the second testing day, with the addition of manual recording of each arm entry, scored only when all four limbs of the rat were inside the arm. After each session the maze was cleaned with 70% ethanol to exclude any olfactory cues that might interfere with subsequent testing. An alternation was considered to have occurred if three successive different arms were entered during an overlapping triplet set. The percentage of spontaneous alternation activity (SAB%) was calculated as the “number of consecutive alternations” divided by “the total number of arms entries minus 2” and multiplied by 100 (37).

Morris Water Maze

The MWM is one of the most frequently applied *in vivo* neurobehavioral tests in neuroscience (38). Its advantage over other neurobehavioral tests is its ability to assess and differentiate deficits in memory formation from other types of deficits unrelated to memory including sensory, motor, motivational, and retrieval processes (39). The test is swimming based model employing distal clues, in which the animal swims from a starting location and navigates to a hidden submerged escape platform. The swimming environment is a large, circular stainless steel pool (150 cm in diameter, 60 cm in height, maintained at a temperature of $25 \pm 1^\circ\text{C}$) half filled with water and divided into four quadrant using two threads positioned perpendicular to each other on the edges of the pool. The platform is positioned 2 cm below the water level in the target quadrant. The platform position remained unchanged throughout the training session, and was rendered invisible by coloring the water using a non-toxic dye. Visual clues, such as colored shapes and stickers, were placed around the pool in plain sight of the animal. The position of the experimenter was constant so as not to disturb the relative location of the water maze with respect to other objects in the laboratory, which serve as prominent visual cues. For four consecutive days, each rat was subjected to two trials where, for



each trial, the rat was allowed to search for the fixed platform for 120 s. The gap between the two trials was 10–15 min. On the fifth day, the platform was removed, and each rat was subjected to a probe test during which it was required to swim for 60 s. The time spent by the animal within the probe (target) quadrant was recorded (the quadrant that held the platform during the training days). Time spent in the target quadrant is considered to be an index of memory retrieval (40–42).

Biochemical Assessments

Tissue Sampling

The rats were sacrificed by cervical dislocation and decapitation, after which, brains were divided into two equal hemispheres in an ice: salt mixture. Each hemisphere was homogenized with the appropriate buffer, according to the assay kits described below.

TABLE 2 | Effect of Glu, Vit E/Glu, TQ 2.5/Glu, and TQ 10/Glu on the mean escape latency (MEL) in Morris water maze (MWM) test.

Days	–ve control	Glu	Vit E/Glu	TQ 2.5/Glu	TQ 10/Glu
1st Day MEL (s)	24.46 ± 4.377	62.666670 ± 14.92	44.42 ± 18.88	61.75 ± 10.20	32.58 ± 5.943
2nd Day MEL (s)	23.54 ± 2.208	63.833330 ± 16.55	16.15 ± 3.975	17.08 ± 2.336	23.42 ± 10.03
3rd Day MEL (s)	19.96 ± 2.792	47.683330 ± 9.323	17.80 ± 4.245	12.92 ± 1.67	19.5 ± 5.583
4th Day MEL (s)	11.46 ± 2.208	24.083340 ± 5.352*	7.80 ± 1.913®	14.58 ± 3.197®	12.92 ± 3.446®

Effect of Glu, Vit E/Glu, TQ2.5/Glu, and TQ10/Glu on the fourth day MEL in MWM. Glu was injected i.p. as a single dose for 7 days. TQ and Vit E were injected as a single dose for 7 days. Animals in each group were subjected to MWM test. Animals were subjected to eight training trials, two trials sessions each day for four consecutive days with an inter-trial interval of 5–15 min and the MEL of each animal was recorded for 2 min in each trial. The average MEL of each animal was recorded for the four consecutive days and statistical analysis of the mean MEL of animals in each group were carried out by one-way ANOVA followed by Tukey's multiple comparison tests, by graph pad prism 5. Each value represent the mean value of 12 rats ± SEM. Significant difference between the groups in MEL on the fourth day was recorded.

*Statistically significant from –ve control group ($p < 0.05$).

®Statistically significant from Glu group ($p < 0.05$).

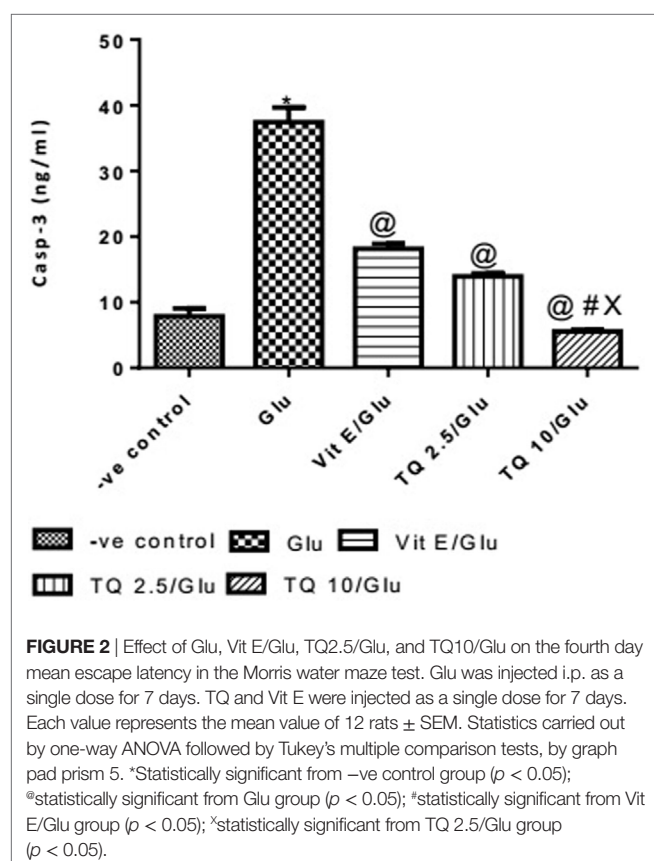


FIGURE 2 | Effect of Glu, Vit E/Glu, TQ2.5/Glu, and TQ10/Glu on the fourth day mean escape latency in the Morris water maze test. Glu was injected i.p. as a single dose for 7 days. TQ and Vit E were injected as a single dose for 7 days. Each value represents the mean value of 12 rats ± SEM. Statistics carried out by one-way ANOVA followed by Tukey's multiple comparison tests, by graph pad prism 5. *Statistically significant from –ve control group ($p < 0.05$); ®statistically significant from Glu group ($p < 0.05$); *statistically significant from Vit E/Glu group ($p < 0.05$); *statistically significant from TQ 2.5/Glu group ($p < 0.05$).

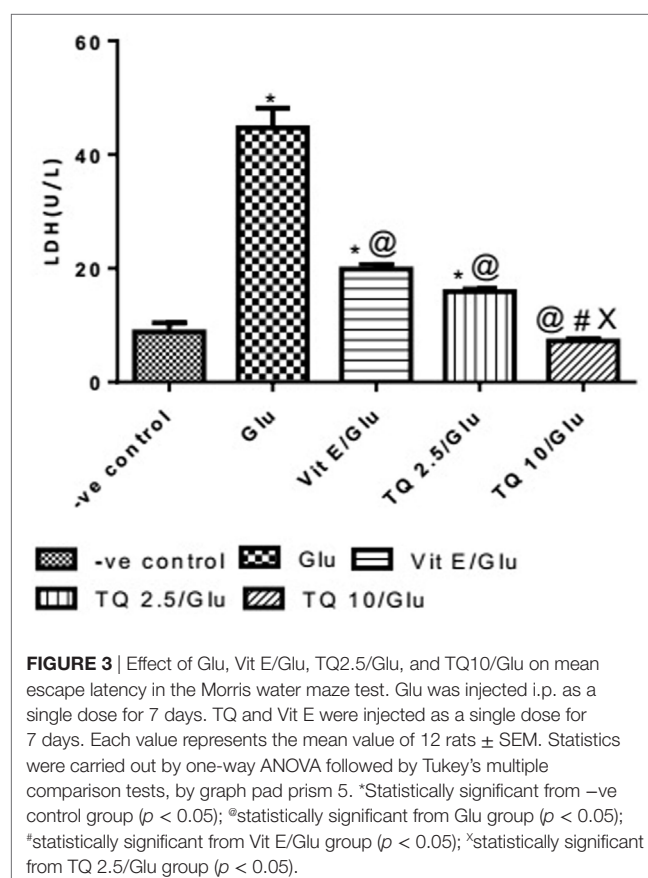


FIGURE 3 | Effect of Glu, Vit E/Glu, TQ2.5/Glu, and TQ10/Glu on mean escape latency in the Morris water maze test. Glu was injected i.p. as a single dose for 7 days. TQ and Vit E were injected as a single dose for 7 days. Each value represents the mean value of 12 rats ± SEM. Statistics were carried out by one-way ANOVA followed by Tukey's multiple comparison tests, by graph pad prism 5. *Statistically significant from –ve control group ($p < 0.05$); ®statistically significant from Glu group ($p < 0.05$); *statistically significant from Vit E/Glu group ($p < 0.05$); *statistically significant from TQ 2.5/Glu group ($p < 0.05$).

Detection of Cyto-c Gene Expression in Brain Tissue

Briefly, total RNA was extracted using the Qiagen tissue extraction kit (Qiagen, USA) from the rat brain tissue samples. RNA concentration was obtained by spectrophotometry (Dual wavelength spectrophotometer, Beckman, USA) and was reverse transcribed to cDNA with a high capacity cDNA reverse transcription kit (Fermentas, USA). The qPCR assay was carried out in which amplification and analysis of the converted cDNA were processed employing an Applied Biosystem with software version 3.1. The qPCR assay was optimized by adjusting the annealing temperature such that the sample, together with the primer sets and SYBR Green I, were able to bind. A typical qPCR run includes roughly 40 cycles. The Ct of a sample is defined

as the number of PCR cycles necessary for the fluorescent signal produced by SYBR Green to cross above a threshold in the linear part of the amplification curve and it is employed in the analysis step. Relative quantification (RQ), using GAPDH gene expression as an endogenous control, was performed for all samples tested. The RQ was calculated using the $2^{-\Delta\Delta Ct}$ method (43).

Determination of Casp-3 Levels in Brain Tissue

Caspase 3 levels in the brain samples were assayed using Casp-3 ELISA kit (Cusabio, China). This assay measures the amount of sample by sandwiching it between two antibodies one of which

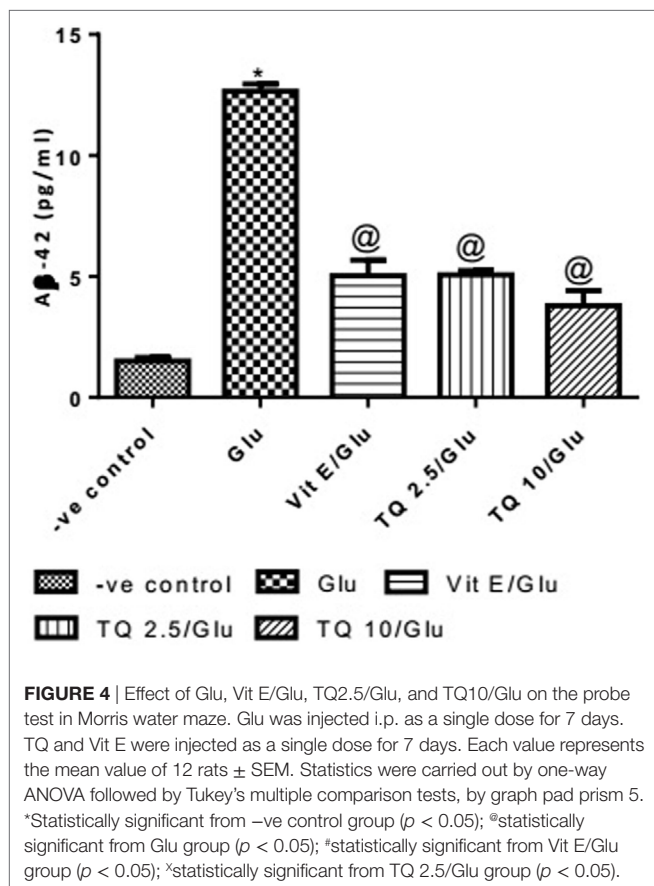
TABLE 3 | Effect of Glu, Vit E/Glu, TQ 2.5/Glu, and TQ 10/Glu on the probe test in Morris water maze (MWM).

Groups	–ve control	Glu	Vit E/Glu	TQ 2.5/Glu	TQ 10/Glu
Mean time spent in the target quadrant (s)	23.75 ± 0.9320	8.500 ± 1.118*	18.75 ± 1.726®	18.25 ± 1.078®	18.5 ± 1.4232®

Glu was injected i.p. as a single dose for 7 days. TQ and Vit E were injected as a single dose for 7 days. Animals in each group were subjected to MWM test. Animals were subjected to probe test and time spent in the target quadrant of each rat was recorded for 60-s period on the fifth day of MWM test. The mean time spent in the target quadrant of all rats in each group was calculated and expressed in seconds. Statistical analysis of mean time spent in target quadrant were carried out by one-way ANOVA followed by Tukey's multiple comparison tests, by graph pad prism 5.

*Statistically significant from –ve control group ($p < 0.05$).

®Statistically significant from Glu group ($p < 0.05$).



is pre-coated to the microtiter plate and the other of which acts as a detector antibody (44). Antibodies specific for Casp-3 were pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Casp-3 present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for Casp-3 is then added to the wells. After washing, avidin conjugated horseradish peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Casp-3 bound in the initial step. The color development is stopped and the intensity of the color is measured. Casp-3 levels in the brain were expressed as nanograms/milliliter and calculated from a constructed standard curve using known concentrations of Casp-3.

Determination of LDH in Brain Tissue

Lactate dehydrogenase levels in the brain samples were assayed using an LDH ELISA kit (Stanbio Lab., USA). The microtiter plate provided in this kit was precoated with an antibody specific to LDH. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for LDH and avidin conjugated to HRP is added to each microplate well and incubated. Then, a TMB (3,3',5,5' tetramethyl-benzidine) substrate solution is added to each well. Only those wells that contain LDH, biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme–substrate reaction is terminated by the addition of a sulfuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 ± 2 nm. LDH levels in the brain were expressed as units/liter and calculated from a constructed standard curve using known concentrations of LDH.

Determination of Amyloid-Beta in Brain Tissue

Aβ1–42 levels in the brain samples were assayed using an Aβ-42 ELISA kit (MyBiosource, USA). The assay measures the amount of sample by sandwiching it between two antibodies one of which is precoated to the microtiter plate and the other of which acts as a detector antibody (44). The microtiter plate provided in this kit was precoated with an antibody specific to Aβ1–42. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for Aβ1–42 and avidin conjugated to HRP is added to each microplate well and incubated. Then, a TMB substrate solution is added to each well. Only those wells that contain Aβ1–42, biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme–substrate reaction is terminated by addition of a sulfuric acid solution and the color change is measured spectrophotometrically at a wave length of 450 ± 2 nm. Brain Aβ (1–42) level was expressed as picograms/milliliter and calculated from a constructed standard curve using known concentration of Aβ (1–42).

Statistical Analysis

Data were represented as mean ± SE. Results were analyzed by one-way ANOVA followed by Tukey's Multiple Comparison Test, by the aid of Graph pad prism 5 software. p -Value ($p < 0.05$) was considered significant. In the present study *, @, #, and X were utilized to denote statistical difference from different groups according to the following scheme: *: statistically significant from negative control group by p value ($p < 0.05$).

TABLE 4 | Effect of Glu, Vit E/Glu, TQ2.5/Glu, and TQ10/Glu on Cyto-c gene expression.

Groups	–ve control	Chi	Vit E/Glu	TQ 2.5/Glu	TQ 10/Glu
Cyto-c gene expression	1.032 ± 0.014	9.67 ± 0.895*	2.77 ± 0.148 [®]	5.407 ± 0.98 [®]	2.40 ± 0.264 ^{×,®}

Glu was injected i.p. as a single dose for 7 days. TQ and Vit E were injected as a single dose for 7 days. Animals in each group were subjected to the neurobehavioral tests. Once they were sacrificed, their brains were retrieved and homogenized in its suitable buffer and used in RT-PCR assay.

Statistical analysis of mean Cyto-c gene expression (RQ) were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests, by graph pad prism 5. Each value represents the mean value of 12 rats ± SEM.

*Statistically significant from –ve control group ($p < 0.05$).

[®]Statistically significant from Glu group ($p < 0.05$).

[×]Statistically significant from TQ 2.5/Glu group ($p < 0.05$).

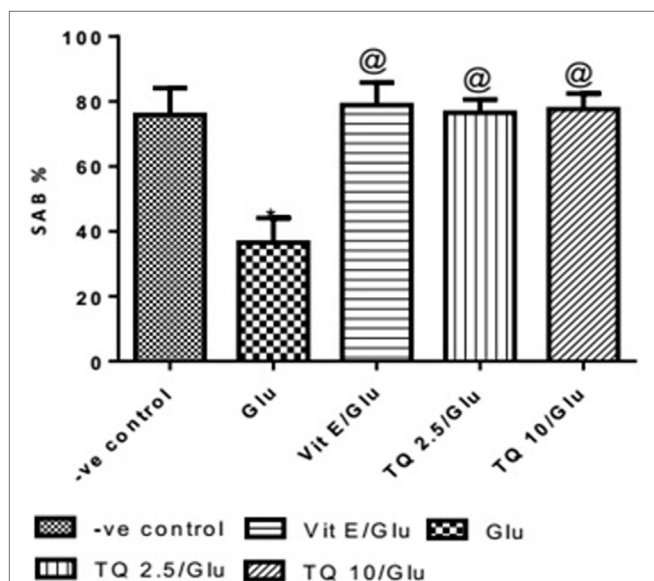


FIGURE 5 | Effect of Glu, Vit E/Glu, TQ2.5/Glu, and TQ10/Glu on Cyto-C gene expression. Glu was injected i.p. as a single dose for 7 days. TQ and Vit E were injected as a single dose for 7 days. Each value represents the mean value of 12 rats ± SEM. Statistics were carried out by one-way ANOVA followed by Tukey's multiple comparison tests, by graph pad prism 5.

*statistically significant from –ve control group ($p < 0.05$); [®]statistically significant from Glu group ($p < 0.05$); #statistically significant from Vit E/Glu group ($p < 0.05$); [×]statistically significant from TQ 2.5/Glu group ($p < 0.05$).

@: statistically significant from Glu group by p value ($p < 0.05$). #: statistically significant from Vit E/Glu group by p value ($p < 0.05$). X: statistically significant from TQ 2.5/Glu group by p value ($p < 0.05$).

Results of TQ10/Glu Group were compared to that of TQ 2.5/Glu, VitE/Glu, and Glu Group. Results of VitE/Glu group were compared to that of Glu group. Results of Glu group were compared to negative control group.

RESULTS

Neurobehavioral Tests

Effect of Different Doses of TQ and Vit E on Spatial Memory in the Y-Maze Test

Glu group showed significant decreased in the SAB% when compared to negative control group by 51.9%. Vit E group showed

significant improvement in SAB% when was compared to Glu group by 116%. TQ (2.5 and 10 mg/kg) showed a significant increase in SAB% when compared to Glu group by 109.5 and 112.6%, respectively (Table 1, Figure 1).

Effect of Different Doses of TQ and Vit E on the Mean Escape Latency (MEL) in the MWM Test

The effect of TQ and Vit E was estimated on the MEL using MWM after four testing days. MEL on the fourth day was recorded for each mouse. Glu showed significant increase in MEL when compared to the negative control group by 110.12%. Vit E caused a significant reduction in MEL when compared to Glu group by 70.5%. TQ 2.5 and 10 mg/kg showed a significant decrease in MEL when compared to Glu group by 39.4 and 46.3%, respectively (Table 2, Figures 2, 3).

Effect of Different Doses of TQ and Vit E on the Probe Test in the MWM Test

The time spent in the target quadrant of the MWM was evaluated by the probe test. Glu group showed a significant decrease in the time spent in the target quadrant when compared to the negative control group by 64%. Vit E group significantly increased the time spent in target quadrant when compared to Glu group by 120.5%. TQ (2.5 and 10 mg/kg) showed a significant increase in the time spent in the target quadrant when compared to Glu group by 114.7 and 117.6%, respectively (Table 3, Figure 4).

Biochemical Parameters

Effect of Different Doses of TQ and Vit E on the Cyto-c Gene Expression in Brain Tissue

Glu group showed a significant increase in the expression of Cyto-c gene, when compared to negative control by 837.01%. Vit E group significantly reduced the expression of Cyto-c gene, when compared to Glu group by 71.35%. TQ (2.5 and 10 mg/kg) produced a significant decrease in the expression of Cyto-c gene when compared to Glu group by 44.08 and 75.18%, respectively. TQ (10 mg/kg) reduced significantly the expression of Cyto-c gene when compared to Vit E group by 14.98%; moreover, it showed a significant decrease when compared to TQ (2.5 mg/kg) by 55.64% (Table 4, Figure 5).

Effect of Different Doses of TQ and Vit E on the Casp-3 Levels in Brain Tissue

The Glu group showed a significant increase in the levels of Casp-3 when compared to negative control group by 378%. Vit

TABLE 5 | Effects of Glu, Vit E/Glu, TQ 2.5/Glu, and TQ 10/Glu on Casp-3 level.

Group	–ve control	Glu	Vit E/Glu	TQ 2.5/Glu	TQ 10/Glu
Concentration of Caspase-3 (ng/ml)	7.833 ± 1.218	37.43 ± 2.260*	18.15 ± 0.7610 [®]	13.95 ± 0.4288 [®]	5.550 ± 0.2742 ^{®,x}

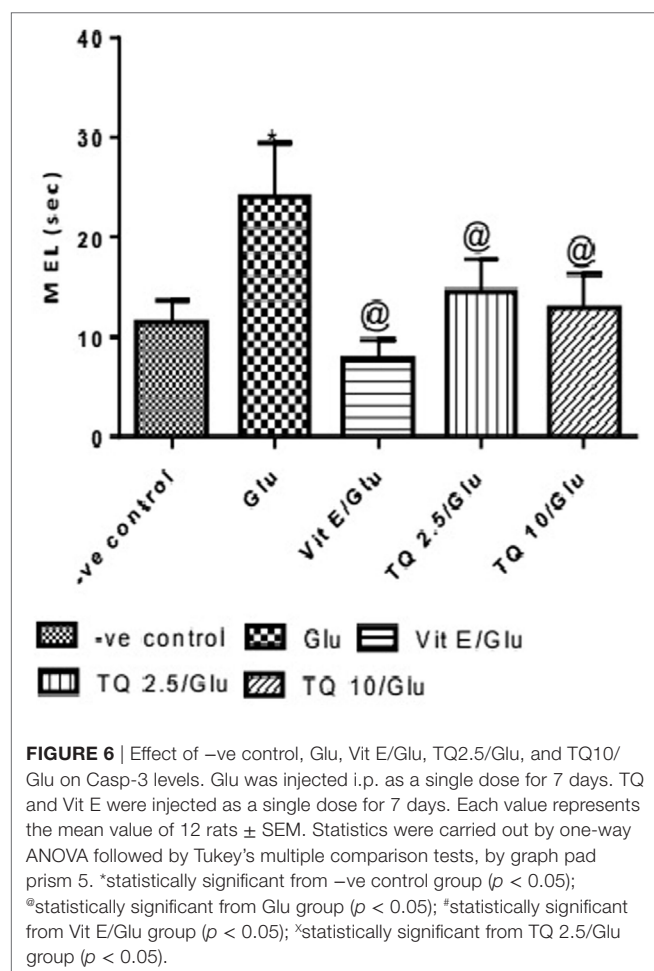
Glu was injected i.p. as a single dose for 7 days. TQ and Vit E were injected as a single dose for 7 days. Animals in each group were subjected to the neurobehavioral tests before being sacrificed. The brains were isolated and homogenized as 10% homogenate in 0.1 M PBS then centrifuged, and supernatants were used in the Casp-3 ELISA kit. Statistical analyses of mean Casp-3 level were performed using ANOVA followed by Tukey's multiple comparison tests, by graph pad prism 5. Each value represent the mean value of 12 rats ± SEM.

*Statistically significant from –ve control group ($p < 0.05$).

[®]Statistically significant from Glu group ($p < 0.05$).

[®]Statistically significant from Vit E/Glu group ($p < 0.05$).

^xStatistically significant from TQ 2.5/Glu group ($p < 0.05$).



E group showed a significant decrease in the level of Casp-3 when compared to Glu group by 51.51%. TQ (2.5 and 10 mg/kg) produced a significant decrease in the level of Casp-3 when compared to Glu Group by 62.74 and 85.17%, respectively. TQ (2.5 mg/kg group) reduced the level of Casp-3, compared to Vit E group by 23.14%. TQ (10 mg/kg) group showed a significant decrease in the level of Casp-3 when compared to Vit E and TQ 2.5/kg groups by 69.44 and 60.22%, respectively (Table 5, Figure 6).

Effect of Different Doses of TQ and Vit E on the LDH Levels in Brain Tissue

Glu group showed significant increase in the level of LDH when was compared to –ve control by 453.04%. Vit E group showed significant decrease in the level of LDH when compared to Glu group by 55.5%. TQ 2.5 and 10 mg/kg groups showed a significant reduction in the level of LDH when compared to Glu group by 64.40 and 83.40%, respectively. TQ 2.5 mg/kg group showed decrease in LDH level when compared to Vit E group by 19.91%. TQ (10 mg/kg) significantly reduced the LDH level than Vit E and TQ 2.5 mg/kg groups by 62.64 and 53.42%, respectively (Table 6, Figure 7).

Effect of Different Doses of TQ and Vit E on the Aβ-42 Levels in Brain Tissue

Glu produced a significant increase in the level of Aβ-42 protein when compared to negative control group by 743.33%. Vit E significantly reduced the Aβ1–42 level when compared to Glu group by 60.21%. TQ (2.5 and 10 mg/kg) showed a significant decrease in the Aβ-42 levels when compared to Glu group by 59.60 and 70.04%, respectively (Table 7, Figure 8).

DISCUSSION

Overactivation of NMDA receptors by Glu induces neuronal apoptosis through excitotoxicity and contributes to many neurological disorders (45). Importantly, overactivation of NMDA receptors by glutamate also leads to an increase in the level of Aβ1–42, by shifting the APP processing toward the amyloidogenic pathway, resulting in subsequent cognitive dysfunction (10, 46).

In the current study, administration of glutamate produced a significant alteration in spatial memory, as measured by the Y-maze test. This coincides with previous studies whereby Glu-excitotoxicity induced by several agents caused alteration in the SAB%, compared to control groups, in the Y-maze test (47–49). Previously, it was shown that bilateral intracerebroventricular administration of Aβ-35 caused impairment in the SAB% and contributed to the neurotoxicity of Aβ through increasing glutamatergic neurotransmission (49). Moreover, it was observed that olfactory bulbectomy in rodents led to deficits in behavioral and cognitive deficits through glutamate-mediated excitotoxicity manifested by impairment in the SAB% (50). Furthermore, NMDA receptor overactivation during ischemia

TABLE 6 | Effect of –ve control, Glu, Vit E/Glu, TQ 2.5/Glu, and TQ 10/Glu on lactate dehydrogenase (LDH) level.

Groups	–ve control	Glu	Vit E/Glu	TQ 2.5/Glu	TQ 10/Glu
Mean level of LDH (U/l)	8.088 ± 1.593	44.73 ± 3.418*	19.89 ± 0.8119 [®]	1S.93 ± 0.4335 [®]	7.423 ± 0.3897 ^{®,#,*}

Glu was injected i.p. as a single dose for 7 days. TQ and Vit E were injected as a single dose for 7 days. Animals were subjected to the neurobehavioral tests before being sacrificed. The brains were isolated and homogenized as 10% homogenate in 0.1 M PBS then centrifuged, and supernatants were used in the LDH ELISA kit. Statistical analyses of mean LDH level were performed using ANOVA followed by Tukey's multiple comparison tests, by graph pad prism 5. Each value represents the mean value of 12 rats ± SEM.

*Statistically significant from –ve control group ($p < 0.05$).

[®]Statistically significant from Glu group ($p < 0.05$).

#Statistically significant from Vit E/Glu group ($p < 0.05$).

*Statistically significant from TQ 2.5/Glu group ($p < 0.05$).

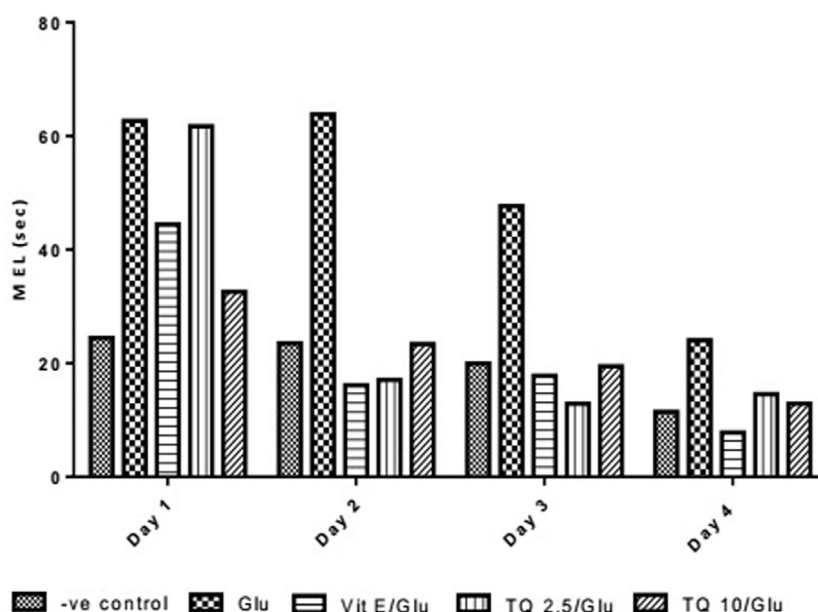


FIGURE 7 | Effect of Glu, Vit E/Glu, TQ2.5/Glu, and TQ10/Glu on lactate dehydrogenase levels. Glu was injected i.p. as a single dose for 7 days. TQ and Vit E were injected as a single dose for 7 days. Each value represents the mean value of 12 rats ± SEM. Statistics were carried out by one-way ANOVA followed by Tukey's multiple comparison tests, by graph pad prism 5. *Statistically significant from –ve control group ($p < 0.05$); [®]statistically significant from Glu group ($p < 0.05$); #statistically significant from Vit E/Glu group ($p < 0.05$); *statistically significant from TQ 2.5/Glu group ($p < 0.05$).

in mice contributed to apoptotic cell death in neuronal cells leading to behavioral impairment, also manifested by a decrease in SAB% in the Y-maze test (51). The MWM is a common test used to assess cognitive and behavioral impairment induced by the administration of monosodium glutamate, which serves as a traumatic brain injury model. Glutamate administration caused spatial reference memory deficits, spatial discrimination deficits and cognitive dysfunction, as shown by an increase in MEL and a decrease in time spent in the target quadrant; these deficits can be attributed to hippocampal cell death induced by excitotoxicity (52–56). Consistent with these findings, the current study confirmed that Glu administration for seven consecutive days in the Glu group caused impairment in the spatial memory function which was evidenced by a decrease in MEL and also in the time spent in target quadrant. These findings could be attributed to the increase in the apoptotic cell death markers (Cyto-c, Casp-3, and LDH) observed in the present study. This coincides with previous

studies showing that either acute or repeated administration of Glu induced the intrinsic pathway of apoptotic cell death through the release of Cyto-c and induction of caspase-dependent cell death evidenced by an increase in Cyto-c and Casp-3 levels (57–59). It was reported the main role of Glu-excitotoxicity in spatial memory impairment induction is through apoptotic cell death, which was confirmed by the increase in caspase activity and demonstrated by the MWM test (60). Taken together, these studies support the view suggested by the current study that the Glu-excitotoxicity contribution to spatial memory impairment occurs mainly through apoptotic neuronal cell death as a downstream mechanism, which was also suggested by the observed increase in the levels of LDH.

In the current study, Glu administration in the Glu group resulted in an increase in Aβ 1–42 concentration. The mechanism by which excitotoxicity increased Aβ production was previously reported by a study which showed that excitotoxicity following

TABLE 7 | Effect of Glu, Vit E/Glu, TQ 2.5/Glu, and TQ 10/Glu on the A β -42 levels.

Groups	–ve control	Glu	Vit E/Glu	TQ 2.5/Glu	TQ 10/Glu
A β -42 pg/ml	1.500 $\pm$ 0.155	12.65 $\pm$ 0.312*	5.033 $\pm$ 0.644 [®]	5.107 $\pm$ 0.150 [®]	3.793 $\pm$ 0.622 [®]

Glu was injected i.p. as a single dose for 7 days. TQ and Vit E were injected as a single dose for 7 days. Animals in each group were subjected to the neurobehavioral tests before being sacrificed. The brains were isolated and homogenized as 10% homogenate in 0.1 M PBS then centrifuged, and supernatants were used in the A β -42 ELISA kit. Statistical analyses of mean A β -42 level were performed using ANOVA followed by Tukey's multiple comparison tests, by graph pad prism 5. Each value represents the mean value of 12 rats $\pm$ SEM.

*Statistically significant from –ve control group ($p < 0.05$).

[®]Statistically significant from Glu group ($p < 0.05$).

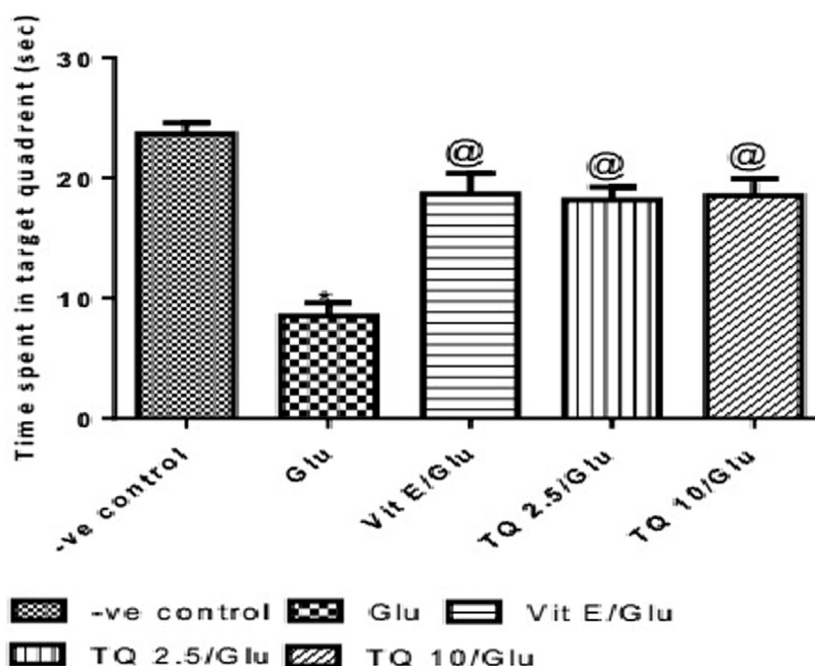


FIGURE 8 | Effect of –ve control, Glu, Vit E/Glu, TQ2.5/Glu, and TQ10/Glu on A β (1–42) levels. Glu was injected i.p. as a single dose for 7 days. TQ and Vit E were injected as a single dose for 7 days. Each value represents the mean value of 12 rats $\pm$ SEM. Statistics were carried out by one-way ANOVA followed by Tukey's multiple comparison tests, by graph pad prism 5. *Statistically significant from –ve control group ($p < 0.05$); [®]statistically significant from Glu group ($p < 0.05$); *statistically significant from Vit E/Glu group ($p < 0.05$); *statistically significant from TQ 2.5/Glu group ($p < 0.05$).

acute brain injury caused an increase in the amyloidogenic processing of APP toward the production of the A β protein (14). This finding is in agreement with a previous report revealing that NMDA receptor activation after acute brain injury may significantly contribute to the development of amyloid plaques (10). Moreover, a recent study showed that monosodium glutamate administration induced an increase in A β accumulation in the rat hippocampus and induced neurobehavioral abnormalities demonstrated by a decrease in SAB% (61), which coincides with the findings in the present study suggesting a role for the A β accumulation in inducing the neurobehavioral deficits observed in rats. In addition, it has been shown that Casp-3 is involved in APP complex proteolysis, causing an increase in A β peptide formation (14). Moreover, A β production and accumulation within neuronal cortices consequently was found to induce neuronal apoptotic cell death (62). Altogether, these findings, together with

the findings of the current study, suggest a reciprocal relationship between A β production and apoptotic cell death relative to the spatial memory impairment shown here to be a result of Glu administration.

In the current study, administration of both doses of TQ showed a dose-dependent improvement in spatial memory function, as demonstrated by the neurobehavioral tests. Previous studies showed that oral administration of TQ was able to improve spatial memory impairment induced in diabetic rats, as shown by an increase in the SAB% (63–65). Recently, it was shown that TQ produced a shortening of the time latency in the MWM (66) and an increase in the time spent in the target quadrant (67). Moreover, administration of TQ at a concentration of 10 mg/kg in rats was also able to restore the cognitive impairment induced by status epileptics shown by improvements in the time latency and time spent in the target

quadrant in the MWM test (68). The current study assumed that the improvement of spatial memory observed is attributed to the inhibition of apoptotic cell death which was evidenced by the significant decrease in the apoptotic markers (Cyto-*c* and Casp-3). A previous study showed that administration of TQ to cultured cortical neurons relieved the apoptotic cell death triggered in fetal alcohol syndrome by increasing the expression of Bcl-2, leading to inhibition of Cyto-*c* release and suppressing the activity of the apoptotic caspases, including Casp-3 (20). In addition, TQ protected against apoptotic cell death following ischemia reperfusion injury in hepatic cells owing to its antioxidant property, reducing NF- κ B and Bcl-2 expression, which was reflected by a decrease in the apoptotic cell markers (21). Moreover, oral administration of TQ attenuated apoptosis of the hippocampus following chronic toluene exposure in rats *via* its antioxidant property, as evidenced by suppression of the activity of Casp-3 (69). Furthermore, the antiapoptotic property of TQ in *Nigella sativa* contributed to the modulation of neuronal cell death in pentylenetetrazol-induced kindling which occurs in part due to NMDA receptor activation (70). These findings further support the assumption proposed by the current study, that the maintenance of spatial memory by TQ, demonstrated by the improved SAB%, MEL and time spent in the target quadrant, can be attributed to its antiapoptotic activity, evidenced by the significant decrease in the apoptotic cell markers and the maintenance of cell viability reflected by the decrease in LDH levels. Regarding A β , both doses of TQ (5 and 10 mg/kg) reduced A β -42 levels. As discussed above, because of the contribution of apoptotic cell death to the increase in A β -42 production (14), the antiapoptotic activity of TQ may contribute to the observed decrease in A β -levels. Nanoemulsion of TQ was found to reduce A β -42 levels through modulating the processing of APP, decreasing β -secretase and γ -secretase levels and increasing the degradation of A β -42 (71). In addition, a previous study showed that TQ reduced A β aggregation as a result of its properties as an antioxidant and antiapoptotic agent (72). In agreement with these findings, the present study showed that administration of TQ decreased A β levels, which could be due to several different mechanisms: (1) the antiapoptotic properties of

TQ, (2) shifting APP processing from the amyloidogenic to the non-amyloidogenic pathway, and (3) increasing the clearance of A β . Thus, the current study could be extended by evaluating the effect of TQ on the levels of β - and γ -secretase.

In conclusion, TQ treatment caused a decrease in the level of Cyto-*c*, Casp-3, LDH, and A β -42 in brain homogenates, thus proving to be a good choice for restoring memory and cognitive deficits induced by Glu-excitotoxicity, as reflected by improvements in SAB%, MEL, and time spent in the target quadrant. We attribute these results to the ability of TQ to counteract the apoptotic cell death and increased A β -42 production-induced by Gluowing to its antiapoptotic properties. As a result, TQ reduced cognitive impairment induced by Glu administration and thus it is a promising therapeutic approach against many neurodegenerative diseases that are induced by Glu-excitotoxicity.

ETHICS STATEMENT

Animal procedures were performed after the approval of the ethics committees of German University in Cairo and Cairo university with the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Cerebral Microcirculation and Histological Mapping After Severe Head Injury: A Contusion and Acceleration Experimental Model

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Background: Cerebral microcirculation after severe head injury is heterogeneous and temporally variable. Microcirculation is dependent upon the severity of injury, and it is unclear how histology relates to cerebral regional blood flow.

Objective: This study assesses the changes of cerebral microcirculation blood flow over time after an experimental brain injury model in sheep and contrasts these findings with the histological analysis of the same regions with the aim of mapping cerebral flow and tissue changes after injury.

Methods: Microcirculation was quantified using flow cytometry of color microspheres injected under intracardiac ultrasound to ensure systemic and homogeneous distribution. Histological analysis used amyloid precursor protein staining as a marker of axonal injury. A mapping of microcirculation and axonal staining was performed using adjacent layers of tissue from the same anatomical area, allowing flow and tissue data to be available from the same anatomical region. A mixed effect regression model assessed microcirculation during 4 h after injury, and those results were then contrasted to the amyloid staining qualitative score.

Results: Microcirculation values for each subject and tissue region over time, including baseline, ranged between 20 and 80 ml/100 g/min with means that did not differ statistically from baseline flows. However, microcirculation values for each subject and tissue region were reduced from baseline, although their confidence intervals crossing the horizontal ratio of 1 indicated that such reduction was not statistically significant. Histological analysis demonstrated the presence of moderate and severe score on the amyloid staining throughout both hemispheres.

Conclusion: Microcirculation at the ipsilateral and contralateral site of a contusion and the ipsilateral thalamus and medulla showed a consistent decline over time. Our data suggest that after severe head injury, microcirculation in predefined areas of the brain is reduced from baseline with amyloid staining in those areas reflecting the early establishment of axonal injury.

Keywords: anemia, amyloid precursor protein staining, histology, microcirculation, microspheres

INTRODUCTION

Severe head injury is commonly the result of a combination of contusion with acceleration–deceleration forces leading to cellular breakdown, cytogenic and vasogenic edema, impaired cerebral autoregulation, and perfusion mismatch (1). Irreversible cellular damage has been described specifically in areas where microcirculation is critically reduced (2, 3) and in areas where critically low levels of partial pressure of tissue oxygenation (P_{tiO_2}) have been sustained (4). While the assessment of cerebral regional microcirculation is primordial experimental in nature, its knowledge is essential in comprehending further the pathophysiology behind severe head injury and its extrapolation to clinical grounds. In the interim, management of head injury patients is commonly based on systemic measures that ensure global perfusion and oxygenation, without specifically targeting cerebral metabolic demands, cerebral tissue oximetry, or regional distribution of blood flow. Despite the previously demonstrated relationship between regional microcirculatory blood flow (RMBF), tissue metabolic demands (5), and cerebral hypoperfusion leading to irreversible cellular damage, there are no studies quantifying the temporal variability of RMBF in different cerebral anatomical regions after severe head injury, its close relation to the degree of tissue damage assessed by amyloid precursor protein (APP) staining or the state of cerebral tissue oxygenation. We hypothesized that after severe head injury cerebral microcirculation heterogeneity may correlate to the severity of the injury with maximal RMBF reduction seen within the areas of severe tissue disruption. We also hypothesized that RMBF and APP show a temporal and spatial distribution, after injury.

This study aims to quantify the temporal changes in cerebral RMBF after severe head injury focusing on the anatomical region such as the ischemic penumbra in comparison with contralateral regions. This study also aims to simultaneously superimpose flow data with histopathology data at each area of interest and at each time point before and after severe head injury to establish the hypothetical pathophysiological relation between perfusion and mechanical related injury to axons.

MATERIALS AND METHODS

Animal Care and Preparation

Sheep were considered to be the optimal experimental model due to their cerebral anatomical similarities with humans, specifically the cerebral gyrencephalic surface allowing better examination of the gray–white matter; a well-defined physiology of the ovine hemoglobin dissociation curve (6) and extensive experimental neuroscience experience using this animal model (7–9). A convenience sample of eight Ovis Aries wethers weighing 40 ± 5 kg were instrumented with a triple lumen central line (Cook Medical Inc., QLD, Australia) and two 16 Fr introducer sheaths in the right internal jugular vein. *Via* the central line, general anesthesia was given using ketamine with an initial bolus of 5 mg/kg and maintenance infusion between 0.5 and 1 mg/kg/h. Sedation was achieved with a combined infusion of midazolam (0.5 mg/kg/h), fentanyl (10 μ g/kg/h), and alfaxalone (6 mg/kg/h), previously used in a mild head injury study (10) demonstrating that this

anesthetic combination maintains cardiovascular stability without altering cerebral microcirculation in sheep (11). Hydration was maintained with an infusion of Hartmann's solution up to a rate of 2 mL/kg/h, titrated to maintain a central venous pressure of 6–10 mmHg. Cardiovascular monitoring included cardiac output (CO) and vascular resistances *via* a Swan-Ganz catheter as previously described (10, 12) and a 5 F umbilical vessel catheter (Argyle, Tyco HealthCare, Mansfield, MA, USA), placed in the right femoral artery to allow a withdrawal of blood at a rate of 10 mL/min. Orotracheal intubation used a size 10 mm endotracheal tube (SIMS Portex, UK). Sheep were ventilated at 12 bpm with tidal volumes of 8 mL/kg and 5 cmH₂O of PEEP with an initial FiO_2 of 1.0 with the FiO_2 and respiratory rate titrated to maintain a partial pressure of oxygen (PaO_2) of >95 mmHg and normocapnia. PEEP levels were maintained at 5 cmH₂O to minimize de-recruitment consistent with common clinical practice and known to have no detrimental effect on cerebral blood flow (10, 13). Neuro-monitoring included a Lycop P_{tiO_2} probe and an intracranial pressure (ICP) monitor, Oxford Optonix Ltd., Oxford, UK. Craniectomies were performed before injury but dura was not pierced to avoid the ICP release if pierced beforehand. Craniotomies for the insertion of both probes were performed exactly 15 mm lateral to the sagittal suture and anterior to the coronal suture (9, 14). Probes were introduced at 35 and 15 mm from the skull, respectively, after piercing the dura with the end of the tip located at the white matter as previously performed (15).

To avoid red blood cell storage into the sheep's spleen and maintaining stable hemoglobin throughout the study (16, 17), the ligation of the splenic artery was performed as reproduced from previous studies (10, 15).

The monitoring and preparation phase was completed with an intracardiac echocardiography guided insertion of a transeptal catheter into the left cardiac atrium (LA). Echocardiography images were obtained using an Acuson Sequoia C512 scanner (Siemens, California). Transeptal puncture and insertion of a pigtail catheter into the LA followed previously described methods (18).

Trauma Model

Under anesthesia, a blunt injury was applied over the left temporal bone using a non-penetrating stunner (model MKL, Karl Schermer, Ettlingen, Germany) with the intention to generate a severe head injury but without leading to brain death. This model followed the exact method as a previous study (10) leading to a final combination of contusion and acceleration injury (19). After injury, burr holes were formalized and the dura was pierced for the insertion of pressure and tissue-oximetry probes as previously described (10). The main difference with the previously reported study (10) was that in this study the goal was to achieve a severe head injury, unlike the former study which aimed to achieve a mild degree of head injury. To achieve this goal, recruited animals were significantly smaller in weight: while for the achievement of a “mild” head injury, animals weighed between 60 and 75 kg, in this study, recruited animals weighed between 40 and 45 kg. Subjects were still adult sheep and wethers of the same ovine species. As the impact forces generated by the same stunner were unchanged; the result of these forces onto smaller brains was

expected to be different. This latter supposition is based upon the theoretical rationale that a reproducible and identical force may lead to greater impact or harm when applied to a smaller area.

Protocol for Microspheres Injection

At each time point (T0 corresponding to baseline, T1–T4 corresponding to first to fourth hours after trauma, respectively) an injection of color-coded microspheres [E-Z TRAC; Interactive Medical Technology (IMT), Los Angeles, CA, USA] was done through the LA pigtail catheter as performed previously (10, 15, 20). Randomly assigned colors at each time point and subject minimized selection biases and allowed the tracking of RMBF at specific anatomical regions for each time point and subject. Five different colors (purple low, purple high, pink high, yellow high, and coral low) were recommended by the manufacturer www.microspheres.net to facilitate cytometric count. Each injection included a homogeneous mixture of one color microspheres of a density equal to five million spheres in a 0.8 ml. This microsphere density has been used (21) without causing microvascular occlusion.

Microspheres were injected 30 s after the initiation of the withdrawal pump. The withdrawal pump was connected to the arterial catheter with the intention to withdraw blood at an established rate of 10 ml/min to obtain the reference blood sample required for the calculation of regional tissue RMBF. Two minutes after commencement of the withdrawal pump, the reference blood sample collection was completed, and the inline catheter was flushed with Tween 80 reagent to recover all the microspheres that may have been entrapped in the line (22). This protocol reproduced the same steps as previously published studies (10, 15).

Euthanasia and Postmortem Tissue Manipulation

After 5 h of continuous monitoring and microsphere injection, sheep were euthanized under non-recovered anesthesia with a bolus injection of 0.5 mL/kg of sodium pentobarbitone. After confirmation of death (asystole arrest), the brain was extracted, weighed, and fixed with 10% formalin for 3 weeks.

Brain Harvesting Technique

Brain harvesting was facilitated with the use of a round reciprocating saw sectioning approximately 5 cm bone sections from the temporal region to the frontal sinuses. These bone sections were removed while simultaneous dissection of the dura avoided parenchymal tearing. Once the brain was fully exposed, the olfactory bulbs, optic chiasm, tentorium, and cranial nerves were progressively sectioned as the brain was lifted from the base of the skull. This approach achieved a controlled dissection avoiding injury to the brain tissue and ensuring a cautious brain removal as previously demonstrated (11). Brains were weighed before insertion into a formalin bath for an immersion fixation during a minimum of 3 weeks.

Tissue Sampling Model

After the period of immersion fixation, brains were macroscopically inspected to assess for cortical impacts, hemorrhages, or

the presence of contra-coup injury. Following external inspection, each brain was sectioned creating 5 mm anteroposterior slices. Each slice was macroscopically inspected to identify regions of maximal contusion. Cone samples were extracted from predefined anatomical regions these labeled as follows: AL corresponding to the core of contusion at the side of the injury; BL, the peri-contusional region at the side of the injury; AR, the mirror region to the core of contusion on the contralateral side; BR, the mirror region to the peri-contusional region on the contralateral side; C, the thalamus at the ipsilateral side to the contusion, and D corresponded to the medulla (Table 1), this method was reproduced from a previous study (10, 15). Adjacent tissue blocks were assigned for both cytometric and histological analysis, to superimpose histology with cerebral blood flow data at the same anatomical region.

Samples from skin, kidney, heart, and spleen were extracted from each sheep to demonstrate systemic distribution of microspheres as well as to confirm the presence of splenic infarct related to a successful spleen ligation, respectively.

Quantification of Microvascular Blood Flow

The total amount of each color microsphere imbedded in each particular region of interest used a cytometric analysis which has been previously validated (22). RMBF was calculated as a mathematical derivation from the known microsphere concentration injected into the arterial supply at each injection time and the amount of each color microspheres found at each “reference sample of blood.” The reference sample of blood represents an arterial blood sample withdrawn at a known rate over a fixed period of time (23). RMBF represents the proportion of microspheres trapped in the targeted tissue in relation to the total quantity of spheres per milliliters of blood per minute of the reference sample using the equation (24):

$$\text{RMBF (mL/min/g)} = \frac{(\text{Total tissue spheres})}{[(\text{Tissue weight, g}) \times (\text{Reference spheres/mL/min})]}.$$

Cytometric analysis was performed at the IMT, Los Angeles, CA, USA, www.microspheres.net.

Immunohistochemistry Processing

Immunohistochemistry analysis was performed at the neuropathology laboratory, Royal Brisbane and Women's Hospital, QLD, Australia. Immunohistochemistry used a Leica Novolink Polymer Detection Systems Kit (Leica Microsystems Pty Ltd., North Ryde,

TABLE 1 | Tissue sampling labeling.

Anatomical regions	Anatomical location
AL	Core of contusion, left side
BL	Ischemic penumbra, left side
AR	Mirror region to core of contusion, on the right
BR	Mirror region to ischemic penumbra, on the right
C	Thalamus ipsilateral to injury
D	Medulla

NSW, Australia) as per manufacturer's instructions, www.leica-microsystems.com. Sections had paraffin removed through a series of xylene immersions and re-hydrations. Antigen retrieval was carried out using Leica BOND ER1 solution. Endogenous peroxidase was neutralized. Sections were incubated with a protein block. The primary antiserum made up in Leica BOND Antibody Diluent was applied to the sections.

Immunohistochemistry and Hematoxylin–Eosin Scoring and Interpretation

Immunohistochemistry analysis using APP antibodies staining was applied to all targeted areas of interest. APP antibody staining was used to identify areas of tissue with high density of APP staining, specifically at regions of interest. APP expression is considered to be a very early marker of neuronal damage (25) and therefore suitable as an early histopathological marker for a 4 h study. A grading system of the density of APP staining previously described (9) was used. APP score was structured into three qualitative categories dependent upon the severity of injury seen: *Mild*: a focal contusion with APP labeling limited to the site of injury or focal APP labeling; *Moderate*: a pattern of APP staining greater than one hemisphere, greater than half a hemisphere or less than half a hemisphere; *Severe*: characterized for the presence of diffuse staining and sub-classified as either diffuse vascular injury, diffuse axonal injury with macroscopic hemorrhage, diffuse axonal injury with microscopic hemorrhage, tissue tears or diffuse axonal injury only (11). Each animal had samples for both cytometric count of RMBF and immunohistochemistry at each anatomical region of interest with the intention to superimpose flow data with histopathology data at each area of interest and at each time point before and after severe head injury.

Statistical Analysis

Regional microcirculatory blood flow raw data for each sheep was plotted over time and then time averaged for the study cohort. The ratio of RMBF from 1 to 4 h after injury (T1–T4) compared with baseline (time 0–T0) was plotted. A ratio of 1 indicated no RMBF changes pre–post-injury. RMBF values below the ratio of 1 indicated that RMBF was reduced over time from baseline. RMBF values above the ratio indicated an increase in RMBF over

time from baseline. To test for statistical differences, we used a mixed effect regression model of the ratios from times T1 to T4 with a random intercept for each sheep to control for repeated responses from the same sheep. We fitted an independent effect at each time (T1–T4) as we were uncertain of how the change in RMBF over time would be. We also examined a simpler model where the RMBF ratio was the same at times T1–T4.

All the plots and regression models were run separately for each area studied (AR, BR, AL, BL, C, and D). We used the R software version 3.1.2 for all analyses.

An additional analysis was performed to compare RMBF ratios from a “mild head injury” cohort used in a previous study (10) and the current “severe head injury” cohort study. We plotted the mean ratio over time grouped by study to graphically compare differences. To test for statistical differences, we used a mixed effect regression model of the ratios from times T1 to T4 with a random intercept for each sheep to control for repeated responses from the same sheep. We fitted an independent effect at each time (T1–T4); the key independent variable was the study (the “mild head injury” cohort study versus the “severe head injury” cohort study). The mean difference and the 95% confidence intervals were shown; the plots and regression models were run separately for each tissue region (AL, BL, AR, BR, C, and D); R software version 3.2.1 was also used for all the analysis.

RESULTS

A convenience sample of eight subjects weighing 40–45 kg was used in this study. Subjects remained cardiovascularly stable throughout the entire study time even after a severe head injury (Table 2), except for one subject (subject number 5) who became profoundly vasoplegic after injury in association with bilateral unreactive midriasis and a comminuted skull fracture. These signs suggested high ICPs leading to herniation state, but due to the significance of his bone fractures, insertion of ICP probes was not feasible, therefore ICP could not be quantified. In addition, this subject showed undetectable RMBF from T1 compatible with a state of global cerebral hypoperfusion (Figure 1).

Systemic variables affecting cerebral perfusion and reflecting peripheral oxygen extraction, such as CO and central venous

TABLE 2 | Mean arterial blood pressure (mmHg), ICP, and cerebral perfusion pressure (mmHg) values in all subjects at each time point.

MAP/ICP/ CPP (mmHg)	T0 preinjury MAP	T1	T2	T3	T4
Subject 1	8,484	80/8/72	81/17/64	86/15/71	99/14/85
Subject 2	100	80/6/72	87/8/79	81/7/73	79/9/70
Subject 3	120	119/7/112	111/7/104	120/8/112	125/9/116
Subject 4	110	124/18/106	122/17/115	120/18/102	119/19/100
Subject 5 ^a	73	60/NA	65/NA	69/NA	72/NA
Subject 6	75	90/11/79	96/18/78	88/20/68	114/18/96
Subject 7	70	92/24/68	100/24/76	102/22/80	81/20/61
Subject 8	110	105/40/65	109/32/77	115/34/81	114/35/79
Mean (SD)	1,143 (2,966)	98.6 (17.8)/21.8 (19.2)/82 (19)	101 (14.3)/23.5 (18.6)/84.7 (17.9)	102 (16.9)/24.1 (20)/83.9 (16.7)	104 (18.5)/24.5 (20.9)/86.7 (18.8)

MAP, mean arterial pressure; ICP, intracranial pressure; CPP, cerebral perfusion pressure, calculated as per MAP–ICP.

Only values of MAP are present at T0 as ICP probe was inserted after injury.

^aSubject 5 showed signs of sudden cerebral tamponade with secondary neurogenic shock requiring vasopressor support to maintain MAP. A comminuted skull fracture did not allow for the insertion of PtiO₂ or ICP probes.

oxygen saturation (SVCO₂), respectively, were maintained homogeneously within each subject for the entire study period and with differences among subjects (Table 3).

Metabolic variables directly affecting oxygen delivery to tissues and cerebral blood volume, such as PaO₂ and partial pressure of arterial CO₂, respectively, remained stable throughout the study period for each subject also with minimal variations among subjects (Table 4).

Metabolic variables reflecting oxygen delivery to tissues as well as impacting into microcirculation rheology, such as the hemoglobin level (Table 5) also remained stable at all times in each subject with minor variations among subjects.

Cerebral PtiO₂ was recorded in every subject from hour 1 after trauma as PtiO₂ probes were inserted after formalizing craniectomies (Table 6). PtiO₂ among subjects showed compromised levels of tissue oxygenation through all times except for subject number three which maintained a hyperemic range of values. PtiO₂ was

reflecting only tissue oxygenation at the ipsilateral side to the injury.

RMBF Analysis

Each sheep had a baseline RMBF measure before injury (time 0–T0) and subsequent hourly RMBF measures every hour during 4 h after injury, corresponding to times T1–T4, respectively. RMBF values for each subject and tissue region over the entire study time are represented in Figure 1.

Regional microcirculatory blood flow values for each subject and tissue region over the 4 h from baseline are represented in Figure 2. The ratio of RMBF from 1 to 4 h after injury (T1–T4) compared with baseline (time 0–T0) was also plotted. A ratio of 1 indicated no RMBF changes pre–postinjury. RMBF values below the ratio of 1 represented a reduction in RMBF over time from baseline; RMBF values above such ratio represented an increase in RMBF over time from baseline.

Regional microcirculatory blood flow means ratio from baseline and for all subjects per anatomical region and time with their 95% confidence intervals represented by the vertical lines were shown in Figure 3. Statistical significance was represented by confidence intervals that would not cross the horizontal reference line of 1. As shown in this figure, the RMBF means at all anatomical regions and times were reduced from baseline but their differences were not statistically significant. Regional flow in the Y-axis is represented as per 1 g tissue weight, showing that in our study, physiological RMBF values were found, when normalized to a 100 mg tissue weight.

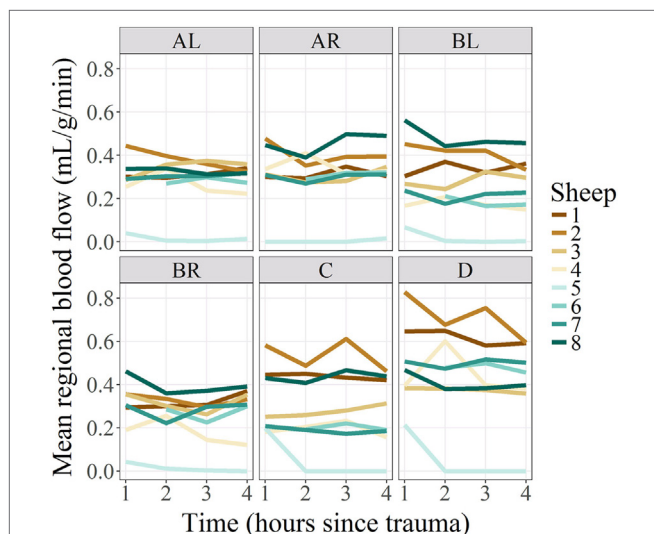


FIGURE 1 | Regional blood flow per regions of interest and subjects, over time. Regional microcirculatory blood flow (RMBF) is represented on the Y-axis on a scale of milliliters per gram per minute; time is represented on the X-axis at four consecutive time epochs corresponding each of them to 1 h from the injury. Mean RMBF is displayed for each subject with subject number 5 being an outlier as his RMBF was severely reduced.

TABLE 3 | Summary statistics for SvO₂ and CCO data by sheep over all times.

Subject number	n	CCO		SvO ₂	
		Mean	SD	Mean	SD
SN 01	14,255	6.30	3.62	88.33	5.31
SN 02	15,394	4.17	0.69	77.93	9.65
SN 03	13,222	4.20	0.40	81.65	4.10
SN 04	14,225	4.62	1.20	41.97	11.28
SN 05	14,188	5.38	1.09	80.33	3.30
SN 06	6,061	3.63	0.36	65.94	17.12
SN 08	17,759	3.90	0.92	68.89	5.35
All subjects	95,104	4.76	1.90	81.72	7.31

Venous saturation of oxygen (SvO₂) expressed in %.

Continuous cardiac output (CCO) expressed in liters per minute.

APP Scoring

Results for APP scoring are represented in Tables 7 and 8. Minimal APP staining pattern was seen predominantly in all anatomical regions with moderate and severe APP staining pattern also present and distributed homogeneously over all anatomical regions. Evidence of axonal damage and axonal retraction balls (ARB) was found (Figure 4). Wider representation of moderate and severe APP scoring was found among subjects in this study, when compared with a mild head injury cohort.

Extracranial Tissues

Direct quantification of RMBF was also performed at extracranial regions, in particular, at the skin, heart, kidney, and spleen (Figure 5). The aim was to demonstrate systemic distribution of color-coded microspheres as a proof of concept during all time points. In addition, RMBF at the spleen aimed to demonstrate that spleen artery ligation had been performed efficiently, demonstrating nearly negligible presence of spheres.

Statistical Comparison With a Previous Study

Statistical comparison of cerebral RMBF at specific anatomical regions of interest and at all times within the “mild head injury” cohort study and the “severe head injury” cohort showed: first, nearly all regions showed a temporal reduction in RMBF from baseline. Second, RMBF at the ischemic penumbra and at the thalamus on the “mild head injury” cohort study had a temporal

TABLE 4 | PH/PCO₂ and PO₂ (at FiO₂ 0.4) values per sheep at each time points.

Subjects	Times				
	T0 PH/PCO ₂ /PO ₂ (mmHg)	T1 PH/PCO ₂ /PO ₂ (mmHg)	T2 PH/PCO ₂ /PO ₂ (mmHg)	T3 PH/PCO ₂ /PO ₂ (mmHg)	T4 PH/PCO ₂ /PO ₂ (mmHg)
Sheep 1	7.40/48/138	7.37/49/138	7.40/49/142	7.40/48/146	7.42/47/136
Sheep 2	7.41/48/141	7.40/49/140	7.43/48/147	7.46/44/152	7.43/41/150
Sheep 3	7.41/48/208	7.40/50/196	7.43/50/193	7.45/48/207	7.45/49/206
Sheep 4	7.49/35/206	7.43/46/220	7.45/45/218	7.49/37/238	7.48/36/239
Sheep 5	7.33/49/128	7.41/44/125	7.35/48/123	7.34/49/103	7.37/48/98
Sheep 6	7.44/38/212	7.43/44/200	7.42/43/222	7.47/40/205	7.44/42/203
Sheep 7	7.35/50/126	7.42/43/171	7.43/47/240	7.49/40/280	7.49/37/266
Sheep 8	7.45/41/203	7.39/49/200	7.42/49/210	7.46/46/217	7.47/44/225
Mean (SD)	7.41 (0.052)/44.6 (5.8)/ 170 (39.9)	7.41 (0.021)/46.8 (2.8)/ 174 (35.5)	7.42 (0.030)/47.4 (2.3)/ 187 (43.6)	7.45 (0.051)/44 (4.5)/ 194 (56.7)	7.44 (0.039)/43 (4.9)/ 190 (57.1)

TABLE 5 | Hemoglobin levels (g/dL) for all subjects over time.

Subjects	Times				
	T0	T1	T2	T3	T4
Sheep 1	7.0	7.1	7.2	7.3	7.1
Sheep 2	8.0	8.2	7.9	7.6	8.0
Sheep 3	8.0	7.7	7.7	7.6	7.7
Sheep 4	7.6	8.1	8.0	8.3	8.3
Sheep 5	8.1	9.3	8.2	7.9	7.7
Sheep 6	7.0	7.3	8.1	7.7	7.9
Sheep 7	8.0	7.7	7.8	7.4	7.4
Sheep 8	9.0	8.7	8.8	8.7	8.9
Mean (SD)	7.8 (0.65)	8.0 (0.73)	8.0 (0.46)	7.8 (0.47)	7.9 (0.55)

Hemoglobin levels were maintained stable from baseline (T0) throughout times (T1–T4) by the completion of a spleen artery ligation.

TABLE 6 | Partial pressure of tissue oxygenation (PtiO₂) expressed (in mmHg) for all subjects over time.

PtiO ₂ (mmHg)	T1	T2	T3	T4
Sheep 1	1.99	2.69	3.03	3.61
Sheep 2	1.16	11.1	15.21	29.21
Sheep 3	34.01	47.11	48.11	46.23
Sheep 4	16.00	20.21	21.12	24.22
Sheep 5	NA	NA	NA	NA
Sheep 6	11.21	13.31	19.54	22.41
Sheep 7	11.12	10.86	10.31	12.61
Sheep 8	11.01	25.12	26.71	26.41
Median	11.12	13.31	19.54	24.2
IQR	7.1	11.7	11.2	10.3

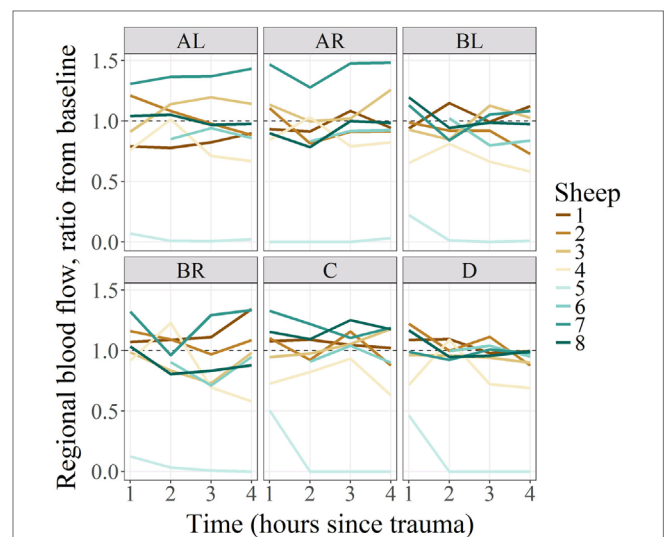
T0 represents preinjury phase in which craniectomies are not formalized.

PtiO₂ probes were inserted after trauma, recording PtiO₂ values only from T1.

NA, not available values.

increase in their RMBF, of uncertain explanation; unlike for the “severe head injury” cohort study in which these areas also showed a consistent reduction of their RMBF over time from baseline (Figure 6) with a contrast of the magnitude of the impact, on two different subjects’ postmortem findings (Figure 7), showing that in the severe injury subject there is an ipsilateral combined with a substantial contra-coup contusion and associated with an evident axial hemorrhage.

The main finding in such comparison was that RMBF in all regions except for medulla, on the “mild head injury” cohort

**FIGURE 2** | Regional blood flow ratio for all subjects and all regions from baseline. All subjects’ regional microcirculatory blood flow (RMBF) (except for subject 5) was distributed along the ratio of 1. Those above the ratio, indicating that their mean RMBF did increase over time from baseline; those with mean RMBF below the ratio indicating that their mean RMBF was reduced from baseline.

study was consistently higher than on the “severe head injury” cohort study, although these differences were not statistically significant.

Mean ratio of RMBF from baseline by tissue region and study (comparing “severe head injury cohort” with “mild head injury cohort” studies) are shown in Table 9, where none of the differences in ratios between groups were statistically significant as every confidence interval contained the 0 value.

DISCUSSION

The main focus of this study is to directly quantify cerebral regional microcirculation in anatomical regions of interest after a severe head injury model which comprises a mixture of contusion and acceleration–deceleration forces, a common mechanism of injury otherwise in real life. In addition, this study contrasts

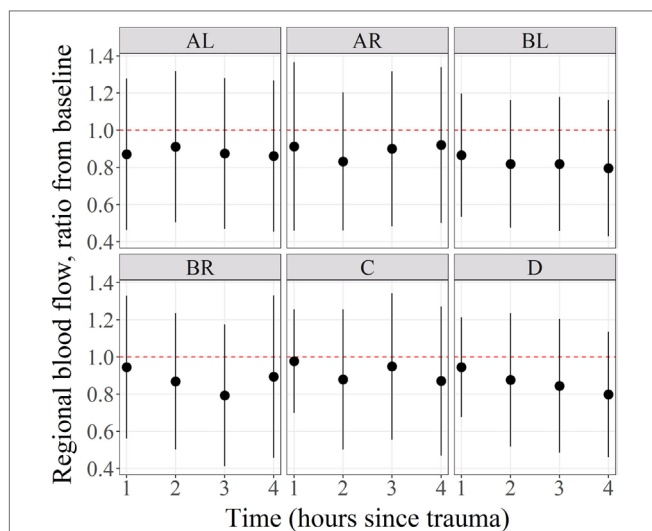


FIGURE 3 | Mean regional blood flow confidence intervals and ratio from baseline. Regional microcirculatory blood flow (RMBF) mean with confidence intervals crossing the ratio of 1 indicated that the changes in RMBF at that region of interest and that time point were not statistically significant.

TABLE 7 | Amyloid precursor protein (APP) staining qualitative scores by tissue region in a “severe head injury” model showing its distribution among six of the eight subjects for the severe head injury study.

Anatomical regions	Amount of subjects categorized on each qualitative APP score		
	Mild	Moderate	Severe
AL	5	0	1
AR	5	1	0
BL	4	1	1
BR	5	0	1
C	4	1	1
D	5	1	0

APP staining is predominantly of “mild” category at all regions, but an expression of “moderate” and “severe” is also present among all regions.

TABLE 8 | Amyloid precursor protein (APP) staining qualitative scores by tissue region in a “mild head injury” model showing its distribution among nine subjects for the mild head injury study.

Anatomical regions	Amount of subjects categorized on each qualitative APP score		
	Mild	Moderate	Severe
AL	9	0	0
AR	9	0	0
BL	9	0	0
BR	9	0	0
C	2	2	5
D	5	2	2

APP staining is predominantly “mild” in category at all regions; categories of “moderate” and “severe” are only present at axial anatomical regions where the highest acceleration–deceleration force mainly concentrates.

regional flow data with histological data facilitated by the novel experimental model design which isolated adjacent micro-layers of tissue from the same anatomical regions to be separately used

for flow and tissue analysis. There are no precedents in this study design. The sequential injection of color-coded microspheres during the experimental phase, allowed a time dependant quantification of cerebral microcirculation at very specific anatomical regions.

This study also captures the hypothetical aim of achieving a “severe” head injury as opposed to a previous study (10) which attempted to recreate a “mild” head injury. Achievement of severe versus mild head injury was intended by using the same impact source (a stunner) while using animals of same gender, age range and species but of smaller weight 40–45 kg on the former versus 65–70 kg weight on the latter and maintaining all other methodological aspects of the study unchanged; those two cohorts of subjects became statistically comparable.

The most important finding in this “severe” head injury study is the reduction of RMBF from baseline (RMBF preinjury or T0) in all subjects, at all anatomical regions of interest and at all times through the study length, as seen in **Figures 2** and **3**. This finding is consistent with the *a priori* expectations regarding cerebral microcirculation after severe head injury; hence, contrasts with the presence of heterogeneity and temporal variability found in a mild head injury study (10). A consistent reduction in cerebral blood flow is found in all targeted regions during the 4 h of study, although this reduction in RMBF was not statistically significant (**Figure 3**) likely due to the small sample size; however, it is important to remark that such finding is pathophysiologically and clinically important as it raises multiple issues. Cerebral microcirculation, for example, is consistently jeopardized even in the very early hours after injury despite the preservation of CO₂, oxygen delivery, and CO₂. This issue is clinically relevant when considering the potential implications during the resuscitation phase of patients. On the other hand, while statistical significance offers methodological robustness, the experimental nature of this study focuses its relevance in its design and clinical plausibility of findings, both of which are demonstrated.

When cerebral RMBF values were normalized to a global unit of measure (ml/100 g tissue/min), a physiological range of RMBF was found through all anatomical regions.

Furthermore, mean cerebral RMBF at baseline was above the ischemic threshold of 15–20 ml/100 g/min despite having elicited a severe head injury (26). This suggests that in this “severe” head injury model, RMBF although consistently reduced from baseline, was maintained above ischemic thresholds without leading to cerebral infarct. For few of our subjects, this was an unexpected finding, specifically in those cases where the impact had led to compound skull fractures, traumatic midriasis or significant acceleration–deceleration drift of the head. However, one subject did become clinically brain dead and that was supported by the presence of critically low levels of RMBF in few regions and undetected RMBF in other anatomical regions of interest. A possible explanation for the relatively preserved RMBF in this cohort was that despite the applied impact and the fact of having been subjected to a higher degree of injury than in the mild study, this was not accompanied by a worse APP scoring throughout the brain, suggesting that the injury was possible lesser than expected.

Regional microcirculatory blood flow data in this severe head injury study were compared with the study corresponding

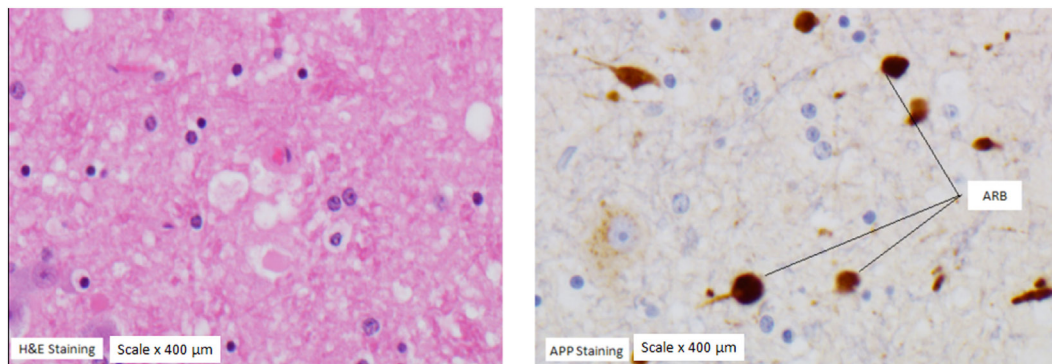


FIGURE 4 | Axonal retraction balls (ARB). ARB under hematoxylin staining (left panel) and under amyloid precursor protein (APP) staining (panel on the right) in a subject with severe APP scoring at the medulla region.

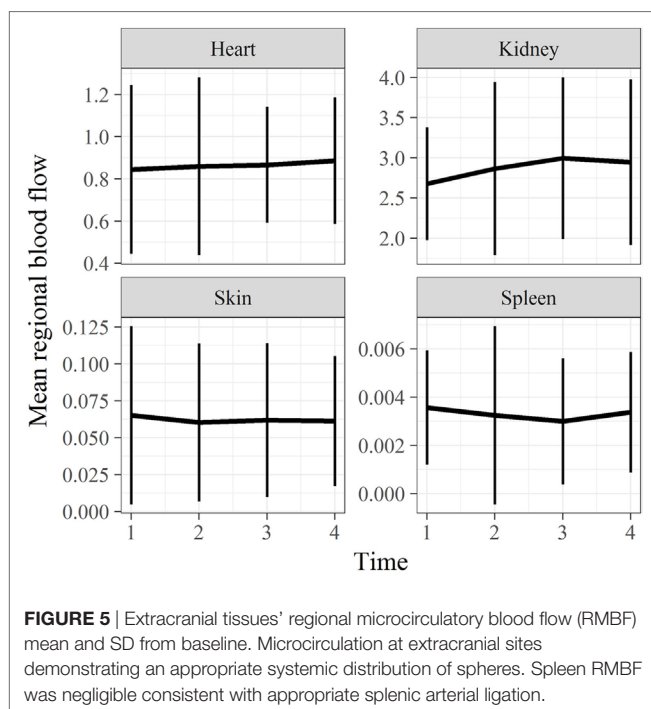


FIGURE 5 | Extracranial tissues' regional microcirculatory blood flow (RMBF) mean and SD from baseline. Microcirculation at extracranial sites demonstrating an appropriate systemic distribution of spheres. Spleen RMBF was negligible consistent with appropriate splenic arterial ligation.

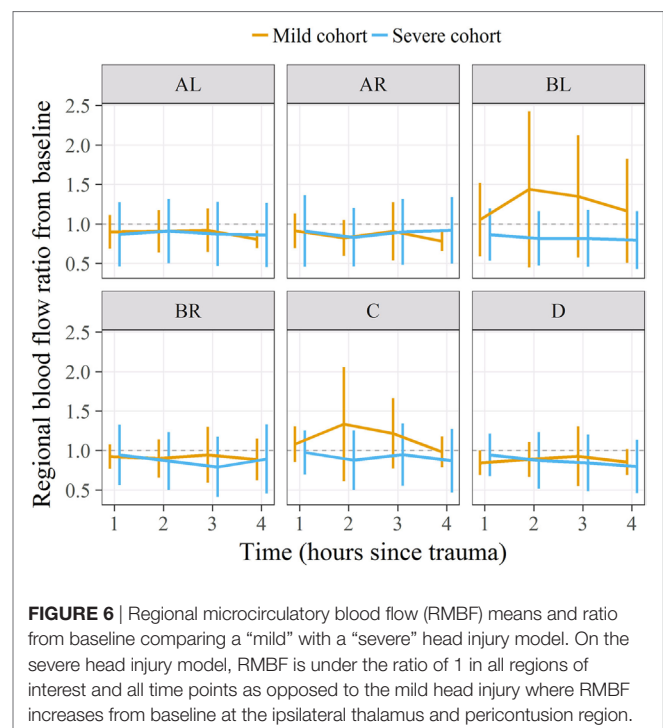


FIGURE 6 | Regional microcirculatory blood flow (RMBF) means and ratio from baseline comparing a "mild" with a "severe" head injury model. On the severe head injury model, RMBF is under the ratio of 1 in all regions of interest and all time points as opposed to the mild head injury where RMBF increases from baseline at the ipsilateral thalamus and pericontusion region.

to a mild head injury (10). No statistical significance was found between the RMBF distribution and quantification throughout all the anatomical regions of interest and during the 4 h of study in either of these two cohorts; however, RMBF in the "severe" head injury study at all regions was lesser than in the "mild" head injury study and the APP staining was more widely distributed, suggesting that our severe injury model could have indeed lead to major degree of injury.

The decline in cerebral RMBF from baseline in this study was not related to an increase in ICP or a reduction in CPP as these parameters were stable and in normal ranges for all subjects except for subjects numbered seven and eight (Table 2). This suggests that reductions in cerebral RMBF postinjury may be also related to early inflammatory changes and tissue disruption after trauma even before the establishment of high

ICP. This finding raises concerns related to cerebral vulnerability to ischemia even in the absence of poor intracranial compliance.

This study's findings and translation into clinical practice are limited by the timeframe of the study, as it focuses on cerebral microcirculation within 4 h after injury. However, as previously emphasized, the novelty of this study design relies on the microcirculatory quantification using cytometric methods when targeting-specific anatomical regions, confirming the reduction of RMBF from the very first hour after trauma. Clinical implications are related to the risk of developing cerebral infarcts even with the co-existence of a preserved ICP, suggesting that efforts should be oriented to optimize cerebral perfusion even within the first hours after head injury, regardless of the level of ICP.

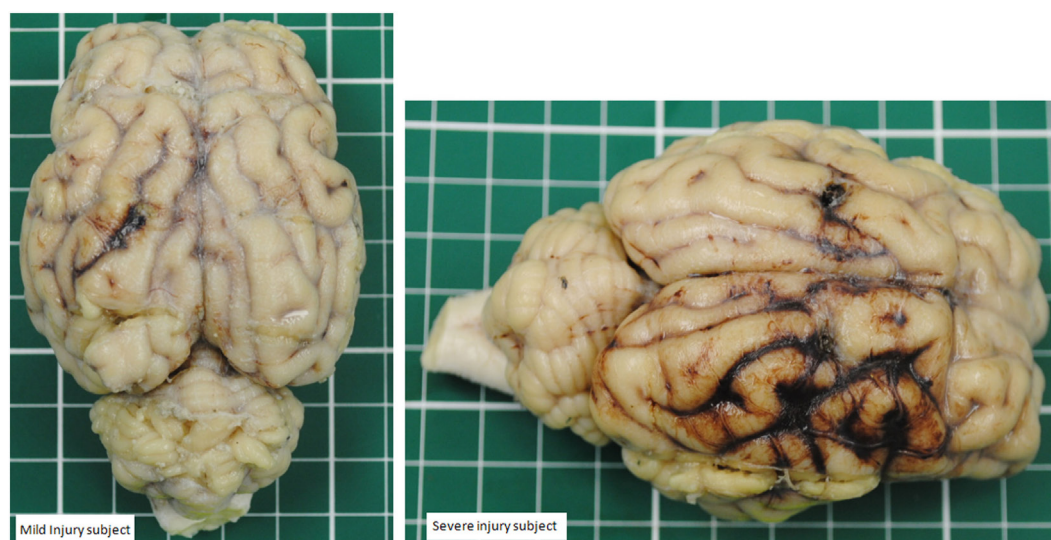


FIGURE 7 | Contrast between a mild head injury contusion (left panel) and a severe contusion (panel on the right) in two different subjects. The mild injury shows a concise contusion at the ipsilateral site of the injury. The severe injury shows a bigger contusion surrounded by hemorrhage and also a contralateral area of hemorrhage.

TABLE 9 | Mean ratio of regional blood flow from baseline by tissue region and study.

Tissue	Mean	95% confidence interval
AL	0.00	−0.28, 0.28
AR	0.05	−0.25, 0.34
BL	−0.46	−0.99, 0.08
BR	−0.04	−0.32, 0.24
C	−0.22	−0.55, 0.11
D	−0.01	−0.26, 0.23
Heart	−0.32	−0.82, 0.17
Kidney	−0.08	−0.32, 0.16
Skin	−0.66	−2.37, 1.04
Spleen	−0.27	−0.91, 0.37

Histopathological analysis using APP staining on the adjacent anatomical regions where flow cytometric count had been performed showed predominantly a minimal degree of APP staining not expected from the severity of the injury applied; however, moderate and severe APP staining scoring was present throughout all regions including the contralateral hemisphere, demonstrating that axonal injury was globally present. In this study, few ARB were found at the medulla region. This is an important finding that supports the higher injury present in this study. ARB are considered to be the hallmark of severe axonal injury. When the axonal cytoskeleton brakes down after injury, axonal transport continues up to the point of such tearing, building up axonal swelling at that disrupted point. When the build-up products alter the cytoskeleton, this retracts back toward the neuronal body leading to a “bulb,” called axonal retraction ball. These findings contrasted with the previous “mild” head injury study (10) in which only medullary and thalamic regions showed a moderate and severe APP staining scoring, confining the axonal injury mainly to axial regions

and not to hemispheres (Tables 7 and 8). Axonal disruption can be histologically graded by microscopic quantification of the amount of APP staining and has a good correlation with the intensity of axonal damage (27–30). In this study, even when mild axonal staining predominates over severe; a time-dependent reduction of cerebral RMBF close to ischemic thresholds was found in all regions, from baseline. This is an important finding as it may indicate that even in situations where severe tissue disruption is not derived from the primary injury, there is still the potential for the development of cerebral infarcts. While this theoretical principle is widely accepted in the neurocritical care arena; this study compares and contrasts for first time the interrelation between tissue damage and microcirculation in experimental models at specific anatomical regions, being this the first time that histopathology and quantification of microcirculation are analyzed simultaneously. This study also brings to light the relation between dynamic blood flow changes and the severity of tissue disruption at very specific anatomical regions showing how, flow and tissue, are spatially affected after trauma.

Amyloid precursor protein staining is not a common anatomopathological marker used in the field of neurotrauma, but it is reproducible and its applicability has been validated. It is used to quantify and define the distribution of axonal damage, as demonstrated in this study.

Cytometric analysis in other organs, in particular the skin and spleen is shown in Figure 6. RMBF in spleen showed negligible perfusion as expected from the arterial spleen ligation technique completed in the preinjury phase. Spleen ligation allowed the maintenance of a steady-state level of hemoglobin, particularly during a stress response phase when splenic red blood cell storage is well described in ovine models (17). Splenic RMBF was expressed in milliliters per gram

per minute, varying between 0.0030 and 0.0036 ml/g/min, a thousand times less than normal controls (12). This finding confirms that our splenic artery ligation was successful as well proven by the necrotic aspect of the spleen after harvesting. The main effect derived from the arterial spleen ligation is found on the stability of hemoglobin in all subjects and over all times after injury (Table 5); suggesting that the temporal changes on cerebral RMBF and PtiO₂ seen in our subjects were not related to anemia. This is an important finding as this reflects the state of cerebral microcirculation after trauma in conditions of baseline hemoglobin, a significant confounder in microcirculation. But, while clinical extrapolations are not possible considering the experimental nature of this study, it is important to highlight that the hemoglobin levels in this cohort of subjects are to a degree higher to those currently accepted within clinical practice, especially among physicians tolerating a restrictive transfusion threshold (4). This raises the concern of the potential risk of cerebral ischemia after trauma if anemia was present.

Cerebral oximetry data were provided by PtiO₂ in all subjects. These probe sensors were introduced *via* a burr hole completed immediately after injury, with the intention not to bias the intracranial compliance, particularly if the opening of the dura had been performed before injury. In this study, PtiO₂ values in four of the eight studied subjects (SN 3, SN 4, SN 6, and SN 8), maintained levels of tissue oxygenation above 15 mmHg, suggesting that oxygen delivery to the brain was preserved despite the reduction of RMBF from baseline. One subject (subject 5) had no recordable PtiO₂ as probes could not be safely introduced due to the presence of compound cranial fractures. These findings may indicate that despite the global reduction in RMBF from baseline, the maintenance of above-ischemic perfusion thresholds may be sufficient to preserve tissue oxygenation, emphasizing the relevance of preserving cerebral perfusion even from early hours post trauma. Of significant relevance is to note that RMBF does not apply to the state of CPP but to the preservation of cerebral microcirculation, as demonstrated in this study.

Study Limitations

The biggest limitation of this study relies on the limited capacity to clinically extrapolate conclusions raised from experimental models, in general. However, it is from experimental studies that direct quantification of cerebral microcirculation, as described in this study, becomes feasible. Another important limitation relates to the restricted length of time during which the animals were monitored and studied (4 h postinjury). Hence, although longer monitoring time could have demonstrated a wider range of pathophysiological processes affecting microcirculation; the focus of this study was to capture the early changes in RMBF at specific regions of interest contrasted to the early histopathology changes derived from axonal injury, directly related to trauma.

An additional limitation relates to the small sample size leading to an under-power study. However, it is worth considering that for this longitudinal study design, five observations per sheep (consistent to the five time points during which data were captured) were performed to maximize the statistical power from the relatively small number of subjects. Therefore, the

authors believe that the methodological aspect of this study is robust and valid. Moreover, the aim of this experimental study lays on the fact of assessing a pathological process in a manner that is not clinically possible or feasible; therefore its findings become of clinical relevance and contribute to the pool of current knowledge.

An additional limitation relies on the concept of “severe” head injury and the comparison with a previous study, considered to be representing a “mild” injury. The authors acknowledge that the assumption of “severe” stands upon a theoretical rationale as the method was a reproduction from the previous study with the only variation being the weight of the subjects. So, while the reduction on the quantification of flow is statistically not significant, when combined with the macroscopic and histologic findings the authors can only suggest that these subjects had been subjected to a bigger impact; however, this does not imply being a “severe” head injury as we could not detect sufficient signs to support it.

Finally, the APP staining score was not statistically compared or correlated to the RMBF quantification because it would have been of an arbitrary value. While APP staining is a validated tool used in clinical settings, RMBF quantification is mainly used at experimental scenarios and therefore the authors found no substantial reason to statistically compare both parameters, as the value of that would be of dubious value. Instead, simply contrasting axonal integrity with cytometric quantification of cerebral microcirculation during the acute phase of head injury, offers a theoretical perspective of an otherwise, not approachable method.

CONCLUSION

After severe head injury, cerebral microcirculation at the ipsilateral and contralateral site of a contusion in addition to the ipsilateral thalamus and medulla shows a consistent decline over the first 4 h after injury, when compared with baseline. The widespread reduction on cerebral microcirculation occurs independently of cerebral perfusion pressure and ICP and is present despite cardiovascular stability. Although not statistically significant, a temporal reduction on cerebral microcirculation contrasts with the relatively spared axonal integrity represented by the APP staining; this may suggest that even in relatively severe head injury, microcirculation is globally reduced from baseline.

ETHICS STATEMENT

Approval for this study was obtained from the Animal Ethics Committee of the Queensland University of Technology and the University of Queensland, Australia.

AUTHOR CONTRIBUTIONS

Primary roles: JB led the study design, surgical procedures, data collection, data interpretation, and manuscript preparation. KC undertook the histopathology analysis. KD led all laboratory support and contributed to the manuscript preparation. SD

orchestrated all surgical procedures and data collection. DP and OR performed the intracardiac echography and transeptal catheterization. LG overviewed the manuscript preparation. AB performed the statistical analysis. JP, RB, and JF reviewed the interpretation and manuscript preparation.

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Virtual Reality for Traumatic Brain Injury

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In this perspective, we discuss the potential of virtual reality (VR) in the assessment and rehabilitation of traumatic brain injury, a silent epidemic of extremely high burden and no pharmacological therapy available. VR, endorsed by the mobile and gaming industries, is now available in more usable and cheaper tools allowing its therapeutic engagement both at the bedside and during the daily life at chronic stages after injury with terrific potential for a longitudinal disease modifying effect.

Keywords: traumatic brain injury, virtual reality, brain protection, neurorepair, rehabilitation

INTRODUCTION

The World Health Organization estimates that traumatic brain injury (TBI) is and will remain the most important cause of neurodisability in the coming years (1). The search for neuroprotective therapies for severe TBI has been extensive but unfruitful over the last few decades, testified by more than 30 failed clinical trials, and we still have no specific neuroprotective therapy, that is, effective in clinical TBI. The burden of mortality and residual disability calls for new approaches to promote recovery of function of TBI patients in the acute and chronic phase (2, 3).

Classically described as a sudden event with short-term consequences, TBI induces dynamic pathological cascades that may persist for months or years after injury with a major impact on outcome (4, 5). Among dynamic mechanisms, the neuroinflammatory response and the accumulation of aberrant proteins may have a critical role in establishing a neuropathological link between acute mechanical injury and late neurodegeneration (6, 7). The close association between post-TBI neurological changes, persistent neuroinflammation, and late neuropathology highlights the fact that the window of opportunity for therapeutic intervention may be much wider than previously thought and that long-term treatment encompassing the acute and chronic phase should be tested to effectively interfere with this complex condition.

Importantly, next to the harmful processes, TBI also induces a neuro-restorative response that includes angiogenesis, neurogenesis, and brain plasticity (8, 9). These spontaneous regenerative mechanisms are short-lived and too weak to counteract damage progression but they could point the way to new therapeutic options if appropriately boosted and amplified. Physical and cognitive exercise increase repair and brain plasticity after injury in experimental models and patients (10, 11). Rehabilitative programs to provide inputs/stimuli to specific sensory or motor neural circuits, could in principle start very early on, and be finely tuned over time to account for the type and degree of injury and the level of motor and cognitive disability.

VIRTUAL REALITY (VR) FOR REHABILITATION AFTER TBI

Cognitive and physical rehabilitation programs are fundamental instruments to improve the clinical outcome of TBI patients optimizing the activities, function, performance, productivity, participation, and quality of life (12). They are based on restitutional, compensatory, and adaptive strategies and vary in relation to the patient potential and disability degree (2, 12).

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Traumatic brain injury encompasses heterogeneous etiology, as well as structural and molecular patterns of injury dictating different prognostic features and potential responses to rehabilitative therapy. Experimental studies indicate that depending on the degree of cognitive and sensorimotor impairment exercise may improve outcome with different window of opportunity, however, evidence supporting the optimal timing, type, and intensity of rehabilitative interventions in patients are scarce (12, 13). For example, rehabilitation is often delayed in patients with severe TBI until their discharge from the intensive care unit, or adopted in the most severe cases with only minimal goals aimed at limiting spasticity (14). Importantly, cognitive rehabilitation in the sub-acute stage of TBI is rarely considered. For these reasons, the use of innovative techniques is advocated to assess the TBI-related deficits and to develop and evaluate new rehabilitative interventions (12).

An emerging technology, VR, represents a new tool for this purpose and might provide TBI care teams with new neuro-restorative strategies readily available at the bedside. Since the late 1980s, this term has been used to describe a 3D synthetic environment created by computer graphics, where the user has the feeling of being inside (15). VR can be described as “an advanced form of human-computer interface that allows the user to interact with and become immersed in a computer-generated environment in a naturalistic fashion” (16). For its flexibility, sense of presence (i.e., the feeling of “being there”) and emotional engagement, VR has been tested in motor and cognitive rehabilitation, with good results. In stroke patients, the number of VR programs is rapidly increasing with compelling data showing an improvement in recovery of motor function and daily living activities (17).

Data on the effects of cognitive function and quality of life are more limited. As underlined by two recent systematic reviews (18, 19), VR allows a level of engagement and cognitive involvement, higher than the one provided by memory and imagination, but is more controlled and can be more easily measured than that offered by direct “real” experience. Its multisensory stimulation means VR can be considered an enriched environment that can offer functional and ecological real-world demands (e.g., finding objects, assembling things, and buying stuff) that may improve brain plasticity and regenerative processes (20–22).

There are several examples in the literature where VR has been successfully used both as assessment instrument and as therapeutic intervention. As assessment tool, VR has been used to detect visual-vestibular deficits in adults after concussion and mild TBI (23, 24). Wright WG et al., developed a Virtual Environment TBI Screen that allows subjects to explore a digitalized setting (i.e., outdoor Greek temple with columns, different kind of floor materials, etc.) performing postural tasks while the system collects data to detect visual-vestibular deficits. Besnard et al. (25) created a virtual kitchen to assess daily-life activity and evaluate executive dysfunctions in subjects with severe TBI. Robitaille et al. (26) developed a VR avatar interaction platform to assess residual executive functions in subjects with mild TBI. The platform can capture real-time subject’s movements translating them in to a virtual body, that is, therefore placed in a simulated environment (i.e., a village). The user is then allowed to explore the simulate surroundings which comprise different navigational obstacles to overcome. Similar approaches have been used by other authors,

TABLE 1 | The most commonly virtual reality (VR) systems.

VR systems									
CAVE		PC based			Mobile based			Console based	
User mobility required	Low/medium/high	Low/medium	Low/medium/high	Low/medium/high	Low	Low	Low	Low	Low/medium
System name (cost)	Proprietary (>100,000 \$)	Oculus Rift (500–1,000 \$)	HTC Vive (500–1,000 \$)	Microsoft (200–500 \$)	Samsung Gear VR (<200 \$)	Google Cardboard (<100 \$)	Google Daydream (<200 \$)	Playstation VR (200–500 \$)	Oculus Go (<200 \$)
Hardware requirements (cost)	High end PCs (>1,000 \$)	High end PC (>1,000 \$)	High end PC (>1,000 \$)	Mid level PC (500–1,000\$)	High end Samsung phone (500–1,000 \$)	Middle/high end Android phone or iPhone (<500 \$)	High end Android phone (<500 \$)	PS4 or PS4 Pro (<500 \$)	None
Body tracking	High	Medium	High	Medium	Low	Low	Low	Medium	Low
User interaction with VR	High	High	High	High	Medium	Low	Medium	High	Medium
Software availability	Custom build applications	Oculus store	Steam store	Windows store	Oculus store	Google Play or IOS store	Google Play	Playstation store	Oculus store
									Google Play

The costs are based on information provided by system vendors in most European and North-American countries and they are expressed in USA dollars. User mobility required: low: static position, medium: limited movements in the space, high: active movements in the space. Body tracking: low: head tracking (rotation), medium: head tracking and positional tracking (forward and backward), high: head tracking and volumetric tracking (up to full room size movements). User interaction with VR: high: using joystick and/or controllers, medium: using gaze, a built in pad or joystick, low: using gaze or a bottom. CAVE: cave automatic virtual environment; PC: personal computer.

whereas simplified settings (i.e., 3D virtual corridor that the subject can explore with a joystick) have been proved useful to assess subclinical cognitive abnormalities in asymptomatic subjects that suffered a concussion (27).

As therapeutic instrument, Dahdah et al. (28) demonstrated that immersive VR intervention can be used as an effective neuro-rehabilitative tool to enhance executive functions and information processing in the sub-acute period, providing evidence of positive effects of a virtual Stroop task over traditional non-VR-based protocol. VR as therapeutic instrument has also been used for attention training in severe TBI with positive results in the early recovery stages (29) with a specific “augmented” task in which virtual and haptic feedbacks were used in a target-reaching exercise to enhance sustained attention. Finally, virtual protocols generated upon commercial available game solutions have been effective in addressing and treating balance deficits (30).

All these works suggest that VR could be useful as assessment instrument and in the rehabilitation of TBI, nonetheless a delineated pattern seems to emerge. VR assessment protocols appear to be primarily implemented for mild TBI, which induce subtle residual deficits hard to detect with traditional instruments (23). Conversely, VR treatment protocols for cognitive rehabilitation are used transversely from mild to severe conditions, although effectiveness of these kinds of interventions needs to be further explored (31).

LIMITATIONS AND FUTURE DIRECTIONS

The use of VR in clinical practice has been limited by two main factors: accessibility and the cost of virtual tools. Nevertheless, VR technology is advancing quickly. Oculus Rift® and HTC Vive™ have showcased high-quality VR experiences at reasonable prices—less than \$3,000 for a fully configured system—that should be widely available to consumers within this year (32), and even more affordable solutions based on smartphones and tablets are on the way (see **Table 1**). New interaction paradigms, like eye tracking, are allowing the use of VR also at the bedside in patients with limited mobility (32). The potential for activity-dependent structural and functional brain remodeling in behaviorally unresponsive brain-injured patients for up to 5 years has recently been shown (33).

Literature evidence suggested that VR protocols can provide innovative assessment and treatment options for TBI, nonetheless possible limitations connected to perception of VR technology and usability, especially in older adults must be taken into account. TBI has a second peak of incidence in the elderly (2). This introduces a challenge related to the limited experience that elderly subjects have with new technological devices. However, evidence indicated the feasibility of VR interventions in elderly

across different pathologies (34) even with active compromised spatial abilities and degenerative cognitive diseases (35), whereas different learning curves due to age-related differences have been effectively addressed through a training phase assisted by an expert (34). Finally, common side effect of VR intervention (i.e., motion sickness and disorientation) did not appear to be specifically related to age (36), thus supporting the feasibility of VR protocols in aged patients.

Time is a key issue in TBI, with a window of vulnerability and opportunity that appears much wider than previously thought: this provides an incentive to look for continuous long-lasting therapeutic interventions to interfere with neurodegenerative processes and promote regeneration. From this viewpoint, VR offers a new strategy to boost and amplify restorative processes in the clinical setting at early stages of the disease, and in daily life at later stages (26). As discussed, VR allows the development of real-life, context-specific experiences, requiring the control of the individual over different cognitive sensorimotor, and social factors, which are usually difficult to reproduce in a clinical setting. For example, VR is effective in assessing a patient's ability to perform everyday activities like cooking in a virtual kitchen, driving a virtual car, or shopping in a virtual supermarket. In these challenging but ecologically valid VR environments, behaviors can be assessed and trained while maintaining experimental control over stimulus measurement and delivery.

In general, the greatest long-term burden to patients are deficits in cognition and behavior (5). Here too, later VR interventions, with a focus on memory, attention, executive function, behavioral control, and regulation of mood, may be helpful in reducing the long-term problems and disabilities experienced by subjects after a TBI.

CONCLUSION

In conclusion, VR has the potential for improving the assessment and treatment of TBI even in cases where the chances of recovery appear poor. The mobile and gaming industries are now significantly endorsing this technology, producing more and more usable and cheaper tools, that can be employed even at the bedside. Thus, collaboration between clinicians, researchers, and technology developers is required to produce VR tools that can fully exploit the terrific potential of this technology in TBI patient.

AUTHOR CONTRIBUTIONS

EZ, TZ, DL, and GR contributed to the conception and writing of the manuscript. All authors read and approved the final manuscript.

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Extended Erythropoietin Treatment Prevents Chronic Executive Functional and Microstructural Deficits Following Early Severe Traumatic Brain Injury in Rats

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Survivors of infant traumatic brain injury (TBI) are prone to chronic neurological deficits that impose lifelong individual and societal burdens. Translation of novel interventions to clinical trials is hampered in part by the lack of truly representative preclinical tests of cognition and corresponding biomarkers of functional outcomes. To address this gap, the ability of a high-dose, extended, post-injury regimen of erythropoietin (EPO, 3000U/kg/dose × 6d) to prevent chronic cognitive and imaging deficits was tested in a postnatal day 12 (P12) controlled-cortical impact (CCI) model in rats, using touchscreen operant chambers and regional analysis of diffusion tensor imaging (DTI). Results indicate that EPO prevents functional injury and MRI injury after infant TBI. Specifically, subacute DTI at P30 revealed widespread microstructural damage that is prevented by EPO. Assessment of visual discrimination on a touchscreen operant chamber platform demonstrated that all groups can perform visual discrimination. However, CCI rats treated with vehicle failed to pass reversal learning, and perseverated, in contrast to sham and CCI-EPO rats. Chronic DTI at P90 showed EPO treatment prevented contralateral white matter and ipsilateral lateral prefrontal cortex damage. This DTI improvement correlated with cognitive performance. Taken together, extended EPO treatment restores executive function and prevents microstructural brain abnormalities in adult rats with cognitive deficits in a translational preclinical model of infant TBI. Sophisticated testing with touchscreen operant chambers and regional DTI analyses may expedite translation and effective yield of interventions from preclinical studies to clinical trials. Collectively, these data support the use of EPO in clinical trials for human infants with TBI.

Keywords: controlled cortical impact, diffusion tensor imaging, diffusivity, infant, touchscreen, cognition, cognitive flexibility

INTRODUCTION

Traumatic brain injury (TBI) is the leading cause of mortality and morbidity for full term infants who are born healthy (1, 2). Pediatric TBI exacerbates social and economic burden throughout the lifespan, and pediatric inpatients accrue an estimated >\$1 billion in total charges for TBI-associated hospitalizations (2, 3). Young children (0–4 years) have the highest rates of TBI of any pediatric age group, though the end result of severe TBI only fully manifests as the central nervous system (CNS) fails to mature with an appropriate developmental trajectory (1, 3, 4). Indeed, children who survive early TBI are at risk for numerous chronic neurological deficits, including impairments in cognition and executive function (3, 5).

Despite the burden of severe chronic sequelae after infant TBI, no treatments are available to enhance the repair of the injured developing brain, beyond supportive therapy offered with typical critical care. A potential emerging intervention for infant TBI is erythropoietin (EPO) (6–9). EPO and its receptor EPOR, have important roles in the nervous system, independent of its hematopoietic actions (10–16). In healthy humans and rodents, recombinant EPO improves cognition and increases hippocampal long-term potentiation (17, 18). EPO is effective after numerous types of insults in the adult CNS (19–21) including TBI (22, 23). Prior data indicate that EPO crosses the blood-brain barrier via non-receptor mediated transport in both humans and rodents (10, 24). After perinatal brain injury, neural cell EPOR expression increases without concomitant EPO ligand expression (6, 7, 25–28), suggesting that exogenous EPO is potentially more effective in the developing CNS. Notably, without ligand present, unbound EPOR triggers neural cell death and exogenous EPO restores balanced EPOR signaling supporting neural cell development (12, 13, 27, 28). Previously, we have reported that extended EPO treatment after infant TBI on postnatal day 12 (P12) in rats, facilitates widespread repair of both gray and white matter, with concomitant prevention of motor deficits (7), similar to reports by other labs demonstrating that EPO improves recognition memory, hippocampal volume, and reduces cell death following TBI in a model of older pediatric TBI on P17 (8, 9).

Translation of emerging neuroreparative agents has been challenging following pediatric TBI, in part due to limited investigation using sophisticated preclinical platforms and outcome measures capable of detecting executive function and chronic diffusion tensor imaging (DTI) abnormalities (1, 29, 30). Further, a lack of sensitive, quantitative outcome measures has been implicated in the failure to detect meaningful differences in clinical improvement in TBI clinical trials (31–33). As neurodevelopmental tests are typically designed to compare age-equivalent groups of infants, and infants suffer TBI at various ages, current neurodevelopmental scales have been deemed inadequate to capture subacute (30 day) and chronic (6 month) outcomes for early phase trials in infant TBI. Thus, sensitive, reliable and reproducible, quantitative imaging measures of damage and recovery can potentially fill this void, and act as a surrogate biomarker for injury and repair. Accordingly,

to fill these gaps in knowledge and more rigorously test the efficacy of potential therapeutic strategies for infants with TBI prior to translation to clinical trials, we tested the hypothesis that a touchscreen platform, analogous to the Cambridge Neuropsychological Test Automated Battery (CANTAB) in humans, could detect sophisticated differences in cognition in rats following early TBI and neurorepair with extended EPO treatment. Taken together, our data demonstrate for the first time the feasibility of sophisticated touchscreen testing of pillars of cognition in a preclinical model of severe infantile TBI. Moreover, we report that extended EPO treatment prevents cognitive and executive function deficits, and concomitant chronic and correlative DTI abnormalities in adult rats following infant TBI.

METHODS

All procedures were performed in accordance with NIH Guide for the Care and Use of Laboratory Animals and were approved by Institutional Animal Care and Use Committees at the Boston Children's Hospital and the University of New Mexico Health Sciences Center. For each experiment, balanced numbers of male and female pups were used, and data represent true *n* (individual pups) from at least 2 different dams. All investigators were blinded to injury and treatment group during the conduct and analyses of each experiment. A power analysis was performed to estimate required sample size (G*Power 3.1.9.3) using published and preliminary data to define expected means and standard deviations for each group (7). We determined the number of samples needed for 80% power in a two-way design to establish the effect of EPO treatment. In order to detect as 20% change with 20% error, an α of 0.05, the number of animals required was 6. Separate cohorts of rats were used for imaging studies at 30 days, and touchscreen plus imaging at 90 day evaluations. For the DTI, primary outcomes were fractional anisotropy, mean diffusivity, axial diffusivity and radial diffusivity in anatomically defined regions of interest. For touchscreen analyses, the primary outcome measures were the number of errors to reach passing criteria, and number of sessions to reach passing criteria in visual discrimination. Secondary outcomes were reaction time and magazine latency. For reversal learning, the primary outcomes were percent passing, and number of correction trials. Secondary outcomes were sessions to passing criteria, errors to passing criteria, correction trials during perseveration, correction trials during learning, reaction time and magazine latency. Visual representation of our experimental design, including the progression through touchscreen stages is provided in **Figure 1A**.

Controlled Cortical Impact

Controlled cortical impact (CCI) was delivered to Sprague-Dawley rat pups on P12 (7). Briefly, rat pups were anesthetized with isoflurane and a 5 mm diameter left craniectomy was performed. Heads were fixed in the prone position, and an air-powered piston (3 mm diameter, Amsci, Richmond, VA) delivered a CCI of 0.6 mm depth, at 6 m/s to the left parietal lobe. Pups were reared with their dams until P21, then weaned

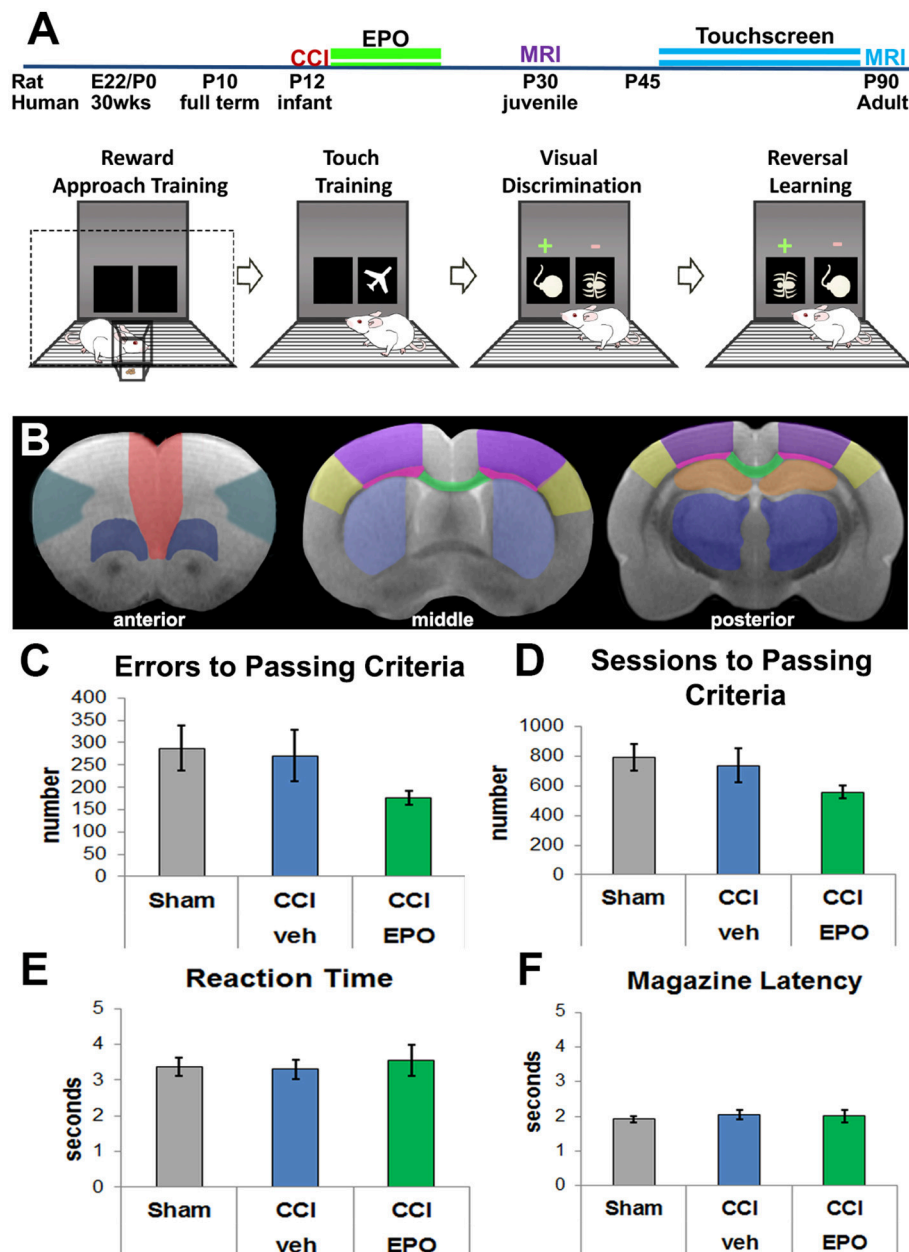


FIGURE 1 | Experimental details and visual discrimination. **(A)** Experimental paradigm including pictorial summary of touchscreen training, visual discrimination, and reversal learning. Rats are first habituated to the food rewards and the touchscreen itself, and then progress to assessment of visual discrimination and cognitive flexibility. Arrows indicate passing of training and testing stages based on *a priori* criteria. The two MRI cohorts are represented by different colors. **(B)** Regions of interest for DTI analyses *anterior* (corresponding to Bregma 3.72 mm): red—medial prefrontal; teal—lateral prefrontal; dark blue—ventral prefrontal; middle (Bregma -0.12 mm) and posterior (Bregma -3.00 mm): yellow—barrel cortex, purple—lesion cortex, neon pink—lateral white matter, green—corpus callosum, light blue—striatum, orange—hippocampus, dark blue—thalamus. **(C)** All 3 groups committed a similar number of errors prior to passing visual discrimination (VD), demonstrating that injured rats can complete the VD task ($n = 6-8$). **(D)** Likewise, all 3 groups required a similar number of sessions to performance criteria. **(E)** Rats in all 3 groups displayed a similar reaction time to respond to touchscreen stimuli. **(F)** Rats also demonstrated a similar motivation to retrieve the reward for a correct response.

and housed in single sex pairs. Housing room lights were on from 7 a.m. to 7 p.m., with food and water available *ad libitum*. Temperature was maintained at $21 \pm 1^\circ\text{C}$ and humidity at $55 \pm 5\%$.

Erythropoietin (EPO) Administration

Injured rats were randomized 24 h following CCI to receive either EPO (3000 U/kg/day, R&D Systems, Minneapolis, MN) or vehicle (sterile saline) (7). EPO or vehicle was injected

intraperitoneally once daily on days 1, 2, 3, 4, 6, and 8 following CCI.

Touchscreen Testing: Pretraining

Visual discrimination (VD) and reversal learning were assessed consistent with prior reports, with minor modifications for rats (34–37). Briefly, operant behavior was conducted in a sound and light attenuating chamber (Med Associates, St. Albans, VT), with a pellet dispenser and a touch-sensitive screen (Conclusive Solutions, Sawbridgeworth UK). Stimulus presentation in the response window and touches were controlled and recorded by KLimbic Software (Conclusive Solutions, Sawbridgeworth UK).

On P28, rats were reduced to and maintained at 90% free-feeding body weight. Prior to training, rats were acclimated to 40 mg food pellet reward by provision of 25 pellets/rat in a home cage. Rats were then habituated to the operant chamber and to eating from the pellet magazine. Rats retrieving at least 48 of 60 pellets in 60 min were moved to a 4-stage training regimen. Beginning on P35, rats first performed autoshaping, followed by VD training 1, 2, and 3 (36).

Touchscreen Testing: Discrimination and Reversal Learning

Following pretraining, all rats were tested on a pairwise visual discrimination-reversal paradigm. Each rat performed daily sessions for a maximum of 60 min. For discriminative learning, two novel equiluminant stimuli were presented in a spatially pseudo-randomized manner over 60-trial sessions (5 s inter-trial interval). Responses at one stimulus yielded a reward, whereas responses at the other stimulus resulted in 5 s time out (signaled by extinguishing the house light). Designation of the initial reward stimulus was randomized across treatment. Stimuli remained on screen until a response was made. Rats were trained *a priori* to a criterion of greater than $\geq 80\%$ correct responses for two consecutive days.

Assessment of reversal learning began after VD performance criteria were attained. For this test, the designation of stimuli as correct vs. incorrect was reversed for each rat. Like VD, rats were tested on daily 60-trial sessions for reversal to an *a priori* criterion of $\geq 80\%$ correct responses for two consecutive sessions. Correction trials following errors were presented, with the same stimuli, in the same spatial orientation, until a correct response was made, or the session ended. Failing criteria were set *a priori* at 24 sessions (days) for both VD and reversal.

We recorded the following dependent measures during VD and reversal: total sessions, correct responses made, errors (incorrect responses), correction errors (correction trials, reversal only), reaction time (time from touchscreen stimuli presentation to touchscreen response) and magazine latency (time from touchscreen response to reward retrieval). Discrimination performance was analyzed across all sessions required to reach criterion. To examine distinct phases of reversal (early perseverative and late learning), we analyzed errors and correction errors for sessions where performance was $< 50\%$ and from 50% to criterion, respectively (36–39).

Magnetic Resonance Imaging (MRI)

At P30 (1 month of age) or P90 (3 months of age), rats were anesthetized and perfused with phosphate-buffered saline, followed by 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde for 1 week, and embedded in 2% agarose containing 3 mM sodium azide for *ex vivo* MRI (7, 40–42). Imaging was performed on a Bruker 4.7T BioSpec 47/40 Ultra-Shielded Refrigerated nuclear MRI system equipped with a 72 mm I.D. quadrature RF coil and a small-bore (12 cm I.D.) gradient set with a maximum gradient strength of 50 Gauss/cm. MR protocols consisted of a echo-planar diffusion tensor imaging (EP-DTI). Images of 12 contiguous coronal 1 mm slices were obtained with a FOV (field-of-view) of 3.00 cm, a TR of 3,000 ms, TE of 40 ms, and *b*-value of 2,000 mm²/s with 30 gradient directions. Brain regions of interest (ROI, **Figure 1B**) were analyzed by observers blinded to treatment status using Bruker's Paravision 5.1 software. Fractional Anisotropy (FA), Mean Diffusivity (MD, $(\lambda_1 + \lambda_2 + \lambda_3)/3$), Axial Diffusivity (AD, λ_1) and Radial Diffusivity (RD, $(\lambda_2 + \lambda_3)/2$) were calculated and analyzed.

Statistical Analysis

Normal distribution was verified in all data sets with Shapiro-Wilk test, with Levene's test to confirm homogeneity of variances. For comparison of nonparametric data (performance criteria), Kruskal-Wallis test with Dunn's *post-hoc* test was performed. For analysis of more than two groups with parametric data (sham, CCI-veh and CCI-EPO), two-way ANOVA (injury X treatment) was performed with Bonferroni's *post-hoc* correction for multiple comparisons using SPSS 21 (IBM, Armonk, NY). To test the strength of correlation between the DTI scalars and the primary cognitive outcome, the number of correction trials on reversal testing, Pearson correlations were calculated. The correlations between the P90 MRI and correction trials were performed using the data from each rat. Because the P30 DTI data was from a separate cohort of rats than the adult rats with cognitive data, the P30 imaged brains were randomly assigned within each group to animals undergoing cognitive testing. This process of random assignment followed by correlational analysis was repeated 5 times. Only those ROI that showed significant Pearson correlation on all 5 random assignments AND showed repair of EPO at P30 were considered robust (41). The correlations for a representative random assignment are shown in **Table 1**. For all analyses, $p < 0.05$ was considered significant.

RESULTS

Adult Rats Subjected to Infant TBI Can Perform VD

We first validated the touchscreen platform in our infant TBI model and assessed whether adult rats subjected to infant CCI could perform VD. Because rats at P10 are approximately equivalent to human infants at term (43, 44), P12 CCI is approximately equivalent to human impact TBI at a few months of age (7). Adult rats in all three groups (sham $n = 8$, CCI-veh $n = 7$, CCI-EPO $n = 6$) successfully completed all aspects of touchscreen habituation and training by P42. Next, we assessed

TABLE 1 | Pearson correlation coefficients between diffusion abnormalities repaired by EPO at P30 and poor cognitive flexibility in adulthood.

		Ipsilateral	Contralateral
MEAN DIFFUSIVITY			
Corpus callosum	0.685 $p < 0.001$		
Ventral prefrontal cortex		0.757 $p < 0.001$	0.758 $p < 0.001$
Striatum			0.772 $p < 0.001$
Hippocampus			0.703 $p < 0.001$
Thalamus		0.756 $p < 0.001$	0.783 $p < 0.001$
AXIAL DIFFUSIVITY			
Corpus callosum	0.644 $p = 0.001$		
Medial prefrontal cortex		0.660 $p = 0.001$	
Ventral prefrontal cortex		0.707 $p < 0.001$	0.727 $p < 0.001$
Lateral prefrontal cortex		0.567 $p = 0.006$	
Thalamus		0.663 $p = 0.001$	
RADIAL DIFFUSIVITY			
Corpus callosum	0.713 $p < 0.001$		
Medial prefrontal cortex			0.693 $p < 0.001$
Ventral prefrontal cortex		0.763 $p < 0.001$	

cognitive performance on VD. All rats were able to successfully perform VD. Specifically, 100% of sham and CCI-EPO rats achieved performance criteria, while 83% of CCI-veh rats passed VD. Of those that completed VD, rats across all treatment and injury groups displayed similar numbers of errors and required similar numbers of sessions to pass (Figures 1C,D). Similarly, all rats had comparable reaction time and magazine latency throughout the VD paradigm (Figures 1E,F). Together, these data indicate that rats suffering TBI as pups had the cognitive capacity as adults to complete VD testing.

Extended EPO Treatment Prevents Deficits in Cognitive Flexibility Induced by Infant TBI

After successful completion of VD, rats in all groups were evaluated for reversal learning. CCI-veh rats were significantly impaired, and fewer CCI-veh rats passed the reversal-learning paradigm compared to sham and CCI-EPO rats (Figure 2A). Only 57.1% of CCI-veh animals successfully passed criteria compared to 100% of Sham, and 83.3% of CCI-EPO treated animals. Notably, CCI-veh animals required more correction trials ($1,114 \pm 132$) compared to sham (730 ± 95 , two-way ANOVA with Bonferroni's correction, $p = 0.039$) and CCI-EPO (652 ± 61 , $p = 0.029$, Figure 2B). As expected, CCI-veh animals also required significantly more sessions (Figure 2C) and committed significantly more errors (Figure 2D) to achieve passing criteria. Thus, poor cognitive flexibility in adult rats can be prevented after early TBI by an extended post-injury EPO dosing regimen.

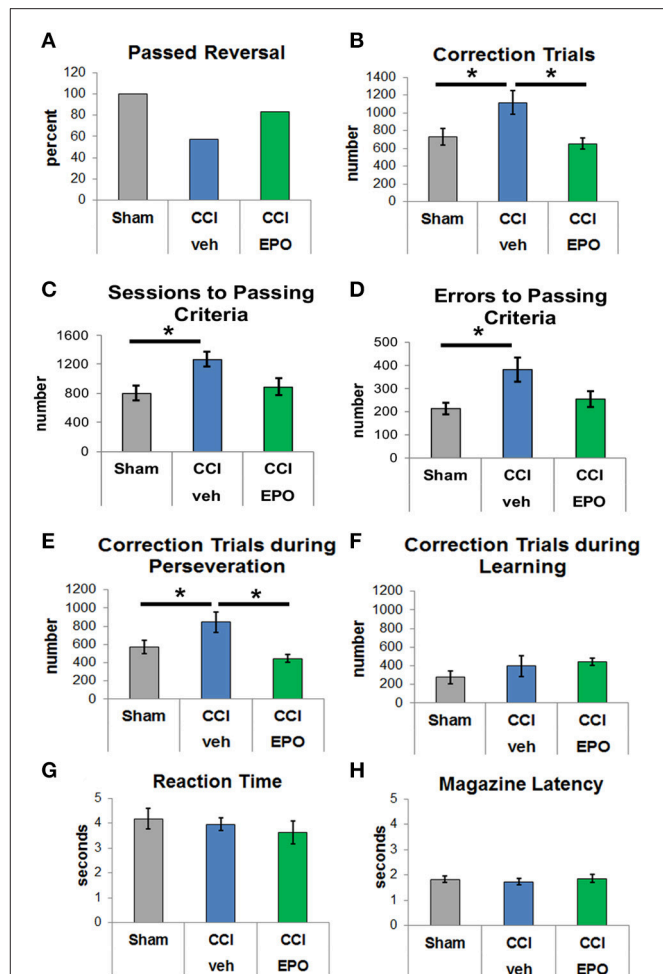


FIGURE 2 | EPO treatment resolves deficits in cognitive flexibility. (A) Fewer vehicle-treated CCI rats passed reversal compared to shams or EPO-treated rats. (B) Vehicle-treated CCI rats required at least 50% more correction trials than shams or EPO-treated CCI rats (two-way ANOVA with Bonferroni's correction). (C) Vehicle-treated CCI rats also required more sessions to achieve passing criteria. (D) Similarly, vehicle-treated CCI rats committed more errors prior to reaching passing criteria. (E) Importantly, vehicle-treated CCI rats showed significant perseveration and required more correction trials during the perseverative phase than shams or EPO-treated CCI rats. (F) By contrast, rats from all 3 groups showed no difference during the learning phase. (G) Similar to VD, rats in all 3 groups demonstrated similar reaction times. (H) Rats in all three groups exhibited similar motivation to retrieve a reward for a correct response ($n = 6-8$, $*p < 0.05$).

Upon establishing this acquired deficit in cognitive flexibility that was ameliorated with EPO treatment, we analyzed different stages of learning. Specifically, we examined phases of reversal, including the perseverative reversal ($<50\%$ correct) and relearning phase ($>50\%$ correct). Infant TBI significantly increases maladaptive perseveration during reversal learning (Figure 2E). CCI-veh rats showed high levels of perseveration by responding to the previously rewarded stimulus over several sessions before re-attaining chance. Importantly, the significant increase in correction trials in CCI-veh rats, a measure of perseveration, was prevented with extended post-injury EPO

treatment (Figure 2E). During the relearning phase, performance was intact across all three groups with no difference in correction trials (Figure 2F). Changes observed were not due to motivation to respond or retrieve reward, as measured by reaction and reward response times, on either phase of the reversal paradigm (Figures 2G,H). Together, these data emphasize that early TBI affects executive function, specifically cognitive flexibility, and that post-injury EPO treatment results in sustained improvement in cognition.

Extended EPO Treatment Yields Sustained Repair of Microstructural Brain Injury

DTI was performed to more specifically quantify the extent of injury from infant CCI, and the efficacy of EPO treatment on microstructural brain injury. First, we quantified subacute injury in rats following infant CCI at P30, approximately 2.5 weeks after P12 infant TBI. Detailed regional analyses of DTI parameters revealed widespread microstructural abnormalities involving the prefrontal cortex, striatum, corpus callosum, hippocampus and thalamus. Similar to prior findings of diffusion abnormalities in bilateral lesional cortex and subcortical white matter after CCI (7), we found widespread reductions in FA in striatum and corpus callosum (Figure 3), and hippocampus and thalamus following injury (CCI-veh $n = 8$),

compared to shams ($n = 6$) (Supplemental Figure 3). We also observed robust increases in MD in these regions, including the prefrontal cortex in CCI-veh (Figure 3). Significantly, EPO treatment ($n = 8$) prevented diffusion abnormalities ipsilateral and contralateral to CCI, and normalized MD, AD, and RD in the corpus callosum (Figures 3I–L). EPO treatment also prevented bilateral abnormalities in directional diffusion in the prefrontal cortex, hippocampus and thalamus (Supplemental Figures 1–3). To determine whether subacute alterations in FA and diffusivity predicted later executive function, we tested the correlation between correction trials during reversal learning with DTI metrics at P30. We found robust correlation between cognitive performance and injury following infant TBI in distinct and diverse brain regions essential for cognition including white matter, prefrontal cortex and deep gray matter that are repaired with EPO (Table 1, Supplemental Figure 4).

We next performed DTI on rats at 90 days, immediately following completion of touchscreen assessments (Figures 4, 5). Color maps showed chronic loss of directionality and confirm long-term reductions in structural coherence in cortex and subcortical white matter ipsilateral to CCI, with improvement following EPO treatment (Figure 4). Specifically, FA in corpus callosum and lateral white matter is reduced in CCI-veh

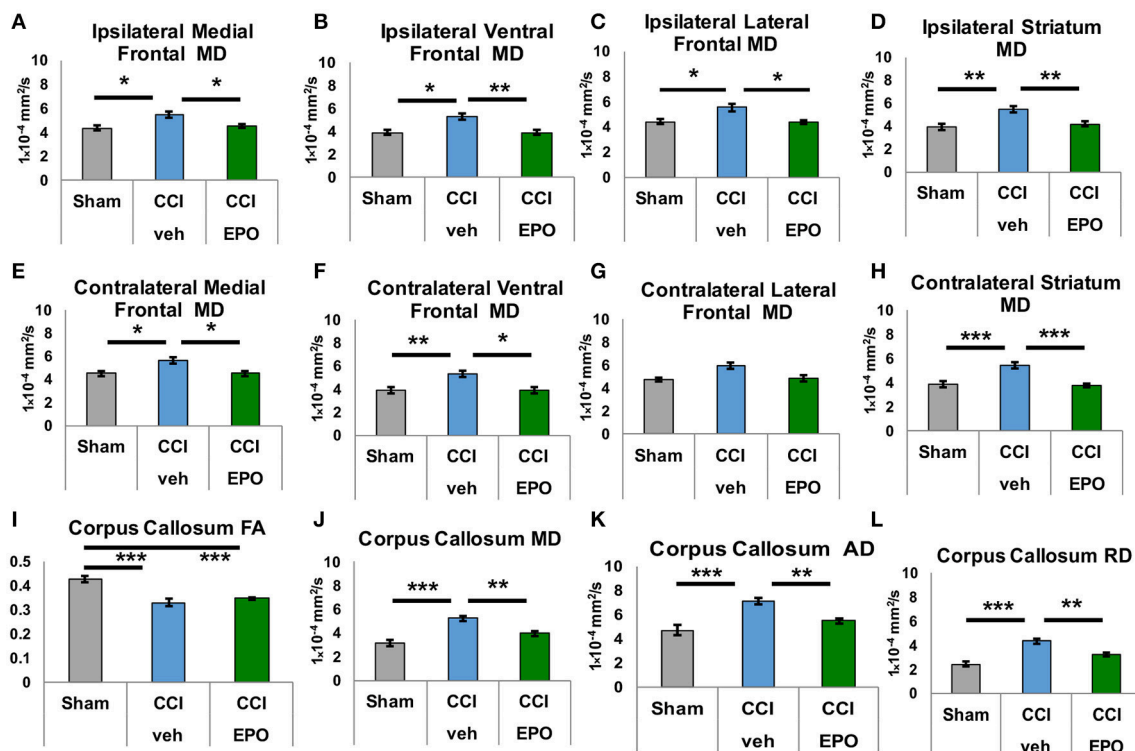


FIGURE 3 | Extended EPO treatment after CCI prevents gray and white matter diffusivity abnormalities in juvenile rats at P30. (A–C) Ipsilateral medial, ventral and lateral prefrontal cortices exhibit abnormal mean diffusivity (MD) after CCI that is prevented by EPO treatment. (E,F) Similarly, contralateral medial and ventral prefrontal cortices also show abnormal MD that is prevented by EPO treatment. (G) Contralateral lateral prefrontal cortex is spared by the injury. (D,H) CCI damages deep gray matter striatum MD bilaterally, and EPO treatment prevents these abnormalities. (I) CCI causes loss of FA in white matter corpus callosum that EPO treatment cannot prevent. (J–L) CCI induces abnormal mean, axial and radial diffusivity in the corpus callosum that extended EPO treatment prevents ($n = 6–8$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

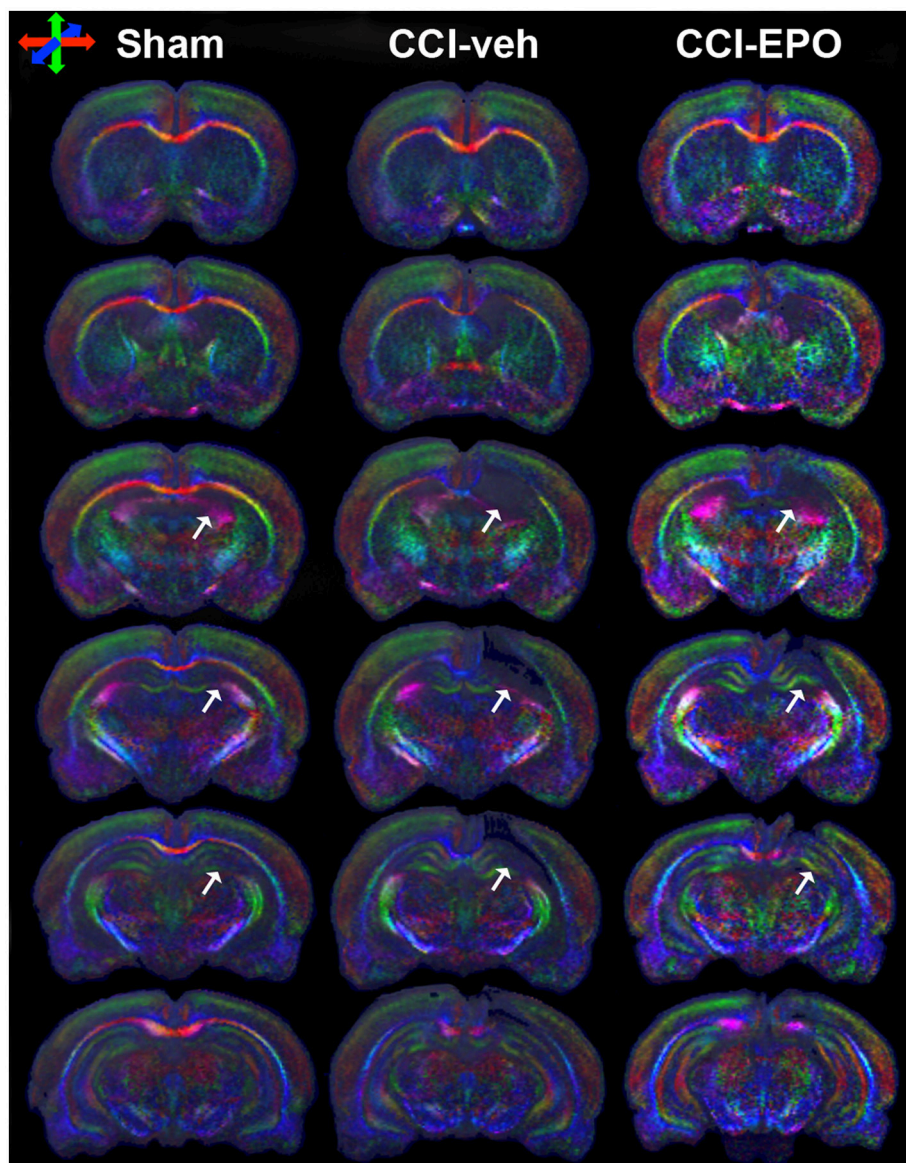


FIGURE 4 | DTI directionally encoded color maps in the coronal plane at P90 demonstrate loss of microstructure in vehicle-treated CCI rats that is not present in EPO-treated CCI rats. White arrows highlight the loss of microstructure and diffusion abnormalities in CCI-veh rats compared to shams, and partial improvement with EPO treatment. Impaired diffusion and loss of microstructure causes loss of directional coherence. (Red–transverse, green–vertical, and blue–orthogonal to the plane).

animals ($n = 7$) compared to shams ($n = 7$). Significantly, EPO treatment ($n = 8$) prevented abnormal diffusion in the corpus callosum and contralateral subcortical white matter at 90 days (**Figure 5B,C**). Assessment of the prefrontal cortex confirms loss of FA in the lateral prefrontal regions ipsilateral to impact (**Figure 5D**), without changes contralateral to impact (**Figure 5E**). Notably, EPO treatment normalized persistent abnormalities in FA in prefrontal regions after infant TBI (**Figures 5D,G**). Together, these results suggest that DTI may reflect damage and recovery of the developing brain following early TBI.

To determine if chronic improvement in microstructural integrity in adult (P90) rats indicated improved cognitive flexibility on touchscreen performance, we tested the correlation between correction trials during reversal learning and prevention of FA abnormalities on 90 day DTI. Indeed, extended EPO treatment repair of FA in the contralateral white matter showed significant correlation with executive function (Pearson coefficient -0.532 , $p = 0.013$), and the corpus callosum demonstrated a similar, non-significant trend (-0.411 , $p = 0.058$). Moreover, the improvement in FA in the ipsilateral lateral prefrontal cortex (-0.471 , $p = 0.042$) and medial

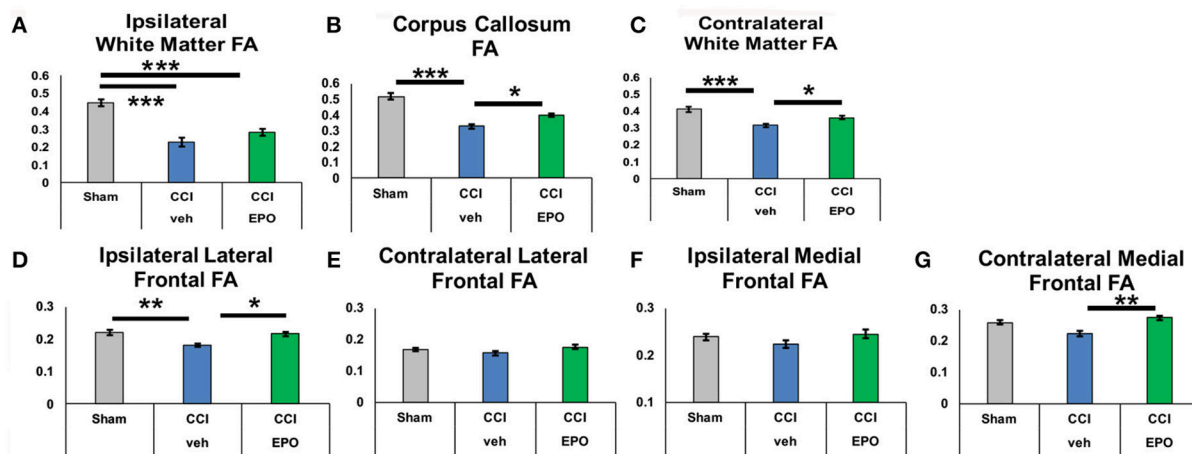


FIGURE 5 | At P90, infant CCI causes widespread chronic damage to the white matter and the ipsilateral lateral prefrontal cortex, and extended EPO treatment prevents sustained injury at a distance from the injury site. **(A)** CCI causes loss of FA in ipsilateral white matter that is not prevented by EPO treatment. **(B,C)** By contrast, white matter loss of FA caused by CCI in the corpus callosum and contralateral white matter is prevented by extended EPO treatment. **(D)** Similarly, EPO treatment after CCI prevents loss of FA in the ipsilateral lateral prefrontal cortex. **(E–G)** CCI does not cause sustained loss of FA in the ipsilateral medial prefrontal cortex, or the contralateral lateral and medial prefrontal cortex ($n = 6–8$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

prefrontal cortex also correlated with improved cognitive performance (-0.557 , $p = 0.011$). Thus, resolution of chronic microstructural abnormalities in white and gray matter DTI is related to improved cognitive performance in adult rats following infant TBI, supporting that DTI may be useful as a subacute and chronic biomarker of cognitive outcomes after infant TBI.

DISCUSSION

In our established model of moderate-severe infantile TBI, extended post-injury EPO treatment prevented widespread bilateral gray and white microstructural injury, concomitant with improved cognitive flexibility. To our knowledge, this is the first demonstration using a translatable, rigorous, touchscreen platform for cognitive testing in a preclinical model of pediatric TBI. Furthermore, EPO, administered in a clinically appropriate extended dosing paradigm compatible with its mechanisms of action, proved efficacious in preventing chronic functional impairment and microstructural brain injury in adult rats following infant TBI. These results emphasize age-appropriate preclinical models with human clinical trial-compatible imaging biomarkers and functional outcome measures. Given the tremendous need for safe, efficacious therapies for neurorepair in the developing CNS after TBI, these data lend support to the testing of EPO in early phase clinical trials for infants with TBI.

Previously, we and others have shown that EPO treatment following perinatal brain injury promotes the genesis, survival, and differentiation of neural cells in the developing and mature CNS, and reduces calpain-mediated injury (7, 19, 21, 35, 40, 41, 45–47). Calpain degrades CNS molecules and proteins essential for the formation of cerebral circuits, including

neurofilaments, myelin basic protein and the potassium-chloride transporter, KCC2 (7, 45, 47, 48). Indeed, it is through EPO's signaling action on structural and functional connectivity, neural networks, and excitatory/inhibitory balance of fundamental circuitry that EPO therapy may improve motor and cognitive function following early brain injury, including TBI. Recently, it has been demonstrated that EPO has an additional novel mechanism in regulation of homeostatic plasticity and synaptic strength (17). Together, with previous reports of EPO's modulation of inhibitory circuitry in brain regions key to higher order brain function and structural connectivity (41, 49, 50), and the beneficial impact on neuronal and oligodendroglial differentiation (35, 46, 51), this effect on synapses provides an additional novel molecular mechanism supporting the improvement in cognition and behavior shown here, and the normalization of the trajectory of brain development after perinatal injury. Using this model of infant TBI, we have reported gait deficits, associated serum and inflammatory brain biomarker abnormalities, and a specific subset of MRI changes that are circumvented with extended EPO treatment (7). Here, we significantly expand on these data by demonstrating that infant TBI leads to impairments in executive control that persist into adulthood. These higher-order cognitive processes, which include attention, working memory, future planning and behavioral flexibility, are essential to adult independence and function in an ever-changing environment (37). Notably, development of executive functions depends on prefrontal cortex maturation and integrity, which provide top-down guidance of posterior cortical and subcortical regions (1, 52). Our analyses of pairwise VD learning showed that rats with infant TBI could learn the paradigm comparable to shams. By contrast, adult CCI-veh rats were significantly impaired on reversal learning, consistent with diminished cognitive flexibility. Extended

EPO administration after injury normalized adult cognitive performance following TBI and offset perseverative behaviors. Notably, perseverative reversal learning in rodents is mediated by cortical subregions, particularly the lateral prefrontal cortex (37, 53, 54), and DTI following the conclusion of touchscreen testing at 90 days revealed amelioration of abnormal diffusivity in both the lateral prefrontal cortex ipsilateral to impact and in the corpus callosum. The corpus callosum is adjacent to frontal regions, and optimal early reversal in the touchscreen paradigm specifically recruits lateral prefrontal cortex, a region functionally necessary for behavioral flexibility in mice (38, 55). Significantly, microstructural and diffusion injury observed in prefrontal cortex here was attenuated by extended post-injury EPO treatment concomitant with improved structural connectivity in multiple essential networks, emphasizing a putative mechanism for the improvement in executive function observed in our studies.

Touchscreen operant chamber platforms for rodents offer an opportunity to use analogous testing paradigms in humans and preclinical models (34, 56). Touchscreen platforms have been used in humans and rodents to test cognitive deficits related to genetic mutations (38, 39, 57, 58), psychiatric disorders (56, 59) and adult TBI (60, 61). However, to our knowledge, use of touchscreen platforms has not been previously been reported for assessing cognition in pediatric TBI. Similarly, while other investigators have shown EPO optimizes cognitive performance in adult rodents with and without brain injury (22, 35), and examined recognition memory following TBI in the immature brain and subacute recovery with EPO treatment (8, 9), the profile of intact discrimination learning and increased maladaptive perseveration shown here via touchscreen is novel. Indeed, together these data support that early TBI extensively alters CNS development, including frontal cortex, major white matter tracts, and fibers of passage in the parietal and the thalamic relays. Additionally, our data supports a loss of top-down precortical control of striatal subregions (37). The striatum receives input from multiple brain areas including prefrontal cortex, and is fundamental in set-shifting, inhibition and cognitive flexibility (1, 62, 63). We also observed a decrease in bilateral striatal FA at 30 days, together with increased MD. These findings corroborate similar findings in children following TBI who exhibited poor cognition and ventral striatal DTI abnormalities (1). EPO treatment also resolved increases in striatal MD. Taken together, these data indicate repair of microstructural brain injury in major gray and white matter brain regions and strengthen the putative clinical utility of EPO in the context of structural and cognitive recovery following infant TBI. Importantly, clinical findings confirm diminished executive function correlating with decreased structural integrity in the striatum and related structures in adults and children who sustained TBI, providing clinical correlation of our observations (1, 64).

Clinical data confirm that DTI is sensitive to time since injury (65), and an accumulation of evidence implicates resolution of cognitive-behavioral function with altered brain architecture after TBI. DTI measures magnitude and directionality of water diffusion in tissue, and may be a sensitive biomarker of

evolving and sustained white matter injury (66). Clinical studies assessing white matter microstructural organization use the same commonly derived diffusion metrics including FA, MD, AD, and RD as investigated here, and confirm children and adolescents with chronic moderate-severe TBI have lower FA and/or higher MD in numerous white matter fiber bundles including the corpus callosum (66–71). Interestingly, MD may index several factors, including fiber density, myelination, and expansion of extracellular space (66, 72). Long-term recovery from TBI is likely dynamic, and the impact of EPO on network function and/or reorganization may be apparent before, or independent from, structural repair. Thus, DTI or similar sophisticated imaging outcomes may serve as a surrogate biomarker to quantify injury and recovery with post-injury interventions.

The strengths of this study include use of high-dose, extended EPO treatment in a clinically relevant dosing regimen after infant TBI. Numerous lines of evidence implicate EPO's utility in the developing brain when administered in a repeated and high-dose regimen and multiple mechanisms of action, including enhanced survival and maturation of oligodendroglial lineage cells (46), reduction in calpain activation (45, 47), decreased inflammation (73), and support of other neural cells, facilitating structural and functional connectivity and contributing to neurorepair in the developing CNS (19, 21, 41). Findings presented here align with neonatal trials using EPO to promote neurorepair (74–76) but are divergent from trials of EPO repair in adult TBI in humans (77, 78) and trials completed in animals without multiple-doses or low dose regimens (79). Notably, EPO repair in the immature brain after injury is distinct from adult TBI trials using EPO due to numerous factors, including developmental mechanisms of action and age-specific pathophysiology related to oligodendroglia, calpain, cell death mechanisms and inhibitory circuit formation, as well as dose, dosing interval, and regimens. Another strength is the use of touchscreen testing to advance the field of cognitive assessment for rodents with early life brain injury. Indeed, a primary challenge in identifying and testing novel interventions to improve cognition is finding paradigms that accurately recapitulate the same function in humans and rodents, and are uncompromised by environmental conditions. An asset of the assessments described here is rigorous control of the performance rules and criteria that distinguish this approach from the use of novel object and water maze assays that use exploration/novelty and stress.

A limitation of the present investigation is that, while both sexes were included throughout, it was underpowered to detect effects of sex in every outcome measure over the developmental time course. Future experiments are warranted with longitudinal, serial multi-modal imaging throughout the acute, subacute and chronic injury periods to fully establish the individual changes in DTI metrics and the correlation with increased cognitive performance. Further studies would also benefit by incorporating advanced examination of networks and connectivity to provide maximum understanding of white matter circuitry following TBI during early and rapid development (1). Future touchscreen investigations in TBI may benefit from the use of a liquid

reward if concerns for stress from a mild diet restriction manifest.

In conclusion, Extended EPO treatment restores executive function and prevents microstructural brain abnormalities in adult rats with cognitive deficits in a translational preclinical model of infant TBI. Together with the use of translational touchscreen testing of cognition, these data support the use of EPO in clinical trials for human infants with TBI.

AUTHOR CONTRIBUTIONS

SR, LJ, RM, and JB conception and design. SR, LJ, LC, JW, AO, TY, JM, YY, and LS acquisition of data. SR, LJ, JB, WM, RM, LS, and NA analysis and interpretation of data. SR and LJ drafting the article. All authors critically revising the article. All authors reviewed submitted version of manuscript. All authors approved the final version of the manuscript. SR and LJ study supervision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fneur.2018.00451/full#supplementary-material>

Supplemental Figure 1 | Fractional anisotropy (FA) shows minimal regional differences at P30 in prefrontal cortex subregions and striatum. **(A–C,E–G)** No differences in FA are present in prefrontal cortex bilaterally. **(D)** CCI reduces FA in the ipsilateral striatum. **(H)** A similar trend is present in the contralateral striatum ($n = 6–8$, $*p < 0.05$).

Supplemental Figure 2 | At P30 CCI causes widespread diffusivity abnormalities in prefrontal cortical subregions that are at least partially prevented by extended EPO treatment. **(A–H)** CCI causes bilateral axial and radial diffusivity abnormalities in medial and ventral cortical subregions that are prevented by extended EPO treatment. **(I,K)** While CCI does not cause ipsilateral damage in lateral prefrontal cortex AD, CCI induces contralateral changes in lateral prefrontal cortex AD that are not prevented by EPO treatment. **(J,L)** By contrast, CCI causes alterations in ipsilateral lateral prefrontal cortex RD that are prevented by EPO treatment, while the contralateral lateral prefrontal cortex RD is not affected by CCI ($n = 6–8$, $*p < 0.05$, $**p < 0.01$).

Supplemental Figure 3 | At P30 CCI causes diffuse deep gray matter loss of microstructural integrity, and extended EPO treatment prevents these abnormalities at a distance from the injury. **(A,E,I,M)** CCI causes loss of FA in bilateral hippocampal and thalamic subregions, and EPO treatment has minimal impact. **(B–D)** In the ipsilateral hippocampus, CCI causes abnormal mean, axial and radial diffusivity that is also not prevented by EPO treatment, likely due to the proximity to the injury. **(F–H)** By contrast, contralateral abnormalities in hippocampal mean, axial and radial diffusivity are prevented by extended EPO treatment. **(J–L,N–P)** Bilateral thalamic abnormalities in mean, axial and radial diffusivity caused by CCI are also prevented by extended EPO treatment ($n = 6–8$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Supplemental Figure 4 | At P30, abnormal mean diffusivity in vulnerable brain regions that is preventable with EPO treatment (green) correlates with poor cognitive flexibility in adult animals. Area of impact during infancy is shown in pink.

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Interferons in Traumatic Brain and Spinal Cord Injury: Current Evidence for Translational Application

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This review article provides a general perspective of the experimental and clinical work surrounding the role of type-I, type-II, and type-III interferons (IFNs) in the pathophysiology of brain and spinal cord injury. Since IFNs are themselves well-known therapeutic targets (as well as pharmacological agents), and anti-IFNs monoclonal antibodies are being tested in clinical trials, it is timely to review the basis for the repurposing of these agents for the treatment of brain and spinal cord traumatic injury. Experimental evidence suggests that IFN- α may play a detrimental role in brain trauma, enhancing the pro-inflammatory response while keeping in check astrocyte proliferation; converging evidence from genetic models and neutralization by monoclonal antibodies suggests that limiting IFN- α actions in acute trauma may be a suitable therapeutic strategy. Effects of IFN- β administration in spinal cord and brain trauma have been reported but remain unclear or limited in effect. Despite the involvement in the inflammatory response, the role of IFN- γ remains controversial: although IFN- γ appears to improve the outcome of traumatic spinal cord injury, genetic models have produced either beneficial or detrimental results. IFNs may display opposing actions on the injured CNS relative to the concentration at which they are released and strictly dependent on whether the IFN or their receptors are targeted either via administration of neutralizing antibodies or through genetic deletion of either the mediator or its receptor. To date, IFN- α appears to most promising target for drug repurposing, and monoclonal antibodies anti IFN- α or its receptor may find appropriate use in the treatment of acute brain or spinal cord injury.

Keywords: traumatic brain injury, interferon alpha, interferon beta, interferon gamma, interferon alpha receptor, anti interferon alpha antibody

INTERFERONS: FAMILIES, SIGNALING AND BIOLOGICAL PROPERTIES

Interferons (IFNs) have been historically identified as autocrine or paracrine factors secreted by a large number of eukaryotic cells in response to viral infections, with the ability to effectively restrict the spreading of viruses (1). However, in the last 50 years extensive research has revealed the existence of a large variety of IFN types displaying a panoply of immunomodulatory effects, independent from a strict anti-viral function (2, 3).

There are three distinct types of IFNs. Type-I IFNs include IFN- α (for which 14 genes are known) and IFN- β and the lesser understood IFN- ϵ , IFN- κ , and IFN- ω . Type-II IFNs include only IFN- γ , which is biologically and genetically distinct from type-I IFNs. A third family (type-III) of IFNs has been more recently described and includes IFN- λ 1, IFN- λ 2, IFN- λ 3 (also known as IL-29, IL-28A, and IL-28B, respectively) and IFN- λ 4 (3, 4). The secretion of type-I IFNs is induced in almost every mammalian cell by the exposure to viruses, double-strand RNA or Toll-like receptor activation (5). IFN- γ , in contrast, is released by a number of activated T lymphocytes and subsets of NK cells but also glial cells (6) and is involved not only in antiviral activity but also in the polarization of the immune response and the regulation of macrophage effector functions (7). The family of IFN- λ proteins are expressed only in myeloid and epithelial cells of the skin and mucosae, where they play a role in the maintenance of epithelial barrier integrity and the innate immunity to bacteria, viruses and fungi (8).

Despite being transcribed from independent genes, type-I IFNs share the same receptor, composed of two chains, IFNAR1 and IFNAR2 [Also known as IFN- α R1 and IFN- α R2c; (9)]. However, different isoforms may have slightly distinct binding sites and affinity, which may account for the only-partially overlapping biological effects (4). Upon binding, the dimerization of IFN receptor leads to the phosphorylation and activation of non-receptor tyrosine kinases, Janus Kinase-1 (JAK1) and TYK2, which, in turn, phosphorylate STAT1 and STAT2 transcriptional regulators. Together with the IFN-regulatory factor 9 (IRF9), phosphorylated STAT1 and STAT2 form the IFN-stimulated gene factor 3 (ISGF3) complex, which is directly responsible for the transcriptional response induced by type-I IFNs. Non-canonical signaling from IFNAR1/2 receptor subunits involves the activation of PI-3K/mTOR and MAPK pathways, as well as the phosphorylation of STAT3, STAT4, STAT5A, and STAT5B (2). Conversely, IFN- γ signaling is mediated by a distinct receptor composed of the two subunits IFN- γ R1 and IFN- γ R2 in a four-chain assembly (10), whose signal transduction cascade involves the activation of JAK1, JAK2 and the phosphorylation of STAT3, STAT5 and the indirect activation of the NF- κ B module (11). Type-III IFNs signal through a dedicated receptor formed by the IFNLR1 subunit (also known as IL-28R1) together with the IL-10R2 subunit, which is shared by several cytokine receptors. Type-III IFNs also use JAK1, TYK2, and JAK2 in their signal transduction cascades.

The transcriptional responses elicited by the three types of IFN are remarkably divergent, despite the commonalities in their signaling cascades. Type-I IFNs activate the transcription of genes displaying IFN-stimulated response elements (ISREs) and provide a large-scale regulation of transcription through chromatin remodeling and epigenetic modulation, often in cooperation with other transcription factors [either co-activators or co-repressors; (2)]. Although IFN- γ has been classically related to the transcriptional activation of genes including a Gamma-interferon Activated Sequence (GAS) elements, (12), gene transcription induced by IFN- γ has been shown to recruit multiple transcription factors beyond the canonical STAT [such as C/EBP β and CREB/AP1; (11)]. The transcriptional responses

activated by type-III IFNs are remarkably similar to type-I IFNs and IFN- λ -induced genes and represent a subset of the transcripts activated by type-I IFNs (13, 14).

INTERFERONS IN NEUROLOGICAL DISORDERS: PATHOGENIC ROLE AND THERAPEUTIC APPLICATIONS

IFNs contribute to pathological conditions unfolding in the Central Nervous System (CNS) in often contrasting roles, either as players in the pathogenic process or as therapeutic agents, revealing the far-reaching impact of IFNs in the CNS. A group of genetically determined conditions (mutations in the genes encoding for MDA5, the double-stranded RNA editing enzyme adenosine deaminase ADAR, SAMHD1, the RNase H2 endonuclease complex and the repair exonuclease TREX1), collectively known under the clinical name of Aicardi-Goutieres syndrome (15) is characterized by aberrant production of IFN- α and clinically resembles congenital infections. In fact, astrocyte-restricted overexpression of IFN- α in murine transgenic models results in brain calcifications, gliosis, leukocyte infiltration of meninges and neuronal loss (16). However, infection with the lymphocytic choriomeningitis virus (LCMV) in the same IFN- α overexpression model, results in a significantly lesser degree of damage, inflammation and improved survival. In a different setting involving the comparison of acute and chronic LCMV infection, suppression of type-I IFN signaling by deletion of the *Ifnar* gene (which encodes the IFN receptor shared by all type-I IFNs) ameliorates the clearing of the LCMV and the resolution of the inflammatory response through a mechanism requiring the recruitment of IFN- γ -secreting T lymphocytes (17). Thus, while acute IFN- α may inhibit virus spreading, chronic IFN- α may prevent the transition to an effective immune-cells-mediated clearing of the virus. Thus, IFN- α is pathogenic or protective depending on the underlying condition and the level and timing of expression.

Type-I IFNs have been shown to be involved in the pathogenic cascades of neurodegenerative diseases, whereby IFN- α contributes to the appearance of amyloid-related cognitive deficits in animal models of Alzheimer's Disease (18) and deletion of the *Ifnar* gene has been shown to ameliorate cognitive deficits and attenuate microgliosis. Conversely, deletion of the IFN- β gene in dopaminergic cells results in the appearance of Parkinson's Disease-like pathological features, in particular synuclein aggregates, as a consequence of impaired autophagy (19).

Besides their role in physiology or pathophysiology, type-I IFNs have made a significant impact as therapeutic agents in neurology. The seminal discovery of the therapeutic effect of IFN- β on relapsing-remitting Multiple Sclerosis [MS; (20, 21)] has led to the clinical use of IFN- β as the first disease-modifying drug approved for relapsing-remitting MS. In several large clinical trials (22), IFN- β decreased the rate of clinical progression and reduced inflammatory lesions in the white matter (as detected by MRI). In the last 20 years, a detailed knowledge of the pharmacokinetics, clinical efficacy, and safety of IFN- β have

accumulated (23–26), and several variants of IFN- β (with distinct pharmacokinetics) have been developed such as longer half-life pegylated-IFN- β (25, 27). The pharmacodynamics of IFN- β in MS is complex and remains poorly understood. However, type-I IFNs (in particular IFN- β) display a significant anti-inflammatory effect on astrocytes, since treatment of astrocytes with IFN- β results in the induction of an anti-inflammatory transcriptional program orchestrated by the Aryl-hydrocarbon receptor (28). In the EAE MS mouse models, a subset of microglial cells appears to be the major source of IFN- β ; exposure of microglia to IFN- β enhances phagocytic activity and loss of IFN- β prevents the clearance of myelin fragments (29). Finally, IFN- β has also been shown to decrease the permeability of the Blood-Brain Barrier (BBB). In fact, deletion of IFN- β in astrocytes facilitated the entry of viruses into the CNS (30). Furthermore, the administration of IFN- β *in vivo* or *in vitro* counteracts the disruption of the BBB caused by inflammatory stimuli (31, 32).

Because of their pivotal role as regulators of neuroinflammation, gliosis and BBB dysfunction, IFNs from all three types may be well positioned to affect the pathogenic cascades in TBI. Although a large number of inflammatory mediators have been reported in the acute neuroimmunological responses to TBI (33) and many have been proposed as possible therapeutic targets (34), only a comparatively small number of studies has addressed the role of IFNs in the pathophysiology of the acute phase of brain or spinal cord traumatic injury (summarized in **Table 1**).

IFNS IN TRAUMATIC BRAIN INJURY: DATA FROM HUMAN PATIENTS

The expression of IFN- α , IFN- β , and IFN- γ has been investigated in biological samples of human patients, including brain microdialysate, brain tissue and cerebrospinal fluid (CSF). A significant fraction of investigations has focused on validating IFNs as prognostic or diagnostic markers. The main focus has been on IFN- γ because of its well-known role in lymphocyte-driven inflammation. Nevertheless, the recent appreciation of the role of type-I IFNs in inflammation, beyond viral infections, has led to the assessment of type-I IFNs in neurotrauma.

In a small cohort study of 12 patients, 42 cytokines, including IFN- α and IFN- γ , were evaluated in the extracellular fluid of the brain (sampled by microdialysis). Although both IFN- α and IFN- γ were detected in microdialysates, their concentrations varied significantly among the patients and over time, and neither cytokine displayed a reproducible peak in concentration (44). The small size of the patient cohort analyzed, and the intrinsic limitations of cytokine recovery and measurement may have contributed to the inconclusive results.

Quantitative analysis of mRNA levels of IFN- α and IFN- β was performed in post-mortem brain samples obtained from TBI patients (27 patients divided in three cohorts according to their survival after injury: <17 min, <3 and >6 h). Interestingly, the levels of IFN- α transcripts were reduced in samples obtained from patients deceased between 17 min and 3 h after trauma

but were comparable to control levels at 6 h or later. Notably, IFN- β mRNA levels were elevated only in samples from patients deceased more than 6 h after TBI and specifically in the injured hemisphere [ruling out an effect of systemic inflammation; (35)].

Changes in IFN- γ , on the other hand, have been investigated in a several clinical cohorts. In a series of patients with severe TBI, the production of eight cytokines were analyzed in the CSF and compared in a group of normoxic individuals (22) to patients suffering from an acute post-traumatic hypoxic episode (20) with the rationale that this severe secondary insult aggravates neuroinflammation, biomarkers of brain damage and long-term outcome (45). When patients were analyzed together ($n = 42$), IFN- γ concentrations were found elevated in CSF at the earliest time point, within 24 h post-TBI and gradually declined to day 5.

Comparison of normoxic and hypoxic TBI patients revealed that both normoxia and hypoxia induced a significant increase in the production of IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN, and TNF, but not IL-8 compared to the control. However, only IFN- γ and GM-CSF were exacerbated by the combination of TBI and post-traumatic hypoxia. In addition, in the hypoxic cohort, the secretion of IFN- γ , and to a lesser extent of TNF, was found to be prolonged up to 4–5 days post TBI compared to the normoxic counterpart. Amplified IFN- γ and inflammation in general, were corroborated by higher levels of the serum injury biomarker S100B and worse outcome scores at 6 months post-TBI using the Glasgow Outcome Scale Extended (GOSE) in hypoxic patients. The secretion of IFN- γ into the CSF is attributed to the upregulation within the injured brain. In fact, in human post-mortem brains, IFN- γ was found significantly overexpressed within a few minutes after TBI, subsequently reaching a >10-fold increase in the brains of individuals dying several hours after TBI. In fact, among the eight cytokines analyzed, IFN- γ reached the third highest elevation after IL-6 and IL-8. Interestingly, the area of the cortex used for cytokine analysis also presented astrogliosis and macrophage activation located in proximity to axonal pathology, implying a direct link between cellular and humoral inflammation (40).

IFN- γ is secreted by glial cells and infiltrating monocytes and is involved in promoting neuroinflammation but also neuroprotective processes such as neurogenesis and brain repair (6). *In vitro* studies have also demonstrated that IFN- γ is a hypoxia-specific mediator induced by T-cells (46). Altogether, these findings suggest that there is an increased secretion of IFN- γ after TBI, its expression and secretion are enhanced after hypoxia and it plays a critical role in secondary brain damage elicited by an acute hypoxic insult following brain trauma. Additionally, IFN- γ plays a critical role in the activation of the kynurenine pathway, which metabolizes the essential amino acid tryptophan leading to the release of the potent neurotoxic factor quinolinic acid, an excitotoxic agonist to the NMDA receptor. In 28 patients with severe TBI some of us reported that critical downstream metabolites of tryptophan were significantly elevated in CSF and that quinolinic acid was higher in the patient cohort with unfavorable outcome and was inversely correlated with the GOSE scores. Furthermore, the overexpression of the upstream enzyme of the kynurenine pathway, indoleamine 2,3-dioxygenase-1 (IDO-1), which is activated by IFN- γ , was

TABLE 1 | Experimental and clinical evidence demonstrating the role of IFNs in neurotrauma.

Interferon under study	TBI model	Intervention/genetic deletion	Effect	Reference
IFN- α	CCI	IFNAR1-KO	• 40% decrease in lesion size • Reduced IL-1β, IL-6; increased IL-10 • Increased M2 microglial polarization	(35)
IFN- α	CCI	IFNAR1 neutralizing antibody MAR1	• 40–50% reduced lesion size (administered up to 2 dpi) • 15–20% improved motor performance	35
IFN- α	• EC-Hypp • Axonal injury	IFNAR1-KO	• Two-fold Increase macrophage infiltration (at 1 dpi) • No effect on IL-1β; increased MMP-9	(36)
IFN- α	CCI	miR-155-KO	• Reduced (50%) IFN induction • Increase (25%) microglial response • Increased neuronal loss	(37)
IFN- β	Hemisection SCI	NSC expressing IFN- β	• 40% decrease in GFAP+ • 50% increase in axon preservation • modest (1 point) increase in Basso score at 4 weeks	(38)
IFN- β	Weight-drop SCI	IFN- β injection	• 50% decrease in MPO levels • 70% decrease lipid peroxidation • 50% decrease in BBB score at 24 h but large spread of the single values.	(39)
IFN- γ	Post-mortem human TBI brain	None	• IFN-γ protein • IFN-γ mRNA	(40)
IFN- γ	SCI	i.p. administration	• Improved motor function • Increased accumulation CD1b⁺ macroph/microglia • Increased MCP-1/CCR3 mRNA • Upregulation GDNF & IGF mRNA	(41)
IFN- γ	SCI	IFN- γ -KO	• Reduced functional recovery • No changes TNF, IL-6, glial responses	(42)
IFN- γ & IFNGR	SCI (contusion)	IFN- γ -KO & IFNGR-KO	• No changes	(43)

The table summarizes the studies reporting the antagonization of various interferon family members in models of neurotrauma, using either pharmacological administration strategies or gene deletion of the IFN or its receptor to determine disease modifying effects. It also includes *in situ* expression of IFN- γ in post-mortem human brain obtained from TBI victims.

detected in post-mortem brains after trauma and associated on tissue pathology (47).

Despite the limitations due to lack of homogeneity between studies in patient selection, cytokine panels and detection methods, converging evidence suggests that all three IFN- α , IFN- β and IFN- γ are induced in human brain after trauma, with different time courses: IFN- α appears to be the first to increase, followed by IFN- γ . However, IFN- α expression appears to be transient, whereas elevation of IFN- γ persists for several days. Because of the complexity of the clinical picture, human studies cannot provide evidence on the role of each IFN in the pathogenic cascade, and consequently on their potential as therapeutic targets. For this purpose, experimental data in murine models need to be evaluated.

IFNS IN TRAUMATIC BRAIN INJURY: EXPERIMENTAL EVIDENCE

Expression levels of both IFN- α and IFN- β have been verified in controlled cortical impact (CCI) murine model of TBI, whereby the former peaks at 2 h post-injury whereas the latter is not

upregulated before 24 h (35). The combined functional role of IFN- α and IFN- β in CCI has been explored in IFNAR1-KO mice since this is a common receptor for both factors resulting in the abolishment of both IFN- α and IFN- β signaling. Notably, in the IFNAR1-KO mice not only is the signaling of IFNs blocked but the transcription of the IFN- α and IFN- β is also reduced, in agreement with the role of IFNAR1 in the positive feedback loop amplifying IFN- α levels and the overall IFN response pathway (48). Overall, upon CCI IFNAR1-KO mice show a significant decrease (40–50%) in lesion size, implying a detrimental impact of type-I IFNs after TBI (35). Mechanistically, loss of type-I signaling results in a significant downregulation of pro-inflammatory IL-1 β and IL-6 and in a marked upregulation of the anti-inflammatory mediator IL-10 upon CCI, suggesting a reduced inflammatory response (35). However, 24 h after TBI, both astrocyte reactivity and microglial density are enhanced in IFNAR1-KO mice by 50 and 20%, respectively. In IFNAR1-KO mice, activated microglia expressed high levels of CD206, a marker of trophic M2 macrophages, suggesting that, although increased in number, microglial cells may have assumed a neuroprotective, anti-inflammatory phenotype, in agreement with the upregulation in

IL-10. Thus, the IFNAR1-KO data supports the hypothesis that in wild-type mice, type-I IFNs contribute to skew the microglial response toward an inflammatory phenotype, increasing the loss of neurons detected as larger lesion size (35).

Comparable results have been obtained with acute suppression of IFNAR1 via the administration of the anti-IFNAR1 monoclonal antibody, MAR1-5A3. Delivery of MAR1-5A3 before CCI reduced the lesion size by 40%, similar to the effect achieved when the antibody was administered 30 min after trauma. Notably, MAR1-5A3 proved to be efficacious even when administered up to 2 days post-injury (dpi) resulting in a lesion size reduction by 40% and enhanced motor recovery, suggesting an extended therapeutic window for type-I IFNs (35). In agreement with the KO data, treatment with MAR1-5A3 suppressed TBI-induced upregulation of IL-1 β , IL-6, and IFN- β , but does not affect the upregulation of IL-10 (35). Although it is not clear whether MAR1-5A3 delivered systemically crosses the BBB, it has been hypothesized that it may act on circulating leukocytes expressing IFNAR1. In fact, chimera mice in which IFNAR1-KO bone marrow was transplanted into a WT recipient causing a specific lack of IFNAR1 only on leukocytes, display a 30–40% decrease in lesion size compared to WT mice, an overall effect similar to what observed in full IFNAR1-KO. In these chimeric mice, the level of microglial activation are actually increased, as observed in IFNAR1-KO, once again suggesting that in the absence of IFNAR1 signaling in leukocytes, microglia activation plays a beneficial role.

Type-I IFN signaling has been shown to regulate the recruitment of leukocytes in a distinct model of acute brain injury, namely the surgical disconnection of the entorhinal cortex (EC) from the hippocampus, leading to the denervation of the Dentate Gyrus and the degeneration of the distal part of the severed EC axons. After injury, the induction of IFN-regulated genes, IRF7 and IRF9 (a molecular signature of type-I IFN pathway activation), was observed from 1 to 7 dpi in microglial cells located in the hippocampus of WT mice but was undetectable in IFNAR1-KO mice (36). The EC-hippocampal disconnection resulted in the accumulation of leukocytes (CD45-bright CD11b⁺ cells) in the hippocampus of WT mice. However, this response was strongly enhanced by 2-fold in IFNAR1-KO mice at 1 dpi. Interestingly, IFNAR1-KO mice displayed reduced levels of the chemokine CXCL10 (and, to a lesser extent, of CCL-2) although, in agreement with the increased leukocyte infiltration, levels of MMP-9 were actually increased (36). Thus, type-I IFNs may not only upregulate local neuroinflammation (toward a detrimental polarization), but also suppress the invasion of immune cells from the periphery.

The effects of downregulating type-I IFNs have been investigated in mice in which the microRNA miR-155 is knocked out, since this is one of the major miRNAs controlling the inflammatory response (49). Upon CCI, the expression of IFN- α and IFN- β is reduced in miR-155-KO mice. In contrast, microglia activation was increased by 25% in miR-155-KO mice together with a reduction in neuronal survival (37). Since miR-155 is strongly expressed in neurons, it cannot be excluded that this effect is unrelated to the suppression of type-I IFNs transcription.

The mechanisms of type-I signaling on neuronal survival have been investigated *in vitro* in an oxygen and glucose deprivation (OGD) model. Upon OGD, IFN- α was strongly upregulated (11-fold) at 2 h whereas IFN- β displayed a milder (2.3 fold) and delayed expression [24 h after OGD; (50)], resembling the time course observed after TBI *in vivo* (35). IFN- α signaling was instrumental in inducing IL-6 and TNF- α secretion in this *in vitro* model, since knocking-down IFNAR1 attenuated both the OGD-induced cytokine upregulation as well as the induction of IFN- α itself. Notably, neuroblastoma cells in which IFNAR1 expression was knocked-down revealed to be more resilient, showing a reduced level of cleaved caspase-3 after OGD (50). Likewise, IFN- α has been reported to have direct pro-oxidative and neurotoxic effects on neurons (51). Thus, based on this *in vitro* model, it can be deduced that IFN- α signaling promotes inflammation in neuroblastoma cells after OGD, leading to an overall detrimental effect on cell survival.

IFN- α / β IN SPINAL CORD INJURY

The investigations on the role of type-I IFNs in SCI have focused mainly on the potential therapeutic application of IFN- β in acute SCI; however no information on IFN- α is available.

In a seminal work on the effect of IFN- β on SCI, Gok et al. (39) administered IFN- β at a dose of 10^7 IU during trauma (weight-drop after laminectomy), followed by a second dose of 0.5×10^7 IU 4 h later. When evaluated at 24 h post-injury, the spinal cord from rats injected with IFN- β displayed a 50% decrease in myeloperoxidase activity compared to vehicle-treated rats. Furthermore, in contrast to the sharp elevation observed in vehicle treated rats, IFN- β treatment reduced lipid peroxidation to sham levels. IFN- β -treated rats, on average, displayed a trend toward an improved motor recovery, although the large variations did not allow to confirm significant differences. Consistently, IFN- β -treated rats could climb steeper slopes in the Inclined Plane test than vehicle-treated counterparts.

A second study investigating the effects of peripheral-administration of IFN- β after SCI, using a single dose of pegylated-IFN- β , given 30 min after SCI, demonstrated a reduced upregulation of inflammatory cytokines (52). However, among all the cytokine tested, a significant effect was only demonstrated for IL-6 with approximately a 25% decrease at 6 and 24 h post-injury, for IL-18 with a 20% increase at 5 dpi and for IL-10, with a modest increase at 6 h. With IFN- β treatment, no difference was found in the extent of the glial scar formation or spinal cord cavitation, and although a statistically-significant improvement was only observed in open-field test, this effect was limited to the first week after injury, after which no difference existed between treated and untreated rats. Taken together, these two studies suggest that IFN- β may be beneficial in reducing secondary damage after SCI, although more robust data are required to support these findings.

An alternative approach to utilize the beneficial role of IFN- β in SCI and enhance its local delivery has been pioneered by Nishimura et al. (38) by engineering Neural Stem Cells (NSC) to constitutively secrete large amounts of IFN- β . After spinal

cord hemisection, NSCs injected intravenously homed within the injury site and displayed a robust expression of IFN- β . The rats injected with IFN- β -secreting cells showed a significant reduction (35%) in astrocyte proliferation and an enhanced preservation of axons (50% more than in NSC secreting beta-galactosidase as control), ultimately resulting in improved motor performance and larger evoked motor potentials 4 weeks after SCI. These effects were markedly reduced when NSCs were depleted by the administration of the cytostatic compound 5-FluoroCytosine (38).

In conclusion, this data suggests that IFN- β may have some beneficial effects in SCI, however the evidence remains limited, possibly due to the restricted CNS penetration of peripherally-administered IFN- β . Thus, more robust experimental data is warranted before IFN- β can be considered as a treatment option in SCI.

IFN- γ IN TBI AND SCI

IFN- γ is upregulated in the tissue affected by blunt TBI or in CCI within a time window spanning 2–12 h after trauma (53, 54). Intriguingly, although IFN- γ is an extensively studied cytokine, it has been mainly used as a readout in TBI studies. Substantial literature is available on the genetic or pharmacological manipulations attenuating the upregulation of IFN- γ in TBI (53, 55–58) but very little is known about the role of IFN- γ *per se*. The majority, if not all studies on the subject assumes a pathological role for post-TBI neuroinflammation and therefore by extension IFN- γ must have a detrimental effect. However, this concept has been challenged. In fact, recent evidence suggests that, at least in SCI, the upregulation of IFN- γ may be beneficial (59). In a model of spinal cord contusion, intraperitoneal administration of IFN- γ (1.0×10^4 UI/day for 14 days), was sufficient to achieve significant levels of this cytokine in the CNS and resulted, unexpectedly, in faster recovery of motor performance from 10 days up to 6 weeks after trauma compared to vehicle-treated mice (41). Interestingly, IFN- γ -treatment did induce a stronger accumulation of CD11b $^{+}$ macrophages/microglia in the spinal cord, but the inflammatory cells were less concentrated in the injury core and more represented in the nearby penumbral and healthy tissue. In agreement with the increased presence of CD11b $^{+}$ cells, the levels of MCP-1 and CCR2 mRNA were upregulated in IFN- γ -treated mice (41). Notably, IFN- γ treatment impacted, unexpectedly on the astroglial response to trauma. Although the activation of astrocytes was increased by IFN- γ treatment, the levels of the chondroitin-sulfate proteoglycans (astrocyte-produced inhibitors of axonal regeneration) were strongly decreased while the levels of GDNF and IGF-I mRNA were upregulated in the injured spinal cord (41).

Interestingly, SCI applied to IFN- γ receptor (IFNGR)-KO mice resulted in worse functional recovery although the local inflammatory response assessed by TNF- α and IL-6 levels as well as astrocyte and microglial responses were not altered (60). In this model, the authors reported that loss of IFNGR resulted in reduced upregulation of adhesion molecules and chemokines in

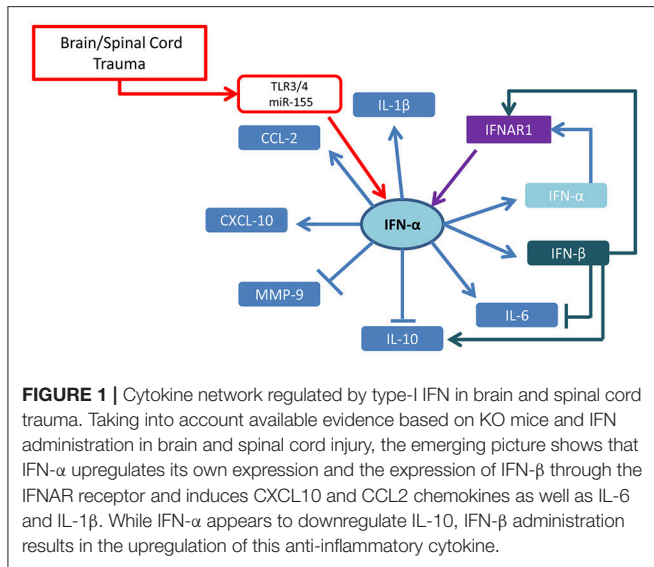
choroid plexus' vascular beds leading to the significant decrease in T lymphocytes in the CSF and in the ependyma as well as in the overall number of CD4 $^{+}$ lymphocytes and monocytes in spinal cord at 7 dpi; the authors suggested that at least one of IFN- γ functions in SCI is to facilitate T-lymphocyte and monocyte migration, and, because of the overall detrimental effect of IFNGR-KO on SCI prognosis, have concluded that this IFNGR should have beneficial net effects (60). Similar effects were observed in mice lacking the transcription factor TBX21, which is key to induce IFN- γ transcription (42). Although this data shows that lack of IFNGR does not necessarily improve outcome in SCI, the proposed model may be only one to represent the multiple mechanisms through which IFN- γ affects prognosis in SCI.

In fact, additional evidence on possible beneficial roles of IFN- γ through a distinct, direct T-cell-dependent mechanism has been reported in studies of adoptive lymphocyte transfer in SCI. While transfer of Th1-polarized CD4 $^{+}$ lymphocytes enhances neurological recovery after contusive SCI, this effect was significantly attenuated when the transferred lymphocytes were unable to secrete IFN- γ (61). In fact, IFN- γ was found to be a key player in this SCI model by inducing IL-10 production by macrophages and microglia, which, in turn, is the actual effector molecule of IFN- γ beneficial effects. In fact, neutralization of IL-10 abolishes the protective action of IFN- γ -producing lymphocytes.

In contrast to these findings, experimental evidence has also been published detailing a net detrimental role of IFN- γ in SCI. In fact, contusive SCI performed on IFN- γ -KO and IFNGR-KO seems to produce a significantly lower degree of impairment (43). A similar degree of improvement was seen in chimeras in which all bone-marrow-derived cells were IFN- γ -KO. This effect was traced down to a population of T cells expressing $\gamma\delta$ TCR whose secreted IFN- γ would act on macrophages to enhance the SCI-associated inflammation and worsen neurological recovery. In fact, chimeras with lack of IFN- γ R expression in macrophages as well as adoptive transfer of T- $\gamma\delta$ cells unable to secrete IFN- γ , displayed an improved motor recovery after SCI (43). In these conditions, loss of IFN- γ resulted in reduced levels of inflammatory cytokines in the spinal cord and a polarization of macrophages toward the so-called M1, proinflammatory phenotype.

Currently, the divergent results obtained in different studies on the role of IFN- γ R/IFN- γ in SCI are not easily reconciled and the issue needs to be revisited taking into account differences in strains and trauma models. It is interesting to note that the level of IFN- γ expression (59, 62) may be an important variable setting the baseline function (mainly inflammatory or anti-inflammatory) in a given mouse strain. Furthermore, the timing of IFN- γ intervention may be critical, since this cytokine may enhance recovery at later stages while still increasing the damage in the acute phase (62). Moreover, the amount of IFN- γ might be affected by additional variables related to trauma, such as hypoxia (46), which should be factored in when assessing the consequence of experimental manipulations of IFN- γ .

Therefore, current data on IFN- γ is not convergent on a specific role of this cytokine. Differences in the amount of the cytokine released and the effects of gene deletion may contribute



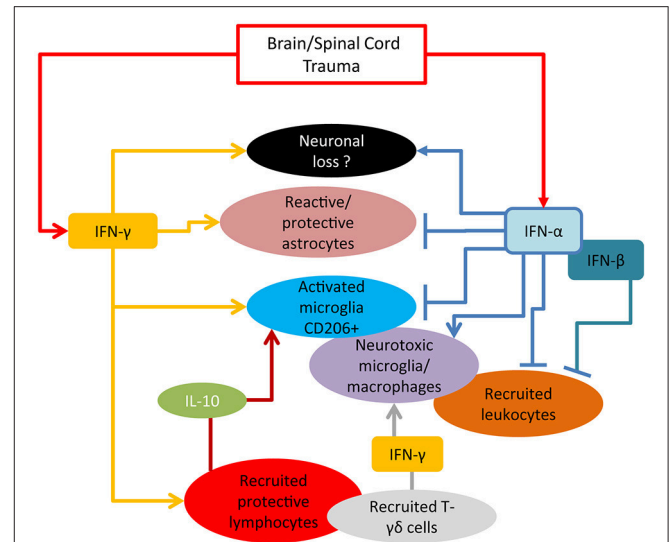
to these conflicting results. Thus, the translational outlook for targeting IFN- γ in SCI remains unclear.

REPURPOSING THERAPEUTIC AGENTS TO TARGET IFN IN TBI AND SCI

Type-I and type-II IFNs appear to play distinct and yet not completely identified roles in neurotrauma pathological cascades. Taken together, the current reports suggest that IFN- α seems to be driving the acute inflammatory process through a self-amplification loop and the induction of inflammatory cytokines and chemokines (summarized in **Figure 1**). On the other hand, IFN- β appears to counteract these effects (at least in SCI model and when administered at pharmacological doses), by upregulating IL-10 and favoring the recruitment of inflammatory cells (cellular targets of IFNs in TBI/SCI are summarized in **Figure 2**). IFN- γ may be protective or detrimental, possibly depending on the cellular source, the stage of the pathophysiological cascade (acute vs. subacute effects) and the concentration of cytokine released.

In order to target therapeutically the action of IFNs in TBI two strategies can be taken into consideration: either to administer a specific IFN based on its known beneficial properties or to selectively block a detrimental IFN through the delivery of neutralizing antibodies (summarized in **Figure 3**).

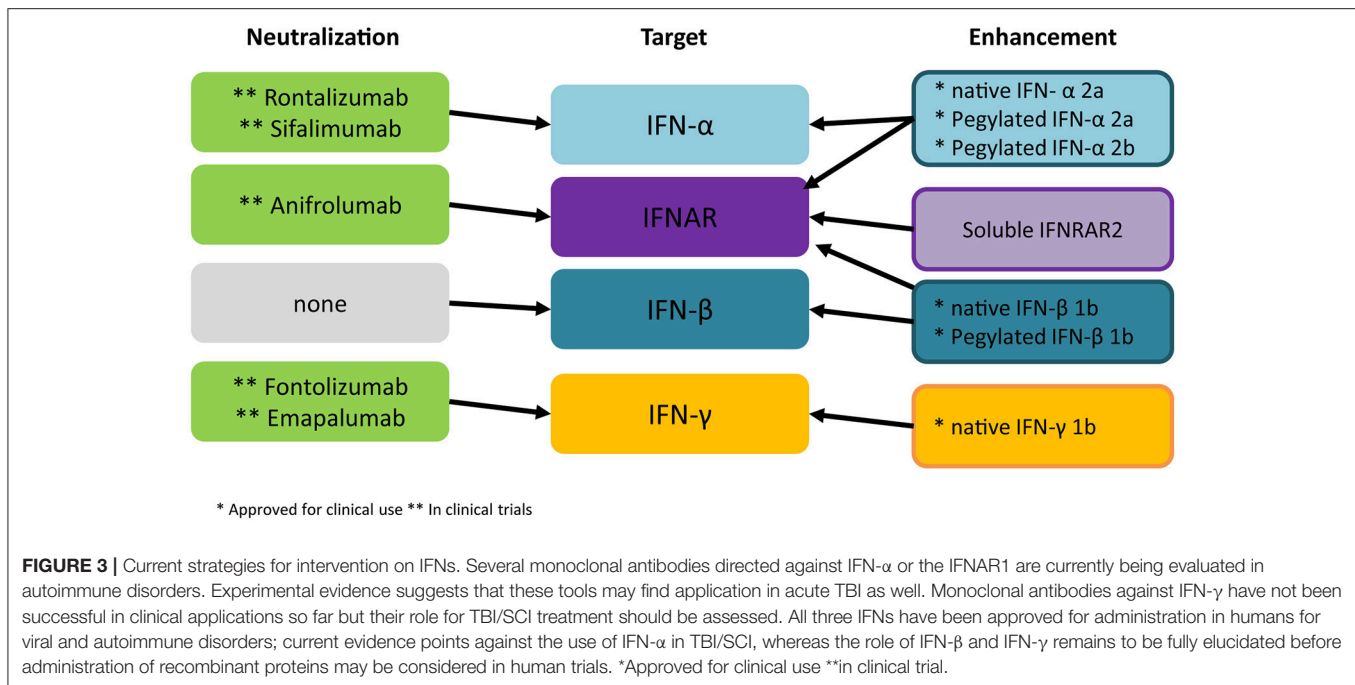
Indeed, extensive knowledge exists on the administration of IFN- α for the treatment of viral hepatitis and lymphoproliferative diseases as well as the use of IFN- β in multiple sclerosis. The pharmacokinetics of both IFNs have been thoroughly investigated. For instance when administered systemically in pharmacological doses, the penetration of exogenous IFN- α in the CNS through the BBB has been observed (63). In contrast, peripheral delivery of IFN- β appears to be completely excluded from the CNS (64). However, the pharmacokinetics may be significantly different relative to the opening of the BBB, such as



in TBI. Recently, the soluble isoform of the IFNAR2 subunit has been found to enhance type-I IFN signaling and to significantly affect pathological conditions. In fact, mice overexpressing IFNAR2 have been reported to be more sensitive to septic shock due to the enhanced IFNAR1 signaling (65), and administration of recombinant IFNAR2 in chronic-progressive Experimental Autoimmune Encephalomyelitis enhances IFN- β signaling, in this case reducing the severity of the disease (66). However, this strategy has not yet been explored for clinical applications since it may enhance detrimental and beneficial effects of IFN signaling with unpredictable effects in TBI.

Despite the high expectations at the time of its discovery, nowadays IFN- γ has limited clinical applications, beside the approval for non-neurological diseases such as Chronic Granulomatous Disease (67), malignant osteopetrosis (68), and as add-on in the treatment of mycobacterial infections. Upon systemic administration, IFN- γ penetrates the brain and spinal cord in significant amounts, although a fraction of IFN- γ in the brain actually binds to the capillary endothelium (69). Thus, being already approved for human use and with known profiles of pharmacokinetics and pharmacodynamics, IFNs would be ideally suited for drug-repurposing efforts in TBI.

Current evidence, based on the deletion of the IFN receptor (35) suggests that enhancing IFN- α signaling by administering IFN- α itself may actually be detrimental, possibly by exacerbating inflammation and gliosis. The effect of administering IFN- β for therapeutic purposes in TBI cannot be assessed because of the deficiency of experimental data on the subject. Nevertheless, the



lack of efficacy of IFN-β in SCI (despite some effects on the neuroinflammatory response) makes it an unlikely target for intervention. The data available on the potential of IFN-γ as therapeutic agent is not univocal: although the administration of IFN-γ is beneficial in one setting (41), other authors (43) have shown genetic data suggesting that suppression, rather than enhancement, of IFN-γ signaling may be favorable. Since IFN-γ displays divergent effects depending on the concentration of cytokine available (59), it is possible that a tight control of IFN-γ levels may be necessary to achieve therapeutic success.

Several approaches have been developed to block type-I and type-II IFNs biological actions. In particular, monoclonal antibodies binding to IFN-α such as rontalizumab [a human anti-IFN-α monoclonal antibody that neutralizes all 12 IFN-α subtypes but not IFN-β or IFN-ω; (70)] sifalimumab [fully human, immunoglobulin G₁ κ monoclonal antibody that neutralizes the majority of IFN-α subtypes; (71)] and IFNAR [anifrolumab, a fully human, IgG1κ monoclonal antibody that binds to IFNAR and prevents signaling by all type I IFN; (72)] have been tested in clinical trials of autoimmune diseases, in particular Systemic Lupus Erythematosus (SLE) and have their safety profiles already investigated (73). Although none of these agents has been tested in TBI clinical settings, the experimental evidence obtained with the MAR1-5A3 (35) suggests that acute neutralization of IFN-α may prove effective. It is unclear how much of the information gained in TBI models (such as CCI) can be transferred to SCI. No experimental data on the neutralization of IFN-α is available for SCI, and the role of IFN-β remains open to question. Therefore, the positive outlook for anti-IFN-α in TBI cannot be extended by default to SCI.

A monoclonal antibody aimed at neutralizing IFN-γ (fontolizumab, a humanized form of a murine anti-human

IFN-γ monoclonal antibody) has been developed and tested for the treatment of autoimmune disorders. However, since the efficacy of fontolizumab proved to be disappointing in Crohn's disease (74) and rheumatoid arthritis, its clinical development has not been refined. More recently, a second anti-IFN-γ antibody, emapalumab [a fully human, anti-IFNγ monoclonal antibody; (75)], has entered clinical trials for the treatment of hemophagocytic lymphohistiocytosis (76). To our knowledge, none of these agents is currently scheduled for investigation in TBI or SCI.

The number of biological agents, in particular monoclonal antibodies developed to target specific cytokines has grown exponentially in last few years. In regard to IFNs, there are already available options for either enhancing IFNs by administering recombinant proteins or blocking IFNs using antibodies directed against these cytokines or against their receptors. In light of the current lack of effective therapies for TBI and SCI, the question to be asked for translational applications is no longer "how to target a given cytokine (or mediator)" but rather "which one of the already available therapeutic agents can be repurposed for treatment." Since drug repurposing offers advantages both to the patients (safer clinical trials, faster entry into clinical applications) and to drug companies (lower development risk, cost and larger return on investment), it is fundamental to provide solid and reproducible rationales to prioritize repurposing efforts. Although both type-I and type-II IFNs appear to be involved in the pathogenic cascade of TBI and/or SCI, their translational outlook appears quite distinct. Evidence available on IFN-β suggests that the net effect of IFN-β administration may be limited. On the other hand, datasets on IFN-γ are inconclusive and both detrimental and beneficial roles have been attributed to this cytokine. Since pharmacological

manipulations are available to either increase or decrease IFN- γ levels in humans, it is fundamental to reach a consensus on its role. Current research suggests that blocking IFN- α signaling or neutralizing IFN- α action may offer the best chance for a positive outcome in clinical trials. Since this hypothesis rests on a comparatively limited amount of studies, strengthening the experimental dataset is a priority to advance future translational applications.

CONCLUSION: AREAS OF UNCERTAINTIES

The role of type-I and type-II IFN in acute traumatic injury of brain and spinal cord remains an active area of investigation, in particular because of the opportunity for re-purposing agents whose pharmacology is well understood, either for enhancing or for neutralizing IFNs effects.

In regard to the basic pathophysiology, the main cellular sources of IFNs and the molecular triggers that activate IFNs' responses in TBI/SCI remain poorly understood while the relationship between IFNs and other alarmins, such as IL-33 [shown in other conditions: (77, 78)] has not yet been investigated.

At the translational level, although it appears that IFN- α neutralization is the most promising prospect for successful therapy, little is known about brain penetration of anti-IFN- α monoclonal antibodies already tested in patients and the relative contribution of peripheral vs. central production of IFN- α , supported by the chimeric mouse experiments remains to be fully understood. In addition, because of the long half-life of monoclonal antibodies, it is not known whether prolonged neutralization of IFN- α is necessary or whether its acute and subacute neutralization may lead to different outcomes.

Finally, the diverging roles of IFN- γ must be clarified before any therapeutic strategy could be sketched; in particular, IFN- γ neutralization experiments in TBI have not been fully investigated to date.

Thus, we are still in the early stages of the understanding of IFNs roles in TBI or SCI. As early responders to tissue damage, IFNs are posited to critically influence the acute neuroimmunological response and possibly shape the phenotype and the net effect of the neuroinflammatory cascade in the subacute phase. To this respect, caution must be exerted in extrapolating possible IFNs roles from other diseases or from *in vitro* models, and in assessing critically the reproducibility of reported findings. Therefore, the future of IFNs manipulation for therapeutic purposes must include the spatiotemporal definition of their roles in models that recapitulate as much as possible the anatomical complexity and the physiological peculiarity of the human condition.

AUTHOR CONTRIBUTIONS

FR and MM-K designed the scope and the structure of the review. FR, AC, and MM-K searched the relevant literature and summarized concepts and results. FR, AC, and MM-K prepared the text and the artwork.

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Cerebrospinal Fluid Biomarkers Are Associated With Glial Fibrillary Acidic Protein and α II-spectrin Breakdown Products in Brain Tissues Following Penetrating Ballistic-Like Brain Injury in Rats

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Treatments to improve outcomes following severe traumatic brain injury (TBI) are limited but may benefit from understanding subacute-chronic brain protein profiles and identifying biomarkers suitable for use in this time. Acute alterations in the well-known TBI biomarkers glial fibrillary acidic protein (GFAP), α II-spectrin, and their breakdown products (BDPs) have been well established, but little is known about the subacute-chronic post-injury profiles of these biomarkers. Thus, the current study was designed to determine the extended profile of these TBI-specific biomarkers both in brain tissue and cerebral spinal fluid (CSF). Protein abundance was evaluated in brain tissue samples taken from regions of interest and in CSF at 24 h, 3 days, 7 days, 1 month, and 3 months following severe TBI in rats. Results showed increased full length GFAP (GFAP-FL) and GFAP-BDPs starting at 24 h that remained significantly elevated in most brain regions out to 3 months post-injury. However, in CSF, neither GFAP-FL nor GFAP-BDPs were elevated as a consequence of injury. Regional-specific reduction in α II-spectrin was evident in brain tissue samples from 24 h through 3 months. In contrast, SBDP-145/150 was robustly elevated in most brain regions and in CSF from 24 h through 7 days. Correlation analyses revealed numerous significant relationships between proteins in CSF and brain tissue or neurological deficits. This work indicates that TBI results in chronic changes in brain protein levels of well-known TBI biomarkers GFAP, α II-spectrin, and their BDPs and that SBDP-145/150 may have utility as an acute-chronic biomarker.

Keywords: traumatic brain injury, biomarker, glial fibrillary acidic protein, α II-spectrin, breakdown product, penetrating ballistic-like brain injury, subacute, chronic

INTRODUCTION

Severe penetrating traumatic brain injury (TBI) from gunshot wounds is of concern to both military and civilians alike. Approximately 5,000 penetrating TBI cases were reported from 2000 to 2017 among military personnel (1). Additionally, a high incidence of gun violence persists among civilian populations (2). Severe TBI patients are at risk for

mortality and reduced life expectancy (3–5) and the incidence and prevalence of chronic debilitating disability among severe TBI patients are substantial (6–8). Further, the lifetime costs of long-term treatment and care for a severely injured TBI patient and the stresses placed on family caretakers are devastating. Neurosurgical interventions may provide benefits such as reduced intracranial pressure (9) and decreased mortality (10). Yet, clinically effective treatments for treating severe TBI at the molecular level remain somewhat limited, which constitutes a critical gap in patient care. Increased understanding of acute-chronic molecular events in multiple brain regions following severe TBI is important to provide guidance regarding targeted solutions for both acute and chronic treatments.

Traumatic brain injury results in a primary injury characterized by immediate destruction of brain tissue with hemorrhage followed by a complex secondary injury cascade during which there is an influx of intracellular calcium (11–13) and subsequent activation of calcium-activated, non-lysosomal proteases such as calpain-II (14). Glial fibrillary acidic protein (GFAP), an intermediate filament protein expressed abundantly by astrocytes that is increased in multiple TBI models during reactive astrogliosis (15, 16), is a well described substrate of calpain-II mediated proteolysis. Another key substrate, non-erythroid α II-spectrin, is a neuronal scaffolding protein expressed abundantly in axons and pre-synaptic terminals (17). Cleavage of GFAP and α II-spectrin by calpain-II results in generation of breakdown products (BDPs) (18, 19). These proteins are highly enriched throughout the CNS and are detectable in biofluids following TBI. Therefore, these proteins and their BDPs have garnered significant interest for their potential utility as indicators of TBI-mediated astrogliosis and neuronal cell death during secondary brain injury cascades and as peripheral biomarkers associated with focal injury severity and negative outcomes (20–22). It should be noted, however, that astrogliosis is not necessarily a maladaptive process and may provide benefit following TBI (23).

The increased presence of these proteins and their BDPs in brain tissues has been well described following acute TBI (24–27). However, these observations may persist chronically and differ across brain regions and CSF. GFAP-BDPs (28) and GFAP autoantibodies (29) are increased in blood derived from chronic TBI patients. CSF levels of α II-spectrin breakdown products (SBDPs) remain elevated compared to controls at 5–7 days after severe penetrating TBI (20), suggesting this increase may continue past the acute timeframe. Additionally, the relative abundance of calpain-II is rarely described within matching cohorts over an extended period although it is a key mediator of BDP generation. Longitudinal characterization of these proteins in brain tissues would provide vital information regarding the duration and extent of secondary injury following severe TBI, while temporal alteration of these proteins in biofluids may prove useful as biomarkers to track injury progression. Further, defining how this secondary injury cascade differs across brain regions that are either proximal or distal to the injury trajectory site as a consequence of both time and injury is of critical importance.

The goal of the current study was to temporally define the abundance of GFAP, α II-spectrin, and their associated BDPs in specific brain tissue regions and CSF following TBI. Injuries were induced using a rat model of penetrating ballistic-like brain injury (PBBi), which mimics the permanent injury tract and temporary cavity generated from a penetrating ballistic round (30–32) and results in well-described pathophysiology as well as behavioral impairment (33–35). This study also explores protein levels of calpain-II in brain tissues. We report that calpain-II mediated proteolysis in brain tissues persists well past the acute post injury window and into the chronic time frame following PBBi. Furthermore, we present evidence that detection of α II-spectrin BDPs is prominent in cerebral spinal fluid throughout acute-subacute injury and has value as a correlative biomarker for these same fragments in brain tissues.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (Charles River Laboratories, Raleigh, VA, United States) weighing ~250–300 g were used in this study. All rats were singly housed with a 12 h normal light/dark cycle. Surgical procedures were performed under isoflurane anesthesia (2–5% delivered in oxygen). For terminal bio-sample collections at 24 hours (h), 3 days (d), 7 days, 1 month (m), and 3m following injury, rats were deeply anesthetized with 70 mg/kg ketamine and 6 mg/kg xylazine. Research was conducted under an approved animal use protocol in an AAALACi accredited facility in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, NRC Publication, 2011 edition.

Brain Injury Procedures

The WRAIR PBBi model captures the injury trajectory and temporary cavity generated by energy dissipation from a high-energy bullet wound to the head (30). Unilateral frontal PBBi was induced as previously described (34). Briefly, anesthetized rats were placed on a stereotaxic frame. Following craniotomy (+4.5 mm antero-posterior, +2 mm medio-lateral from bregma), a probe was inserted through the right frontal cortex and striatum to 1.2 cm from dura and a computer-controlled pulse generator was activated to rapidly inflate and deflate a balloon on the end of the probe, creating a temporary cavity equivalent to 10% brain volume. Probe injured rats received identical procedures except balloon inflation/deflation. Sham rats received craniotomy without probe insertion.

Neuroscore Assessment

Neurological deficits were assessed using a modified testing paradigm (36) at 24 h following injury or sham manipulation by an experimenter who was blinded to the injury condition. A composite score was generated from four separate parameters: contralateral forelimb flexion, body upward curling behavior during tail suspension, open-field circling behavior, and impaired resistance to lateral push. Each parameter was scored from 0

(normal) to 3 (severely impaired) for a maximum composite score of 12 for each animal.

Cerebral Spinal Fluid and Brain Tissue Collection and Preparation

A 4-cm midline incision was made from 0.5 cm anterior to the interauricular line. The atlanto-occipital dura mater was exposed by separating the nuchal muscles and CSF was collected with a 30-gauge syringe needle through the membrane. CSF was stored on ice, supplemented with 1xHALT protease/phosphatase inhibitor mix (Thermo Fisher Scientific, Grand Island, NY, United States), and centrifuged at 1,400 g for 10 min at 4°C. The resulting cell free CSF was stored at –80°C until use. To increase the concentration of antigens in CSF, the entire collected volume of each CSF sample was concentrated with Amicon Ultra Centrifugal Filter Units with Ultracel-3 membranes (EMD Millipore, Billerica, MA, United States) for 30 min at 14,000 × g at 4°C (normalization method presented in “Statistical Analysis”). Ipsilateral brain tissues were washed with 0.9% saline and dissected on ice to isolate the frontal cortex, striatum, hippocampus, and residual midbrain. All tissues were flash-frozen in liquid nitrogen. Concentrated CSF and flash-frozen brain tissues were stored at –80°C until use.

Western Blotting

Brain tissues were sonicated in 1xRIPA buffer containing 1xHALT protease/phosphatase inhibitors (Thermo Fisher Scientific, Grand Island, NY, United States). Protein concentrations were determined using the BCA assay kit (Thermo Fisher Scientific, Grand Island, NY, United States) and crude homogenates were stored frozen until use. All samples for western blotting were prepared in 1x LDS NuPAGE sample buffer and 250 μM DTT and denatured for 5 min at 95°C. Equivalent amounts of total protein (20 μg for calpain-II, 25 μg for GFAP and αII-spectrin) for tissue samples or 10 μL of concentrated CSF prepared as described above were loaded onto 12% (GFAP in tissue) or 4–12% bis-tris gels (GFAP in CSF, αII-spectrin in tissues and CSF, calpain-II in tissues) for separation by SDS-PAGE (Invitrogen NuPAGE, Carlsbad, CA, United States). Proteins were transferred to PVDF membranes and blocked in 5% milk in 1xPBS (GFAP and αII-spectrin in tissues) or to nitrocellulose membranes and blocked with Odyssey Blocking Buffer – PBS (GFAP and αII-spectrin in CSF, calpain-II in tissues; LI-COR Biosciences, Inc., Lincoln, NE, United States). Individual membranes were probed with the following primary antibodies: GFAP (ab7260, Abcam, Cambridge, MA, United States), αII-spectrin (in tissues: MAB1622, EMD Millipore, Billerica, MA, United States; in CSF: BML-FG6090, ENZO, Farmingdale, NY, United States), Calpain-II – Large Subunit (#2539, Cell Signaling Technology, Danvers, MA, United States), and washed with phosphate buffered saline containing 0.01–0.1% Tween 20 (PBST). For tissue GFAP and αII-spectrin blots, membranes were incubated with horseradish peroxidase (HRP)-linked secondary antibodies and bands were detected with Clarity Western ECL (Bio-Rad, Hercules, CA, United States). Blots were visualized and quantified using the ImageQuant LAS4000 and ImageQuant TL v7.0 software (GE Healthcare, Pittsburgh, PA, United States).

For calpain-II and GFAP and αII-spectrin blots of CSF, InfraRed (IR)-Dye labeled secondary antibodies were used to visualize bands on the Odyssey CLx imaging system (LI-COR Biosciences, Lincoln, NE, United States) and bands were quantified using ImageStudio v5.2 software (LI-COR Biosciences, Lincoln, NE, United States).

GFAP Electro-Chemiluminescent Enzyme-Linked Immunosorbent Assay (ELISA)

Concentrated CSF samples were evaluated for GFAP content using an in-house assay developed for detection with the Mesoscale Discovery (MSD) platform (37). Samples and standards were loaded in duplicate. Samples were diluted in 1xPBS, pH 7.8 (Bio-Rad, Hercules, CA, United States) then incubated in plates manually coated with 25 μg/mL polyclonal anti-GFAP (ab7260, Abcam, Cambridge, MA, United States) in 1xPBS, pH 7.8. Plates were then incubated with mixed monoclonal anti-GFAP detection antibodies (BD556330, BD Biosciences, San Jose, CA, United States) and anti-mouse sulfo-tag antibody (MSD, Rockville, MD, United States) at 0.5 μg/mL each in 0.5% Blocker B prepared in 1xPBS, pH 7.8. Protein content was derived from standard curves using recombinant human GFAP protein in PBS (Banyan Biomarkers, Alachua, FL, United States; standard range: 0.156–10 ng/mL) fit by a cubic third order polynomial function. Values derived from PBS were used as blanks and subtracted from all samples. Analyte quantitation (ng/mL) was determined by electro-chemiluminescent signal with a Meso QuickPlex SQ120 (Meso Scale Discovery, Rockville, MD, United States).

Data Management and Statistical Analysis

For tissue western blots, mean target band densities for probe and PBBI groups are presented as a percent of the sham control value (100%) calculated from individual membranes. For CSF western blots, target bands at each time point were normalized to the mean value of a positive control band generated from frontal cortex tissue lysates collected 24 h post-PBBI. Since variable volumes of CSF were used to concentrate proteins, these normalized band values were subsequently normalized by the original μL of CSF loaded for protein concentration. Concentrations of GFAP (ng/mL) obtained in ELISA experiments were also normalized by the original μL of CSF. For CSF western blots and ELISAs, any negative values that were generated (i.e., band signal equal to less than the calculated background signal or sample signal less than blank) were set as ‘0’ for the purpose of data analysis. Sample sizes are detailed in the figure legends. Please note that 9–10 rats/group and time point were initiated on study, however, due to inability to collect CSF or insufficient collection volumes from all subjects, additional rats were injured for acute collections time points when necessary. All values are presented as mean ± SEM. Outliers as determined by ROUT analysis ($Q = 0.1\%$) were excluded from all data sets. For neuroscore, western blotting experiments, and ELISAs, significant injury

effects were determined at each time point using a one-way ANOVA followed by Tukey's multiple comparisons test to compare experimental groups. Two-tailed Pearson correlation coefficients (r) were determined using all available data points from Western blot experiments with sham, probe, and PBBI rats. For all analyses, results were considered statistically significant when $p < 0.05$. All statistical analyses were performed using GraphPad Prism v6 (La Jolla, CA, United States).

RESULTS

Neurological Impairment

To evaluate neurological deficits following the probe injury and PBBI, neuroscore values were assessed at 24 h after injury compared to sham control procedures for all cohorts. Both probe injury (3.38 ± 0.42 , range 0–12) and PBBI (6.75 ± 0.31 , range 1.5–12) resulted in significantly elevated cumulative neuroscore values compared to sham (0.43 ± 0.10 , range 0–1.5) (**Figure 1**), indicating that both injuries result in acute neurological impairment. Additionally, PBBI animals had significantly elevated neuroscore values over probe-injured rats.

Quantitation of GFAP, α II-spectrin, and Calpain-II in Brain Tissues

To determine the abundance of both full length GFAP (GFAP-FL) at 50 kDa and its breakdown products (BDPs) from 37 to 48 kDa, the brain regions that directly encompass the injury trajectory (frontal cortex, striatum) and adjacent distal regions (hippocampus, residual midbrain) were examined by western blotting at 24 h, 3 days, 7 days, 1 month, and 3 months following injury (**Figure 2A**). Quantitative analysis indicated that GFAP-FL and GFAP-BDPs were elevated over time in both the probe injury and PBBI groups compared to sham. Probe injury alone resulted in significantly increased GFAP-FL in the brain regions which encompass the injury trajectory starting at 3 days (frontal cortex: peak of 589% at 7 days; striatum: peak of 397% at 7 days) (**Figure 2B**). This elevation was sustained through 3 months

in the frontal cortex. PBBI resulted in a slight, but significant, increase in GFAP-FL in the striatum starting at 24 h. Beginning at 3 days, GFAP-FL was significantly increased following PBBI in all brain regions and persisted in the frontal cortex (peak of 995% at 7 days), striatum (peak of 912% at 1 month), and residual midbrain (peak of 276% at 1 month) through 3 months, whereas increases in the hippocampus (peak of 276% at 3 days) were resolved by 1 month.

As with GFAP-FL, significant elevations of GFAP-BDPs following probe injury compared to sham were detected only in the frontal cortex and striatum at 3 days (3247%) or 7 days (11,123%), respectively (**Figure 2C**). In contrast, PBBI robustly increased GFAP-BDPs at all time points in the frontal cortex (peak of 25,450% at 7 days), striatum (peak of 38,230% at 7 days), and residual midbrain regions (peak of 525% at 1 month). PBBI significantly increased GFAP-BDPs in the hippocampus at 3 days (1026%) and 7 days (485%). The consequence of increased injury severity on GFAP-FL and GFAP-BDPs was also determined by evaluating differential protein abundance between probe and PBBI injured rats. The abundance of GFAP-FL and GFAP-BDPs following PBBI was significantly increased over probe injury in a number of brain regions and time points assessed (**Figures 2B,C**), although this observation was less frequent than comparisons with sham.

Next, the abundance of α II-spectrin and spectrin breakdown products (SBDPs) was assayed in the same manner as GFAP. Visual inspection of the western blots confirmed that full length α II-spectrin was detectable at 280 kDa as expected. After probe injury and PBBI, SBDP-145/150 was robustly evident, while SBDP-120 was minimally detected and poorly resolved (**Figure 3A**). Quantitative analysis revealed that probe injury decreased α II-spectrin compared to sham at 1 month in the striatum only (–24%) (**Figure 3B**). PBBI acutely reduced α II-spectrin in the areas of the immediate injury trajectory (frontal cortex: maximum decrease of –64% at 3 days, striatum: –26% at 24 h only) compared to sham. In distal injury areas, no acute reductions in α II-spectrin were observed compared to sham. However, PBBI lowered α II-spectrin levels at subacute and chronic time points (hippocampus: –57% at 3 month only, residual midbrain: maximum decrease of –72% at 7 days).

Concomitant increases in SBDP-145/150 were also observed (**Figure 3C**). Probe injury increased SBDP-145/150 in the frontal cortex at 24 h and 3 days (peak of 1091% at 24 h). Following PBBI, SBDP-145/150 increased starting at 24 h and remained elevated through 7 days in the frontal cortex (peak of 1742% at 24 h), striatum (peak of 1731% at 3 days), and residual midbrain (peak of 1230% at 7 days). Levels detected in the striatum remained elevated until 1 month. In the hippocampus, PBBI led SBDP-145/150 to increase at 3 days, only (182%). Quantitative analysis of SBDP-120 revealed that this fragment was elevated, albeit at a much smaller magnitude than observed with SBDP-145/150 (Supplementary Figure S1). Probe injury increased SBDP-120 at 3 month in the frontal cortex only (262%). PBBI resulted in significantly increased SBDP-120 in the frontal cortex at 24 h and 3 days (peak of 357% at 24 h) and delayed increases in the hippocampus at 3 months (434%) and the residual midbrain at 7 days and 1 month (peak of 349% at 1 month). Additionally,

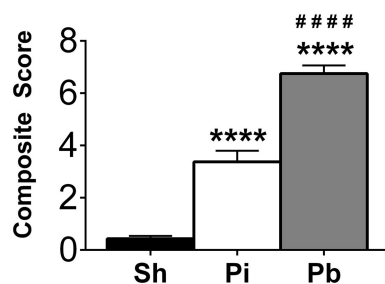


FIGURE 1 | Penetrating brain injury results in significant acute neurological deficits. Neuroscore evaluations were performed 24 h following injury in Sham (Sh, black bar), Probe Injury (Pi, white bar), and PBBI (Pb, gray bar) groups. Composite values are presented here from each injury condition regardless of the tissue collection time point. $N = 44 - 50$ per group. **** $p \leq 0.0001$ Pi or Pb vs. Sh; ##### $p \leq 0.0001$ Pb vs. Pi, one-way ANOVA with Tukey's multiple comparisons test.

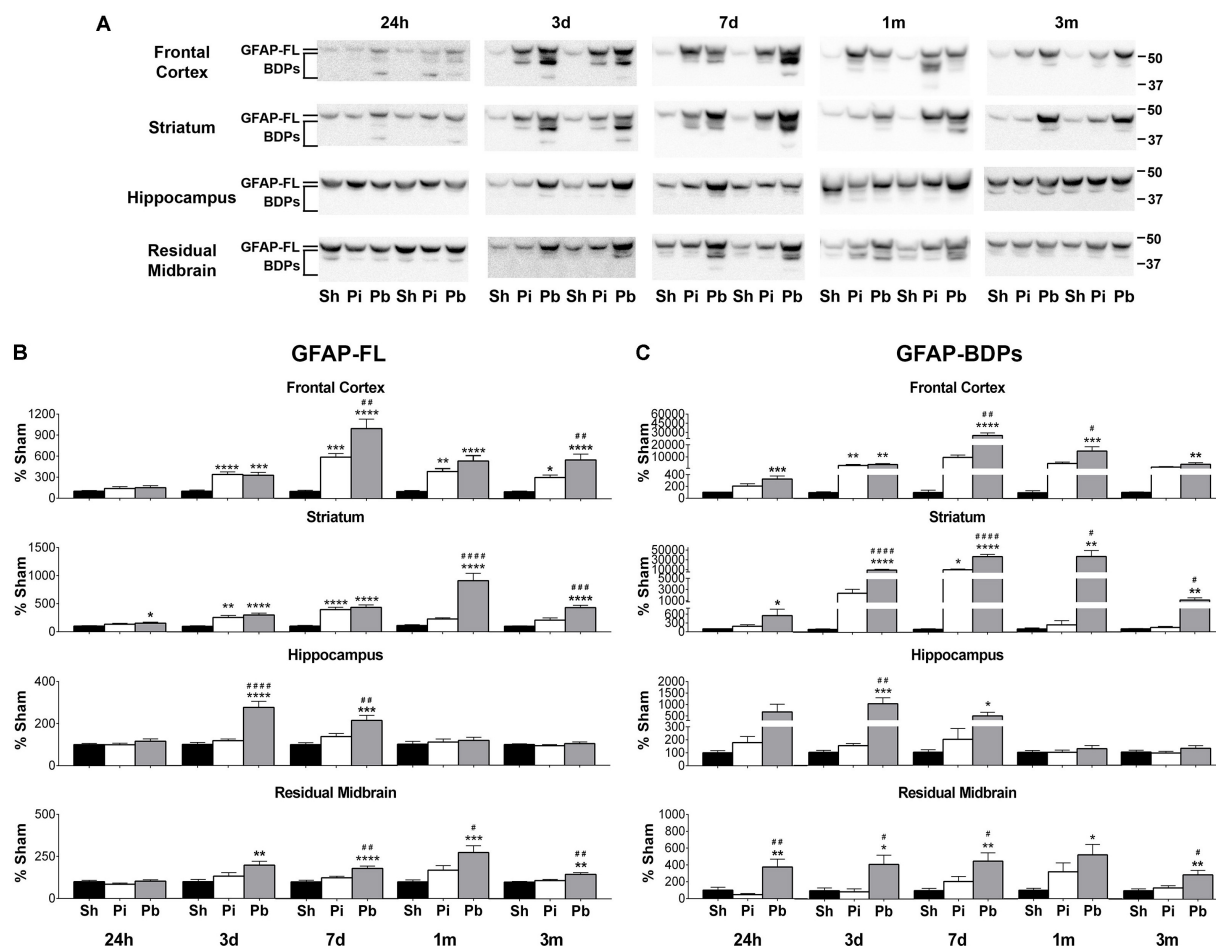


FIGURE 2 | Penetrating brain injury results in robust longitudinal increases in GFAP and GFAP-BDPs in brain tissue. **(A)** Representative western blots to illustrate GFAP and its BDPs at 24 h, 3 days, 7 days, 1 month, and 3 months post injury or sham manipulation in the frontal cortex, striatum, hippocampus, and residual midbrain areas. Full length GFAP (GFAP-FL) was detected as indicated at 50 kDa, with breakdown products ranging from 37 to 48 kDa. Depicted gels were loaded in the order of Sham (Sh), Probe (Pi), PBBI (Pb) as indicated. Quantitation of **(B)** GFAP-FL at 50 kDa and **(C)** GFAP-BDPs at 37–48 kDa is presented as percent change from sham for each time point and brain region examined. Values are presented as mean $\pm$ SEM for Sh (black bars), Pi (white bars), or Pb (gray bars) groups. $N = 9 - 10$ per group and time point, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p \leq 0.0001$ Pi or Pb vs. Sh; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$, $\#\#\#\#p \leq 0.0001$ Pb vs. Pi, one-way ANOVA with Tukey's multiple comparisons test. Full blot images are available in Supplementary Figures S3–S6.

PBBI altered levels of α II-spectrin, SBDP-145/150, and SBDP-120 compared to probe insertion alone.

To determine if calpain-II was differentially abundant after brain trauma, protein levels were assessed by western blot in the cohorts used to define GFAP, α II-spectrin, and respective BDP levels (**Figure 4**). Prominent bands corresponding to full-length calpain-II were detected at 73 kDa (**Figure 4A**). Compared to sham, probe injury reduced calpain-II abundance in the frontal cortex (-34% at 3 days) and the hippocampus (-51% at 7 days) (**Figure 4B**). PBBI acutely reduced calpain-II abundance in the frontal cortex (maximum decrease of -44% at 3 days), striatum (maximum decrease of -34% at 24 h), and residual midbrain (maximum decrease of -30% at 3 days) compared to sham. Surprisingly, at 7 days after PBBI, increased calpain-II was observed in the frontal cortex and striatum (150 and 142% respectively) compared to both sham and probe-injured rats.

Detection of GFAP, GFAP-BDPs and SBDPs in CSF

Cerebral spinal fluid samples were used to determine the abundance of GFAP, α II-spectrin, and their BDPs after injury. Total GFAP ELISAs (representing both GFAP-FL and GFAP-BDPs) indicated that neither probe nor PBBI resulted in significantly altered total GFAP at 24 h or 3 days after injury (**Figure 5A**). Total GFAP in CSF collected 7 days through 3 months was not readily detectable (data not shown). To discern if either GFAP-FL or GFAP-BDPs, rather than total GFAP, may change significantly as a result of injury in these samples, western blots were performed (**Figure 5B**). Visual inspection revealed the presence of GFAP-FL and the characteristic banding pattern for GFAP-BDPs at 24 h and 3 days. Quantitation of this data revealed no significant alteration in GFAP-FL and GFAP-BDPs as determined by western blot (Supplementary Figure S2). As

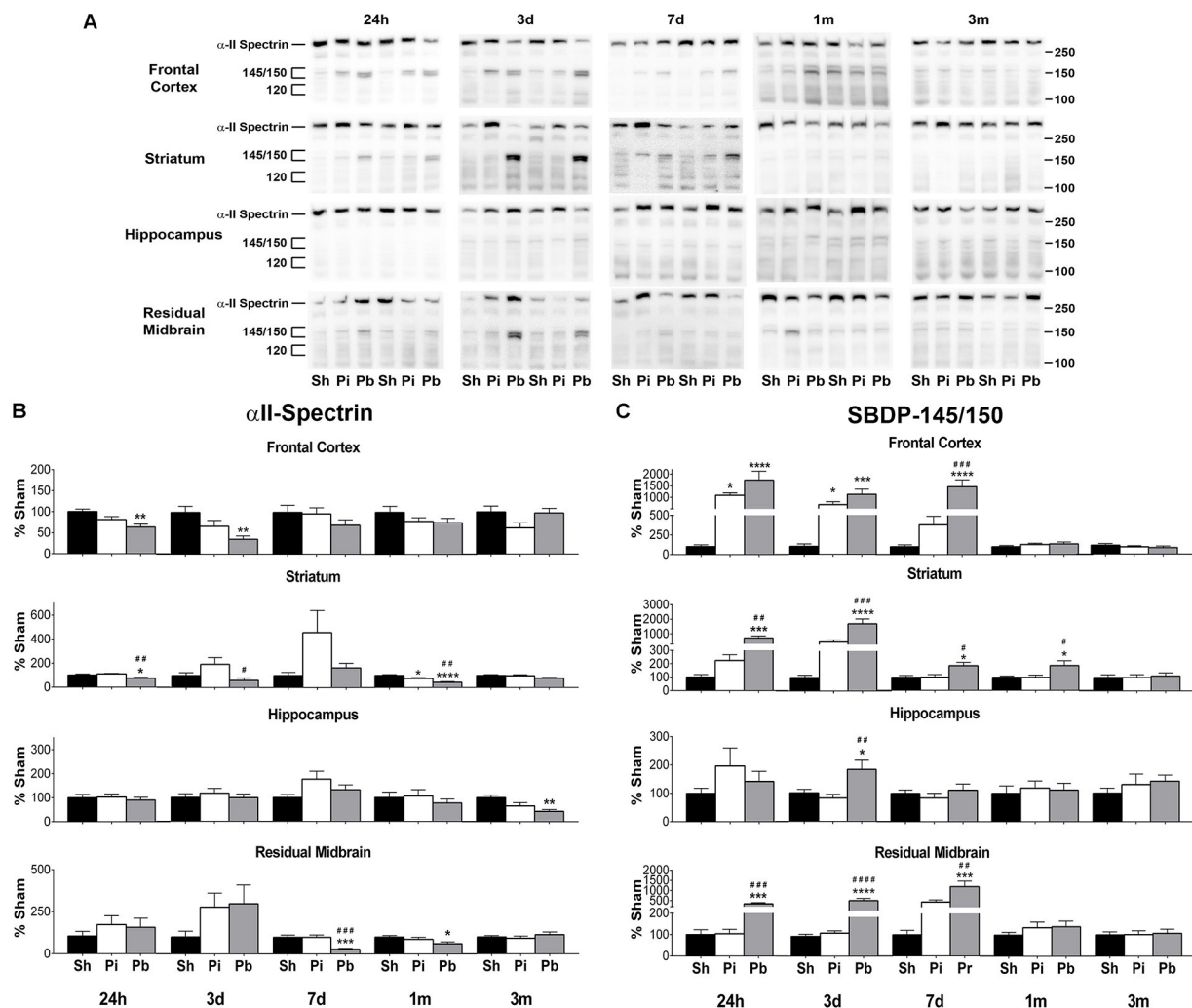


FIGURE 3 | Penetrating brain injury reduces the abundance of α II-spectrin with concomitant increases in SBDP in brain tissues. **(A)** Representative western blots to illustrate α II-spectrin and SBDP at 24 h, 3 days, 7 days, 1 month, and 3 months post injury or sham manipulation in the frontal cortex, striatum, hippocampus, or residual midbrain regions. Alpha-II-spectrin was detected as indicated at 280 kDa, with SBDPs present at approximately 145/150 and 120 kDa. Depicted gels were loaded in the order of Sham (Sh), Probe (Pi), PBBI (Pb) as indicated. Quantitation of **(B)** α II-spectrin and **(C)** SBDP-145/150 is presented here as percent change from sham for each time point and brain region examined. Quantitation of SBDP-120 is available in Supplementary Figure S1. Values are presented as mean $\pm$ SEM for Sh (black bars), Pi (white bars), or Pb (gray bars) groups. $N = 9 - 10$ per group and time point, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p \leq 0.0001$ Pi or Pb vs. Sh; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p \leq 0.0001$ Pb vs. Pi, one-way ANOVA with Tukey's multiple comparisons test. Full blot images are available in Supplementary Figures S7–S10.

with ELISA analysis, levels in most samples were not readily detectable (data not shown) although GFAP-FL and GFAP-BDPs were evident in certain animals from 7 days to 3 month time points. Interestingly, GFAP-BDPs were detectable in a limited number of sham animals at acute through chronic times after injury.

Western blotting of CSF indicated that α II-spectrin, SBDP-145/150, and SBDP-120 were present as late as 3 months after injury (Figure 6A). Quantitative analysis demonstrated that while probe injury did not alter α II-spectrin or SBDP levels, PBBI resulted in significant elevation of these analytes compared to both sham and probe injured groups (Figure 6B). Alpha II-spectrin was increased to 352 and 967% at 24 h and

3 days, respectively, while SBDP-145/150 was elevated by 2248, 84,068%, and by 649% at 24 h, 3 days, and 7 days post-PBBI, respectively. SBDP-120 rose by 1035% at 3 days.

Correlations With Protein Levels in Brain Tissue Regions

To determine if CSF levels of GFAP, α II-spectrin, and their BDPs may have utility to predict abundance of the same protein levels in brain tissues, correlation analyses were performed using grouped data points from all three conditions as described in materials and methods. These correlations between specific protein levels in CSF and the corresponding protein levels in brain tissues were performed for each brain region and time

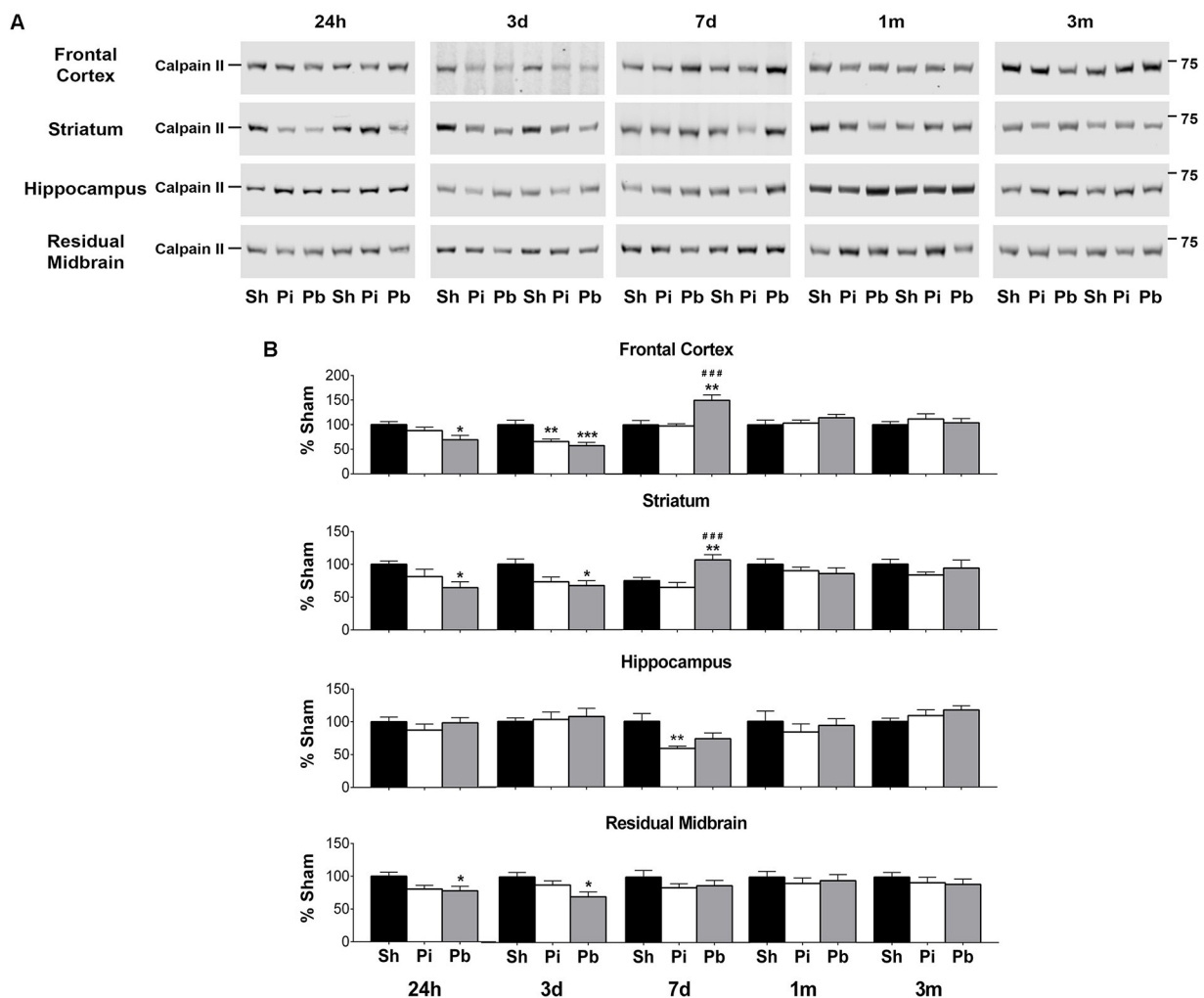
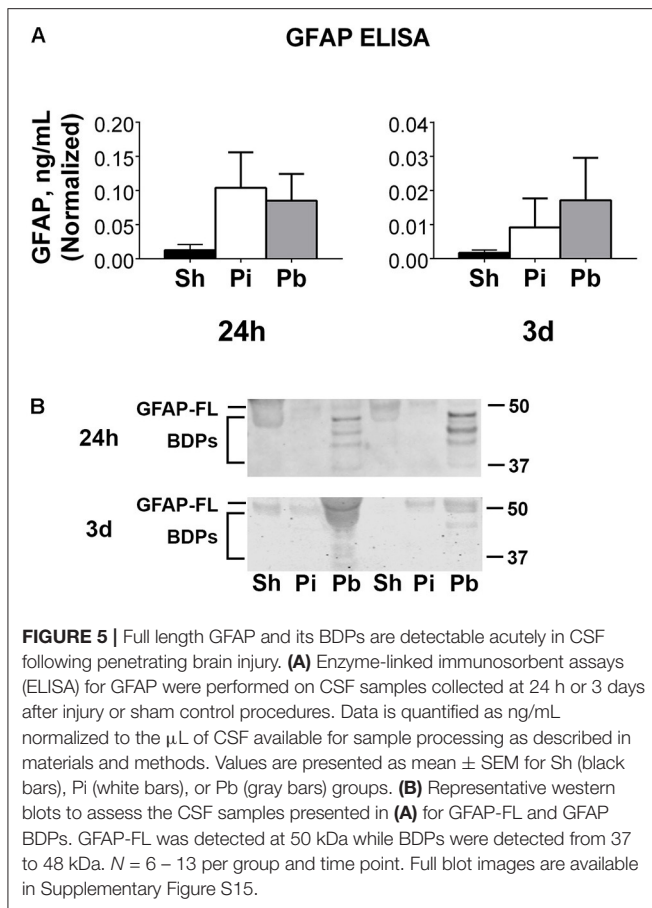


FIGURE 4 | Penetrating brain injury alters the abundance of full length calpain-II in brain tissues. **(A)** Representative western blots to illustrate full length calpain-II at 24 h, 3 days, 7 days, 1 month, and 3 months post injury or sham manipulation in the frontal cortex, striatum, hippocampus, or residual midbrain. Full length calpain-II was detected at approximately 72 kDa. Depicted gels were loaded in the order of Sham (Sh), Probe (Pi), PBBI (Pb) as indicated. **(B)** Quantitation of calpain-II is presented as percent change from sham for each time point and brain region examined. Values are presented as mean $\pm$ SEM for Sh (black bars), Pi (white bars), or Pb (gray bars). $N = 9 - 10$ per group and time point, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Pi or Pb vs. Sh; ### $p < 0.001$ Pb vs. Pi, one-way ANOVA with Tukey's multiple comparisons test. Full blot images are available in Supplementary Figures S11–S14.

point. GFAP-BDPs, but not GFAP-FL, revealed many significant positive correlations between CSF and tissues (Supplementary Table S1). GFAP-FL in CSF correlated with levels detected in the hippocampus at 3 days alone ($r = 0.579$). Starting at 24 h, GFAP-BDPs in CSF correlated with these same cleavage products in the frontal cortex, striatum, and residual midbrain (r range: from 0.476 to 0.569). This observation persisted at 3 days with the frontal cortex, striatum, and hippocampus (r range: 0.511–0.797). Similar to the results for GFAP, the relationship between SBDP, but not α II-spectrin, in CSF and brain tissues was significant in many instances (Table 1). This result was overwhelmingly true for SBDP-145/150 where significant correlations between CSF and brain tissue abundance were observed at 24 h in the striatum and

residual midbrain ($r = 0.582$ and 0.655, respectively) and at 3d in the striatum, hippocampus, and residual midbrain (r range: 0.498–0.918). This correlation persisted at 7 days in the frontal cortex, striatum, and residual midbrain areas (r range: 0.507–0.777). CSF SBDP-120 was positively associated with values detected in the frontal cortex at 7 days post injury only ($r = 0.560$).

Additional correlations were performed to determine the relationship between acute neurological deficits and brain tissue protein abundance grouped from all three injury conditions. Overall, acute neuroscore values were positively associated with GFAP-FL and GFAP-BDPs in brain (Table 2). At 3 days (r range: 0.498–0.749) and 7 days (0.476–0.828), GFAP-FL and GFAP-BDP abundance in all brain regions correlated



significantly with neurological deficits. Acute neuroscore values and brain α II-spectrin levels were negatively associated with one another throughout acute-chronic injury (Table 3). This negative association was strongest at 7 days ($r = -0.632$ in residual midbrain). In accordance with the gain of SBDPs in brain tissues, analysis indicated that these levels were positively associated with acute neurological deficits. This was overwhelmingly true of SBDP-145/150 detected at 24 h–7 days, wherein this correlation was significant among all four brain regions at 3 days, but was greatest with the residual midbrain ($r = 0.742$). Fewer instances of significance were observed with analysis of SBDP-120 levels, where the strongest correlation was with the residual midbrain at 7 days ($r = 0.664$).

DISCUSSION

This study provides a comprehensive evaluation of well-described TBI biomarkers (GFAP, α II-spectrin, and their BDPs) in brain tissue regions and CSF throughout acute-chronic (24 h to 3 months) injury progression. A representation of this timeline following PBBI is indicated for brain tissues (Figure 7A) as approximated from the immediate areas of the primary injury trajectory (frontal cortex and striatum) and CSF (Figure 7B). PBBI resulted in widespread time-dependent changes in protein

abundance, which was especially robust for BDPs as opposed to intact, full-length counterparts within several brain regions. While effects were also evident to some degree in animals exposed to insertion of the probe alone, the majority of injury effects appear to result from the ballistic-like force of the injury captured by the rapid inflation/deflation of the balloon inside the rat brain. This study indicated that CSF SBDP-145/150 levels may have utility during acute-subacute injury to serve as an indicator of neuronal degradation in the brain. Further, this study indicated that acute neurological deficits were associated with levels of GFAP, α II-spectrin, and BDPs as late as 3 months after injury.

Temporal Progression of Astroglial and Neuronal BDPs

GFAP and α II-spectrin proteolysis and subsequent BDP generation in brain tissue is well-established following TBI but has focused on the acute time frame (24–27). This study indicates that GFAP and α II-spectrin degradation, and the resulting increased generation of BDPs, is progressive and dominant during subacute-chronic time frames. The increase in GFAP-BDPs and SBDPs after both probe injury and PBBI provide evidence that both GFAP-FL in astrocytes and α II-spectrin in neurons undergo long-term proteolysis as a consequence of PBBI, albeit with distinct temporal profiles. These diverging profiles indicate that reactive astrogliosis (GFAP-FL) and astroglial protein degradation (GFAP-BDP) are comparatively delayed following PBBI and persist chronically. In contrast, neuronal cytoskeletal protein degradation (SBDP-145/150) is prominent within acute-subacute time frames but resolves afterward, which is likely due to the resolution of the core lesion into an intracranial cavity by 7 days following PBBI (38). This pattern may extend to other injury models as well, since acute elevation of SBDP within 7–14 days (39) and chronic upregulation of GFAP up to 1 year (40, 41) have been previously reported. These distinct temporal profiles associated with GFAP and α II-spectrin inform the duration of drug treatment paradigms targeted toward specific mechanisms, such as the chronic sustenance of astrogliosis and proteolysis of GFAP-FL into GFAP-BDPs.

Calpain- or caspase- mediated protein degradation is proposed to be a key factor in TBI mediated neurodegeneration. Generation of GFAP-BDPs and SBDP-145/150 are products of calpain-mediated proteolysis (26), whereas proteolysis by caspase-3 activity results primarily in generation of SBDP-120 (42). The results presented here indicate the predominance of calpain-mediated cleavage products in the acute-chronic periods following PBBI. Additionally, this study demonstrated an overall loss in full length calpain-II, an indirect marker of calpain activation (43), following PBBI within brain regions that were proximal as well as somewhat distal to the injury tract. Calpain-II is decreased during truncation and activation prior to degradation of protein substrates (42), however, calpain-II fragments that represent the active form of the enzyme were not detectable in this study. As a minor point, full-length calpain-II increased 7 days after PBBI, a finding that has previously been reported in a blast model of TBI (44) and may reflect a

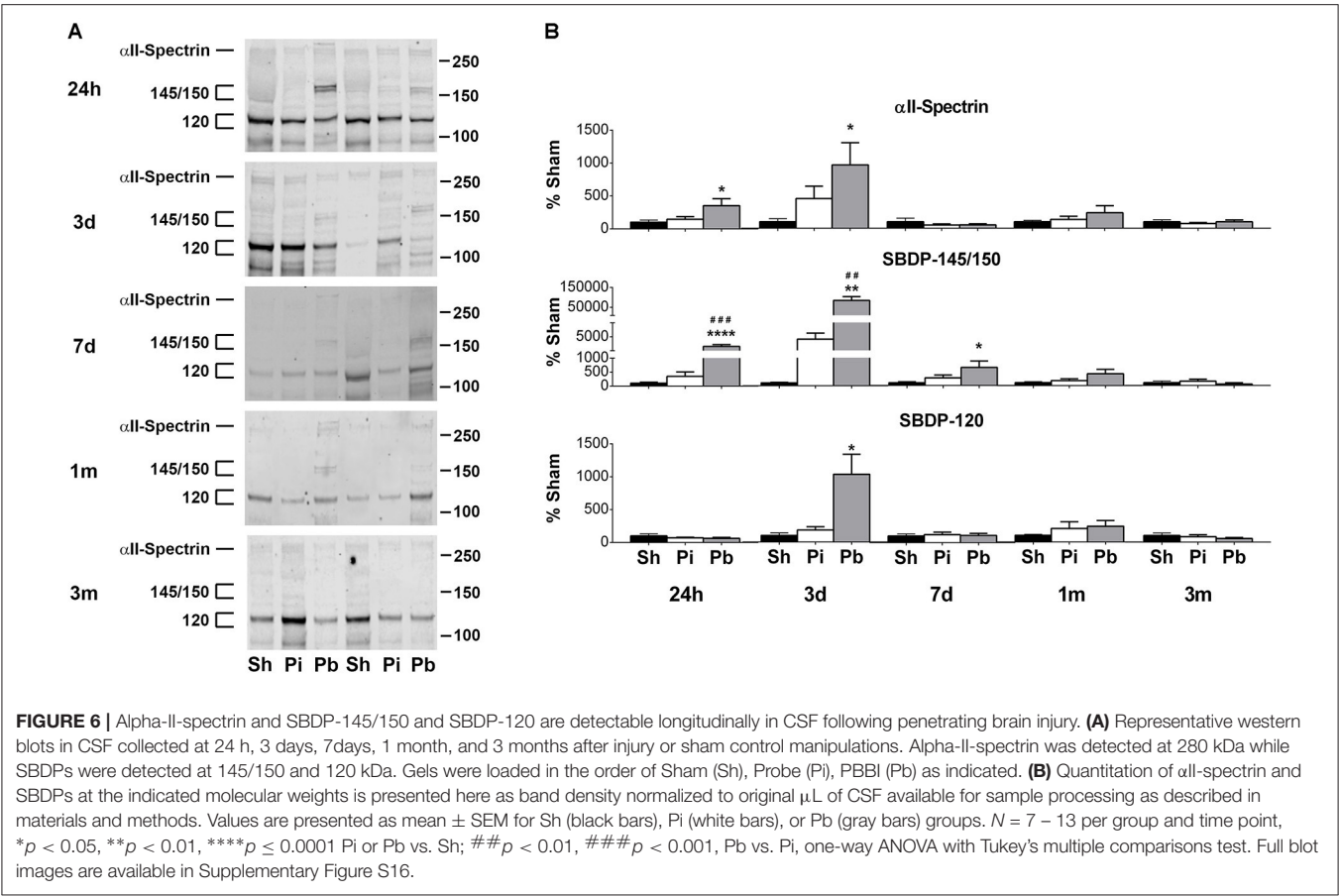


TABLE 1 | Correlation results between full length α II-spectrin or SBDP protein levels in CSF and tissues specified by brain region and time point.

	24 h		3 days		7 days		1 month		3 months	
	<i>r</i>	<i>p</i> -value	<i>r</i>	<i>p</i> -value	<i>r</i>	<i>p</i> -value	<i>r</i>	<i>p</i> -value	<i>r</i>	<i>p</i> -value
<i>αII-Spectrin</i>										
FCX	−0.338	0.078	−0.417	0.096	0.141	0.483	0.273	0.153	−0.338	0.079
STM	−0.315	0.102	−0.172	0.509	−0.082	0.691	−0.145	0.454	−0.221	0.249
HC	−0.017	0.931	−0.256	0.322	0.049	0.808	−0.169	0.380	−0.030	0.881
RMB	−0.229	0.251	0.470	0.057	0.075	0.717	0.168	0.384	0.014	0.942
<i>SBDP-145/150</i>										
FCX	0.287	0.139	0.295	0.206	0.777	<0.0001****	−0.065	0.738	0.398	0.066
STM	0.582	0.001**	0.880	<0.0001****	0.507	0.008**	0.124	0.523	0.251	0.249
HC	0.095	0.631	0.498	0.030*	−0.188	0.348	−0.219	0.254	−0.016	0.943
RMB	0.655	0.000***	0.918	<0.0001****	0.559	0.002**	0.081	0.678	−0.179	0.413
<i>SBDP-120</i>										
FCX	−0.083	0.673	−0.094	0.693	0.560	0.002**	−0.188	0.358	−0.070	0.733
STM	0.155	0.450	0.105	0.660	−0.059	0.776	−0.299	0.138	0.247	0.235
HC	−0.240	0.229	0.030	0.902	−0.361	0.064	0.059	0.775	−0.200	0.317
RMB	−0.359	0.066	−0.241	0.307	0.181	0.365	−0.047	0.821	−0.073	0.731

Pearson *r* correlation analyses were performed between levels of α II-spectrin or SBDPs in CSF and brain tissues by region and time point post injury. Protein levels were determined by Western blot at each time point post injury and were expressed as normalized values for CSF markers and as a percent sham for tissue markers for analysis. Sham, Probe, and PBBI injured rats were all included for analysis. Significant correlations are marked with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), or **** ($p \leq 0.0001$). CSF, cerebrospinal fluid; FCX, frontal cortex; STM, striatum; HC, hippocampus; RMB, residual midbrain.

TABLE 2 | Correlation results between GFAP-FL and GFAP-BDP protein tissue levels and neuroscore values specified by brain region and time point.

	24 h		3 days		7 days		1 month		3 months	
	<i>r</i>	<i>p</i> -value	<i>r</i>	<i>p</i> -value	<i>r</i>	<i>p</i> -value	<i>r</i>	<i>p</i> -value	<i>r</i>	<i>p</i> -value
GFAP-FL										
FCX	0.293	0.116	0.498	0.005**	0.749	<0.0001****	0.587	0.007**	0.392	0.036*
STM	0.482	0.007**	0.521	0.003**	0.559	0.002**	0.677	0.002**	0.361	0.054
HC	0.243	0.196	0.731	<0.0001****	0.647	0.000***	−0.001	0.995	0.109	0.573
RMB	0.104	0.586	0.695	<0.0001****	0.665	<0.0001****	0.601	0.005**	0.250	0.190
GFAP-BDPs										
FCX	0.655	<0.0001****	0.528	0.003**	0.800	<0.0001****	0.431	0.074	0.272	0.162
STM	0.630	0.000***	0.698	<0.0001****	0.828	<0.0001****	0.467	0.044*	0.357	0.058
HC	0.264	0.202	0.476	0.010*	0.404	0.027*	0.105	0.659	0.236	0.217
RMB	0.427	0.021*	0.701	<0.0001****	0.594	0.001***	0.553	0.012*	0.424	0.024*

Pearson *r* correlation analyses were performed between neuroscore and levels of GFAP-FL and GFAP-BDPs in brain tissues by region and time point post injury. Neuroscore assessment was performed at 24 h after injury. Protein levels were determined by Western blot and were expressed as percent sham. Sham, Probe, and PBBI injured rats were all included for analysis. Significant correlations are marked with *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), or ****($p \leq 0.0001$). FCX, frontal cortex; STM, striatum; HC, hippocampus; RMB, residual midbrain.

TABLE 3 | Correlation results between α II-spectrin and SBDP protein tissue levels and neuroscore values specified by brain region and time point.

	24 h		3 days		7 days		1 month		3 months	
	<i>r</i>	<i>p</i> -value	<i>r</i>	<i>p</i> -value	<i>r</i>	<i>p</i> -value	<i>r</i>	<i>p</i> -value	<i>r</i>	<i>p</i> -value
αII-Spectrin										
FCX	−0.470	0.009**	−0.568	0.001**	−0.274	0.143	−0.290	0.214	−0.133	0.499
STM	−0.353	0.055	−0.327	0.078	−0.123	0.525	−0.520	0.019*	−0.095	0.623
HC	−0.041	0.831	−0.169	0.374	0.159	0.401	−0.058	0.807	−0.465	0.013*
RMB	0.160	0.408	0.228	0.226	−0.632	0.000***	−0.412	0.071	0.431	0.020*
SBDP-145/150										
FCX	0.681	<0.0001****	0.565	0.001**	0.692	<0.0001****	0.136	0.567	−0.243	0.213
STM	0.628	0.000***	0.590	0.001***	0.731	<0.0001****	0.293	0.211	−0.246	0.198
HC	0.039	0.837	0.434	0.019*	0.123	0.518	0.266	0.257	0.499	0.007**
RMB	0.517	0.004**	0.742	<0.0001****	0.715	<0.0001****	0.554	0.011*	0.196	0.307
SBDP-120										
FCX	0.410	0.025*	0.456	0.011*	0.249	0.184	0.146	0.540	0.115	0.560
STM	−0.134	0.497	0.037	0.844	−0.086	0.658	0.133	0.575	−0.351	0.073
HC	0.208	0.278	0.054	0.776	−0.125	0.511	−0.090	0.713	0.347	0.065
RMB	0.031	0.872	−0.278	0.138	0.664	<0.0001****	0.267	0.255	−0.273	0.169

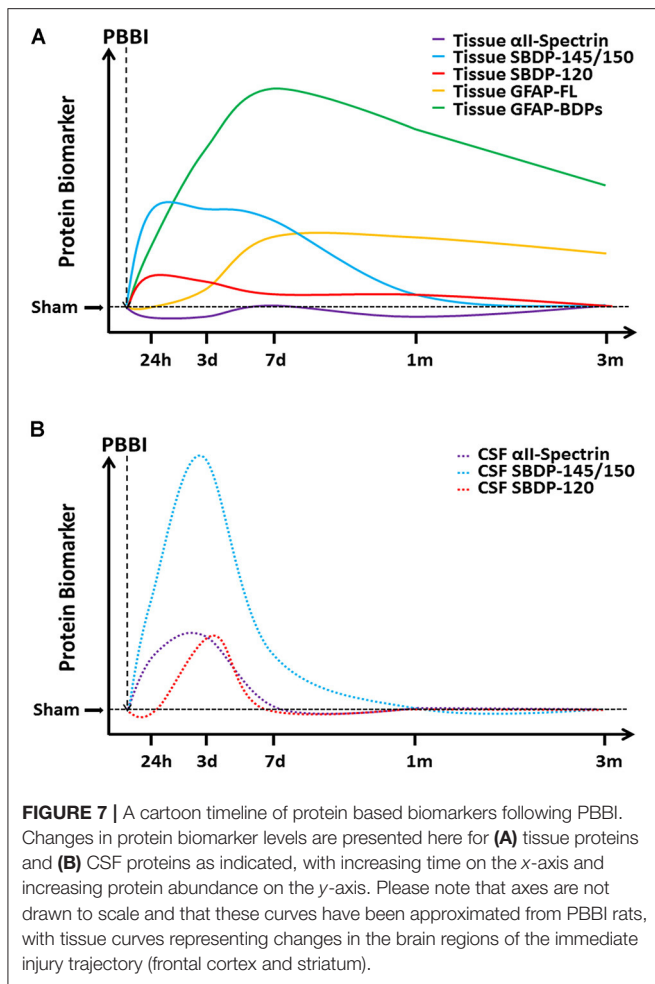
Pearson *r* correlation analyses were performed between neuroscore and levels of α II-spectrin and SBDPs in brain tissues by region and time point post injury. Neuroscore assessment was performed at 24 h after injury. Protein levels were determined by Western blot and were expressed as percent sham. Sham, Probe, and PBBI injured rats were all included for analysis. Significant correlations are marked with *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), or ****($p \leq 0.0001$). FCX, frontal cortex; STM, striatum; HC, hippocampus; RMB, residual midbrain.

compensatory response to injury that has yet to be clearly defined based on TBI mechanism and/or severity.

Variation Across Multiple Brain Regions

This study indicates that chronic, progressive degradation occurs not only in brain regions containing the primary lesion as previously reported (30, 32, 45) but also within areas that are adjacent to the primary wound, albeit with differing magnitudes. In this study, the effects of PBBI were not only more severe in the immediate regions of the primary injury trajectory (i.e., frontal cortex) as opposed to more distal areas (i.e., hippocampus), but

also appeared more quickly and persisted for a longer duration. Interestingly, the temporal profiles of GFAP-FL and GFAP-BDPs revealed that peak levels varied with time and between regions containing the primary injury lesion and distal injury areas. These divergent temporal profiles likely reflect a more rapid resolution of secondary injury effects in the less severely injured areas, whereas astrogliosis and proteolysis continue to increase progressively past 3 days before improving somewhat by 3 months in the more severely injured brain regions. Distinct profiles of protein abundance by brain region have been reported with SBDP-145/150 following CCI (39) and the data presented



here are consistent with previous work following PBBI showing delayed injury induced responses in the thalamus compared to the primary lesion (38).

This work corroborates and expands upon previous work from our laboratory, which defined the presence of GFAP, α II-spectrin, and their BDPs in a coronal brain section containing the primary injury lesion through 7 days after PBBI (27) with a few noteworthy exceptions. First, our previous data indicated that GFAP-FL abundance declines at 3 days after injury, which contrasts with the elevations observed in this study. Second, levels of α II-spectrin were nearly ablated by 3 days following PBBI previously, whereas here we see reductions of a smaller magnitude. Finally, significantly increased SBDP-145/150 were not seen previously at 24 h after PBBI as opposed to the robust increases in most brain regions assessed at this time-point in the current study. Whereas the first study isolated an ipsilateral coronal brain section combining both cortical and subcortical structures in each analysis, this study dissected specific ipsilateral brain regions with differing anatomical locations in relation to the primary injury trajectory. These data indicate that differential protein abundance of these tissue markers varies greatly between these distinct brain regions; thus, a greater resolution within each

region of interest is likely to account for the differences observed here compared to our previous work. In addition, evaluating specific regions of interest provides useful information regarding regional injury progression as opposed to examination of a single brain section containing the primary injury lesion (19, 46).

The Role of the Ballistic-Like Injury Component on Magnitude and Distribution of Neurodegeneration

The differential effects of penetrating injury alone compared to a penetrating ballistic-like injury were examined here by inclusion of both the probe injury and PBBI groups, respectively. While both injuries resulted in acute neurological deficits compared to sham rats, these deficits were of a greater magnitude following PBBI compared to probe injury. Greater elevation in the tissue abundance of GFAP, α II-spectrin, and their BDPs in PBBI compared to probe injured rats was also observed longitudinally across multiple brain regions. This is consistent with previous work that has indicated elevated SBDP-145/150 following more severe CCI (47). These data provide both functional and molecular outcome measures to better distinguish how these different injuries may alter the progression of molecular pathology. For example, while significant changes in GFAP, α II-spectrin, and their BDPs following probe injury were observed, these instances were limited to the frontal cortex and striatum only with no evidence for the spread of this pathology to distal brain regions as seen following PBBI. This finding indicates that while probe insertion alone causes localized injury effects, the temporary cavity mimicking energy dissipation from a high-velocity bullet round plays a critical role in the severity of injury and the propagation of PBBI pathology to areas beyond the primary injury trajectory.

Biofluid Based Biomarkers as Direct Correlates of Tissue Protein Abundance

The identification and validation of biofluid based biomarkers for use in informing individualized treatment plans and/or monitoring the efficacy of treatment over time holds great potential for improving TBI patient outcomes. Both GFAP-BDPs (48) and SBDPs (49) have been reported in CSF samples taken from acute and subacute human TBI patients. These studies indicate that acute levels of CSF SBDP-145/150 are elevated in severe TBI patients who fare worse across multiple outcome metrics, including acute Glasgow Coma Scale and chronic Glasgow Outcome Scale scores. The results presented in this study suggest that SBDP-145/150, rather than GFAP, GFAP-BDPs, or SBDP-120, may have utility as a monitoring biomarker through subacute-chronic stages following injury. Additionally, multiple correlations between CSF GFAP-BDPs and SBDP-145/150 and tissue levels of these same proteins were significant. These data suggest that CSF levels of these BDPs may reflect their presence in brain tissues following injury. While some studies have assessed serum GFAP-BDPs specifically (50), others have evaluated a combination of both GFAP and GFAP-BDPs as biomarkers (51, 52). Our data demonstrated that CSF BDPs of GFAP and α II-spectrin, rather than their full length counterparts,

were overwhelmingly correlated to these same protein forms in tissues, indicating that future studies evaluating the efficacy of CSF TBI biomarkers may benefit from measuring BDPs specifically. Whether these CSF biomarkers precede, co-occur, or follow injury-induced changes in these tissue proteins is unclear from our data. Interestingly, even in the absence of significant group effects, numerous significant correlations of acute and subacute levels of GFAP-BDPs and SBDP-145/150 between CSF and tissues were obtained, emphasizing the importance of these CSF biomarkers to predict molecular changes in individual subjects.

Neurological Deficits as Predictors of Protein Degradation

Acute neurological deficits correlated significantly with acute-chronic brain tissue levels of GFAP, α II-spectrin, and BDPs, however, these associations presented in a different pattern than the CSF correlations discussed above. While significant CSF correlations resulted primarily from GFAP-BDPs and SBDP-145/150, significant relationships between neuroscore values and brain tissue protein levels were more generalized and not as limited to BDPs specifically. This was especially true for GFAP. One explanation for this difference is that more severe injuries that cause greater neurological deficits are more likely to result in generalized injury induced alterations in protein abundance. Thus, in clinical practice, the assessment of neurological deficits with rating scales such as the NOS-TBI may have more benefit for classification of injury severity (53) and prediction of extended outcomes (54) rather than predictive ability for specific chronic neurodegeneration.

CONCLUSION

This study is the first to characterize acute through chronic profiles of the well-known TBI biomarkers GFAP, α II-spectrin, and their BDPs in distinct brain regions of interest, identify the presence of these proteins and their BDPs in CSF, and demonstrate significant correlations between the levels of BDPs present in CSF and brain tissues following PBBI in rats. These

results indicate that a sustained period of reactive astrogliosis but finite period of axonal cytoskeletal degradation follow penetrating brain injury and that the detection of calpain generated BDPs in CSF may predict the underlying presence of these tissue proteins in real time. Additionally, the evidence for chronic proteolysis and astrogliosis presented in this study informs the design of drug treatment paradigms following penetrating TBI, indicating that prolonged treatments into subacute-chronic periods should be considered.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

KD, CC, AB, and DS designed the experiments and prepared the manuscript. KD, CC, and HH performed the experiments and conducted data analysis.

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SUPPLEMENTARY MATERIAL

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Neuroprotective Effect of Artesunate in Experimental Model of Traumatic Brain Injury

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Traumatic brain injuries (TBI) are an important public health challenge. In addition, subsequent events at TBI can compromise the quality of life of these patients. In fact, TBI is associated with several complications for both long and short term, some evidence shows how TBI is associated with a decline in cognitive functions such as the risk of developing dementia, cerebral atrophy, and Parkinson disease. After the direct damage from TBI, a key role in TBI injury is played by the inflammatory response and oxidative stress, that contributes to tissue damage and to neurodegenerative processes, typical of secondary injury, after TBI. Given the complex series of events that are involved after TBI injury, a multitarget pharmacological approach is needed. Artesunate is a more stable derivative of its precursor artemisin, a sesquiterpene lactone obtained from a Chinese plant *Artemisia annua*, a plant used for centuries in traditional Chinese medicine. artesunate has been shown to be a pluripotent agent with different pharmacological actions. therefore, in this experimental model of TBI we evaluated whether the treatment with artesunate at the dose of 30 mg/Kg, had an efficacy in reducing the neuroinflammatory process after TBI injury, and in inhibiting the NLRP3 inflammasome pathway, which plays a key role in the inflammatory process. We also assessed whether treatment with artesunate was able to exert a neuroprotective action by modulating the release of neurotrophic factors. our results show that artesunate was able to reduce the TBI-induced lesion, it also showed an anti-inflammatory action through the inhibition of Nf-kb, release of proinflammatory cytokines IL-1 β and TNF- α and through the inhibition NLRP3 inflammasome complex, furthermore was able to reduce the activation of astrocytes and microglia (GFAP, Iba-1). Finally, our results show that the protective effects of artesunate also occur through the modulation of neurotrophic factors (BDNF, GDNF, NT-3) that play a key role in neuronal survival.

Keywords: traumatic brain injury, neuroinflammation, neurodegeneration, artesunate, artemisin

INTRODUCTION

Artemisinin is a sesquiterpene lactone obtained from a Chinese plant *Artemisia annua* a plant used for centuries in traditional Chinese medicine (1) and known for its antimalarial properties (2). Artesunate is a more stable derivative of its precursor artemisin, and is considered more the most effective drug for treating severe and chloroquine-resistant malaria, seen also its excellent safety profile (3, 4). Artesunate also has anti-tumor properties and thanks to its good tolerability profile is used in combination with standard chemotherapeutic (5, 6). Therefore, artesunate has been shown to be a pluripotent agent with different pharmacological actions, in fact in addition to these properties, has also shown to have an anti-inflammatory activity, in different inflammatory model like allergic asthma (7) and sepsis (8). Artesunate is the most effective drug for the treatment of cerebral malaria, in addition recent studies show that the treatment with artesunate also reduces inflammation of the brain, associated with this disease (9, 10). Artesunate is able to reach and maintain a high concentration in the brain, and this makes it a good candidate for the treatment of diseases of the central nervous system and neurological disorders (11), as an anti-neuroinflammatory agent (12). Traumatic brain injuries (TBI) is a major public health challenge and may affect people in a wide range of ages. In addition, events following TBI can compromise the quality of life of these patients (13). In fact, TBI is associated with several complications both long and short term (14). In fact, some evidence shows how TBI is associated with a decline in cognitive functions (15, 16), such as the risk of developing dementia and cerebral atrophy (17) and can increase the risk to developed Parkinson's disease (PD) (18). The damage from TBI is partly due to a direct consequence of the primary injury, and later with an indirect mechanism (secondary injury). Indeed, different mechanisms contribute to secondary damage. A key role is played by the acute inflammatory response, with the release of many inflammation mediators and the activation of different pro-inflammatory pathways, such as NLRP3 inflammasome (19). Another significant contribution to secondary damage is given by oxidative stress (20). For this series of complex events that are involved in TBI injury, a multi-target pharmacological treatment is necessary. Therefore, as artesunate is compound with more than one protective effect, the aim of this paper was to study if artesunate should be a powerful candidate for the treatment of brain trauma.

MATERIALS AND METHODS

Animals

Male CD1 mice (25–30 g, Envigo, Italy), aged between 10 and 12 weeks, were used for all studies. Mice were placed in a controlled location with standard rodent chow and water. Animals were kept at $22 \pm 1^\circ\text{C}$ with a 12-h light, 12-h dark cycle. The study was permitted by the University of Messina Review Board for the care of animals. All animal experiments were performed following the regulations in Italy (D.M. 116192), Europe (O.J. of E.C. L 358/1 12/18/1986).

Controlled Cortical Impact (CCI) Experimental Traumatic Brain Injury TBI

Mice were anesthetized under intraperitoneal (i.p.) ketamine and xylazine (2.6 and 0.16 mg/kg body weight, respectively). TBI was induced in mice by a controlled cortical impactor (CCI) as previously described (21). In brief, a craniotomy was made in the right hemisphere, encompassing bregma and lambda, and between the sagittal suture and the coronal ridge, with a Micro motor hand piece and drill. The resulting bone flap was removed, and the craniotomy enlarged further. A cortical contusion was produced on the exposed cortex using the controlled impactor device Impact One™ Stereotaxic impactor for CCI (Leica, Milan, Italy), the impact tip was centered and lowered over the craniotomy site until it touched the dura mater. Then, the rod was retracted, and the impact tip was advanced farther to produce a brain injury of moderate severity for mice (tip diameter: 4 mm; cortical contusion depth: 3 mm; impact velocity: 1.5 m/s). Immediately after injury, the skin incision was closed with nylon sutures, and 2% lidocaine jelly was applied to the lesion site to minimize any possible discomfort.

Experimental Groups

Mice were randomly distributed into the following groups: ($n = 10$ for each group, calculated using the statistical test a priori power analyzes of the G-power software)

TBI + vehicle: mice ($n = 10$) were subjected to CCI and vehicle (saline) was administered at 1 after craniotomy.

TBI + Artesunate: mice ($n = 10$) were subjected to CCI and Artesunate (30 mg/kg) was administered at 1 after craniotomy. Sham + vehicle: mice ($n = 10$) were subjected to the surgical procedures as above group (anesthesia and craniotomy) except that the impact tip was not applied, and vehicle was administered at 1 after craniotomy.

Sham + artesunate: mice ($n = 10$) were subjected to the surgical procedures as above group (anesthesia and craniotomy) except that the impact tip was not applied and Artesunate (30 mg/kg) was administered at 1 after craniotomy.

As describe below mice ($n = 10$ from each group for each parameters) were sacrificed at 24 h after TBI to evaluate the various parameter.

Histology

Coronal sections of 5- μm thickness were sectioned from the perilesional brain area of each animal and were evaluated by an experienced histopathologist. Damaged neurons were counted and the histopathologic changes of the gray matter were scored on a six-point scale (22): 0, no lesion observed; 1, gray matter contained one to five eosinophilic neurons; 2, gray matter contained five to 10 eosinophilic neurons; 3, gray matter contained more than 10 eosinophilic neurons; 4, small infarction (less than one third of the gray matter area); 5, moderate infarction (one third to one half of the gray matter area); 6, large infarction (more than half of the gray matter area). The scores from all the sections of each brain were averaged to give a final score for individual mice. All the histological studies were performed in a blinded fashion.

Assessment of Lesion Volume

At 24 h after injuries, mice were euthanized, and brains were frozen. The brains were sectioned in coronal sections (14 μ m). Sections were stained with hematoxylin. Area of the undamaged and injured hemisphere was measured on each section using image analysis software. The hemispheric volume was obtained by summing area of each section and multiplying it by 0.5. Lesion volume (mm³) was expressed as difference between the uninjured and injured hemisphere volume.

Western Blot Analysis

Western blot analysis was performed on tissues harvested 24 h post-TBI. Cytosolic and nuclear extracts were prepared as described previously (23). The filters were probed with specific Abs: anti- NF- κ B p-65 (1:1,000; Santa Cruz Biotechnology) or I κ B- α (1:1,000; Santa Cruz Biotechnology), or anti-NLRP3 (1:500; Santa Cruz Biotechnology), or anti BAX (1:500; Santa Cruz Biotechnology) anti-Bcl-2 (1:500; Santa Cruz Biotechnology) anti-ASC (1:500; Santa Cruz Biotechnology), or anti Caspase-1 (1:500; Santa Cruz Biotechnology) in 1 $\times$ PBS, 5% w/v nonfat dried milk, 0.1% Tween-20 at 4°C, overnight. To ascertain that blots were loaded with equal amounts of proteins they were also incubated in the presence of the antibody against β -actin protein (cytosolic fraction 1:500; Santa Cruz Biotechnology) or lamin A/C (nuclear fraction 1:500 Sigma–Aldrich Corp.). Signals were detected with enhanced chemiluminescence (ECL) detection system reagent according to the manufacturer's instructions (Thermo, USA). The relative expression of the protein bands was quantified by densitometry with BIORAD ChemiDoc™ XRS+ software and standardized to β -actin and lamin A/C levels. Images of blot signals (8 bit/600 dpi resolution) were imported to analysis software (Image Quant TL, v2003). The blot was stripped with glycine 2% and reprobed several times to optimize detection of proteins and to visualize other proteins without the need for multiple gels and transfers.

Immunohistochemistry

Tissue segments containing the lesion (1 cm on each side of the lesion) were fixed in 10% (w/v) buffered formaldehyde 24 h after TBI and sliced in 7- μ m sections for paraffin-embedding previously described (24). After deparaffinization, endogenous peroxidase was quenched with 0.30% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin, respectively. Afterwards, the sections were incubated overnight with one of the following primary antibodies diluted in PBS: polyclonal anti-glial cell line-derived neurotrophic factor (GDNF) (1:500, Santa Cruz Biotechnology), polyclonal anti-brain-derived neurotrophic factor (BDNF) (1:500, Santa Cruz Biotechnology), anti- IL-1 β (1:500, Santa Cruz Biotechnology), anti-TNF- α (1:500, Santa Cruz Biotechnology), anti-iNOS (1:500, Santa Cruz Biotechnology), anti-VEGF (1:500, Santa Cruz Biotechnology). The immunohistochemical images were

collected by Zeiss microscope using Axio Vision software. For graphic representation of densitometric analyses, we measured the intensity of positive staining (brown staining) by computer-assisted color image analysis (Leica QWin V3, UK). The percentage area of immunoreactivity (determined by the number of positive pixels) was expressed as percent of total tissue area (red staining) as seen previously (18).

Immunofluorescence

After deparaffinization and rehydration, detection of GFAP, Iba1, neurotrophin-3 (NT-3), BDNF, GDNF, IL-1 β was carried out after boiling the tissue sections in 0.1 M citrate buffer for 1 min as described previously (18). Non-specific adsorption was minimized by incubating in 2% (vol/vol) normal goat serum in PBS for 20 min. Sections were incubated with one of the following primary antibodies: rabbit polyclonal anti-GFAP (1:100, Santa Cruz Biotechnology), rabbit polyclonal anti-Iba1 (1:100, Santa Cruz Biotechnology), rabbit polyclonal anti-NT3 (1:100, Millipore), polyclonal anti-glial cell line-derived neurotrophic factor (GDNF) (1:100, Santa Cruz Biotechnology), polyclonal anti-brain-derived neurotrophic factor (BDNF) (1:100, Santa Cruz Biotechnology), anti- IL-1 β (1:100, Santa Cruz Biotechnology) in a humidified oxygen and nitrogen chamber overnight at 37°C. Sections were then incubated with secondary antibody: fluorescein isothiocyanate-conjugated anti-mouse Alexa Fluor-488 (1:2,000, Molecular Probes, Monza, Italy) or Texas Red-conjugated anti-rabbit Alexa Fluor-594 (1:1,000, Molecular Probes) for 1 h at 37°C. For nuclear staining, 2 μ g/ml 4', 6'-diamidino-2-phenylindole (DAPI; Hoechst, Frankfurt, Germany) in PBS was added. Sections were observed using a Leica DM2000 microscope (Leica, Milan, Italy). GFAP+, Iba1+, and NT- 3+, BDNF+, GDNF+, IL-1 β + cells were counted stereologically on sections cut at a 40 μ m thickness and every 4th section was counted using a grid of 100 $\times$ 100 μ m. Optical sections of fluorescence specimens were obtained using a HeNe laser (543 nm), an ultraviolet laser (361–365 nm) and an argon laser (458 nm) at a one-mi, 2s scanning speed with up to eight averages; 1.5 μ m sections were obtained using a pinhole of 250. The same settings were used for all images obtained from the other samples that had been processed in parallel. Digital images were cropped, and figure montages prepared using Adobe Photoshop 7.0 (Adobe Systems; Palo Alto, California, United States). Both brightfield (NeuN or BDNF or GDNF or NT3 or GFAP or IBA1 or IL1 β) and fluorescent photographs (DAPI) were taken of 3 representative fields per slide in a blinded fashion using a fluorescent microscope (Leica DM 2000, 100 $\times$ objective). The total number of nuclei per field were quantified by counting the DAPI-positive nuclei using ImageJ software (25–29).

Materials

Artesunate was obtained by Santa Cruz Biotechnology. All compounds used in this study, except where differently specified, were purchased from Sigma-Aldrich Company Ltd. All solutions used for *in vivo* administrations were made using no pyrogenic saline (0.9% wt/vol NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, UK).

Statistical Analysis

All values in the figures and text are expressed as mean $\pm$ standard error of the mean (SEM) of N number of animals. In those experiments involving histology or immunohistochemistry, the pictures exhibited are representative

of at least three experiments performed on different days. Results were analyzed by one-way ANOVA followed by a Bonferroni *post-hoc* test for multiple comparisons. Histological score was analyzed by Kruskal-Wallis test followed by a Dunn's multiple comparisons test. A *p*-value < 0.05 was considered significant.

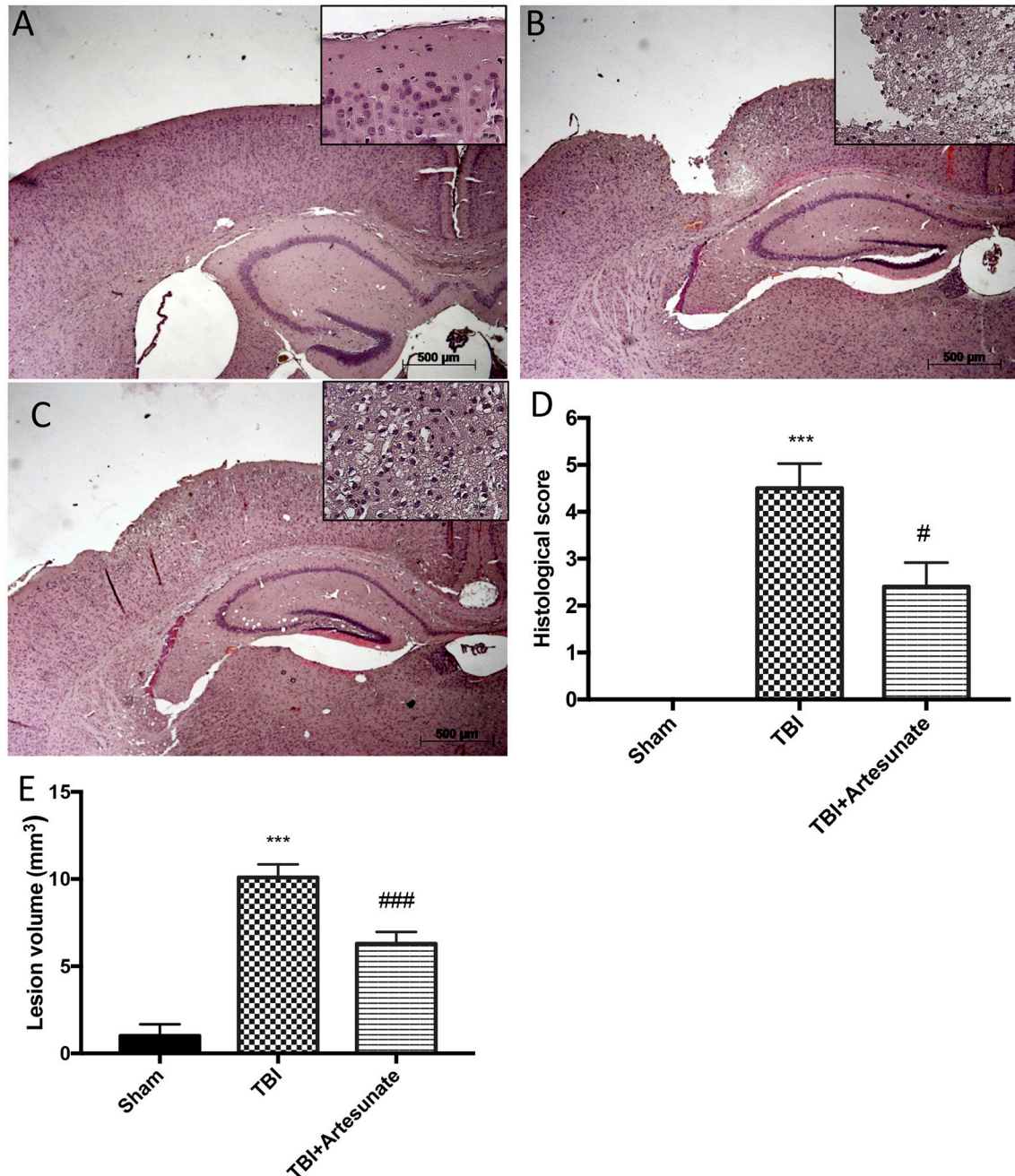


FIGURE 1 | Protective effect of artesunate treatment on histological analysis after TBI. (A) Show brain section from mice of sham group. Brain section at the level of the perilesional area showed that 24 h after TBI injury the TBI group showed an evident tissue disorganization and inflammation in the perilesional area (B). The group that has been treated with artesunate (C) at dose of 30 mg/Kg showed a significant reduction in degree of brain injury compared with the TBI group (D). Moreover treatment with artesunate significant reduce the lesion volume compared to TBI group as show in (E). Data are expressed as Mean $\pm$ SEM from *N* = 10 Mice for each group, ****P* < 0.001 vs. corresponding sham group. #*P* < 0.05 vs. corresponding TBI. ###*P* < 0.001 vs. corresponding TBI. (*F*-value for lesion volume = 433.9. *df* = 2. *r*² = 0.9698).

For one-way ANOVA statistic test, a single “F” value indicated as variation between sample means/variation within the samples was shown.

RESULTS

Protective Effect of Artesunate Treatment on Histological Analysis After TBI

Histological analysis of brain section at the level of the perilesional area showed that 24 h after TBI injury, in the TBI group there is a significant tissue damage, inflammation in, compared with brain from sham group (Figure 1A) the perilesional area and white matter alteration (Figure 1B). The treatments with artesunate (30 mg/Kg) significantly reduce the degree of brain injury when compared to the TBI group, (Figure 1C see histological score Figure 1D). Moreover, treatment with artesunate significantly reduce the lesion volume compared to the TBI group (Figure 1E).

Protective Effect of Artesunate Treatment on I κ B- α Degradation, Nf- κ B Translocation

To test the anti-inflammatory proprieties of artesunate, we evaluated by western blot analysis the I κ - α degradation and Nf- κ B translocation, that play a significant role in inflammation. Figure 2A showed that after TBI injury in the vehicle group there is a significant degradation of I κ B- α compared to the sham group, the treatment with artesunate can significantly prevent the I κ B- α degradation compared to the vehicle group (Figures 2A,A1). Consequently, western blot analysis in the vehicle group showed a significant increase of Nf- κ B translocation, compared to

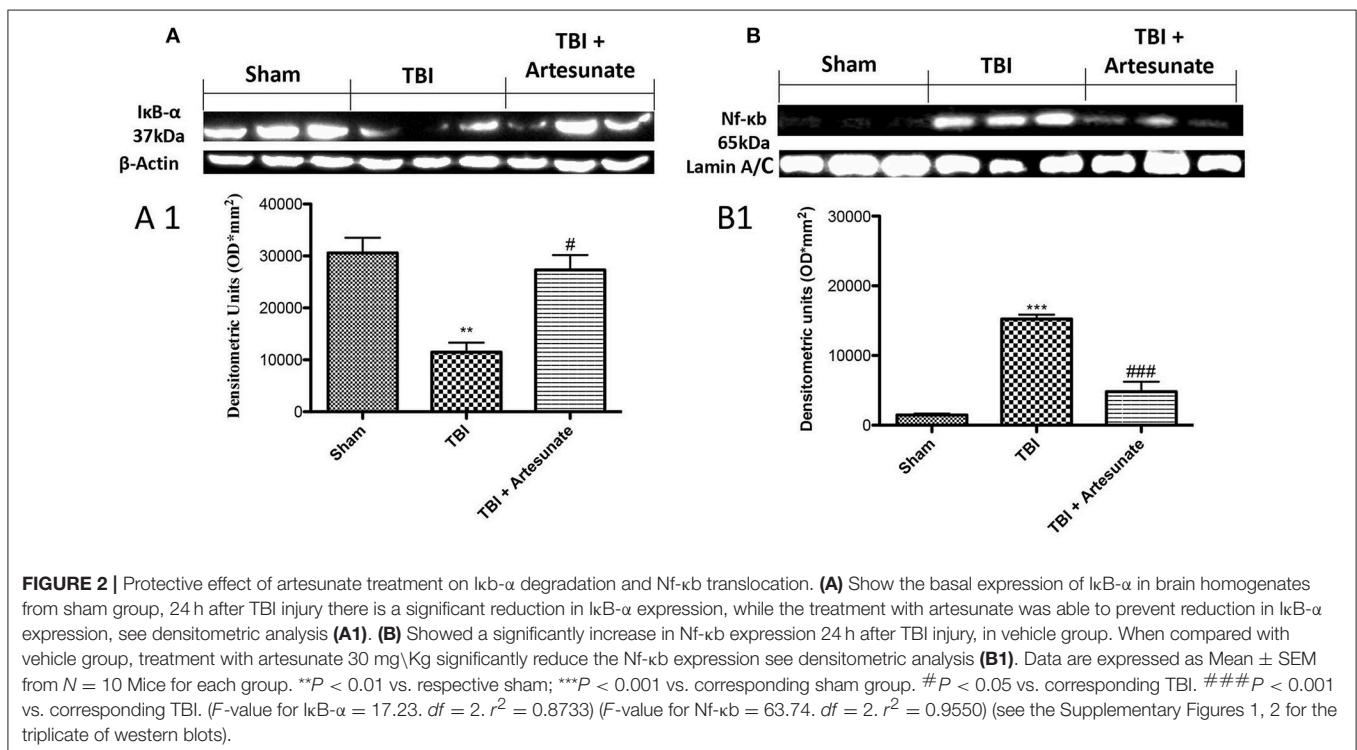
the sham group (Figures 2B,B1). On the contrary artesunate treatment significantly reduce the Nf- κ B translocation as show in Figures 2B,B1.

Protective Effect of Artesunate Treatment on Cytokines, and iNOS Expression

Next, we evaluated the anti-neuroinflammatory effect of artesunate treatment post TBI, in cytokines expression. The immunohistochemistry analysis for IL-1 β and TNF- α showed that, 24 h after TBI there is a significantly increase in IL-1 β and TNF- α expression in brain tissue from vehicle group (Figures 3B,F respectively), when compared with brain from sham group (Figures 3A,E respectively), instead the treatment with artesunate significantly reduce the expression in IL-1 β and TNF- α (Figures 3C,G respectively) when compared to vehicle as show in Figures 3D,H respectively. Moreover, we also assessed iNOS is expression in response to pro-inflammatory cytokines. Immunohistochemical analysis for iNOS expression shows that 24 h after TBI injury. In the vehicle group there is a significant increase in iNOS expression (Figure 3J see densitometric analysis Figure 3L) compared to sham group. (Figure 3I see densitometric analysis Figure 3L), showed that after treatment with artesunate 30 mg/Kg significantly reduce the iNOS expression when compared to the vehicle group, as show in Figure 3K (see densitometric analysis Figure 3L).

Effect of Artesunate on Inflammasome Components

NLRP3-inflammasome, which also includes adaptor proteins such as the apoptosis-associated speck-like protein containing



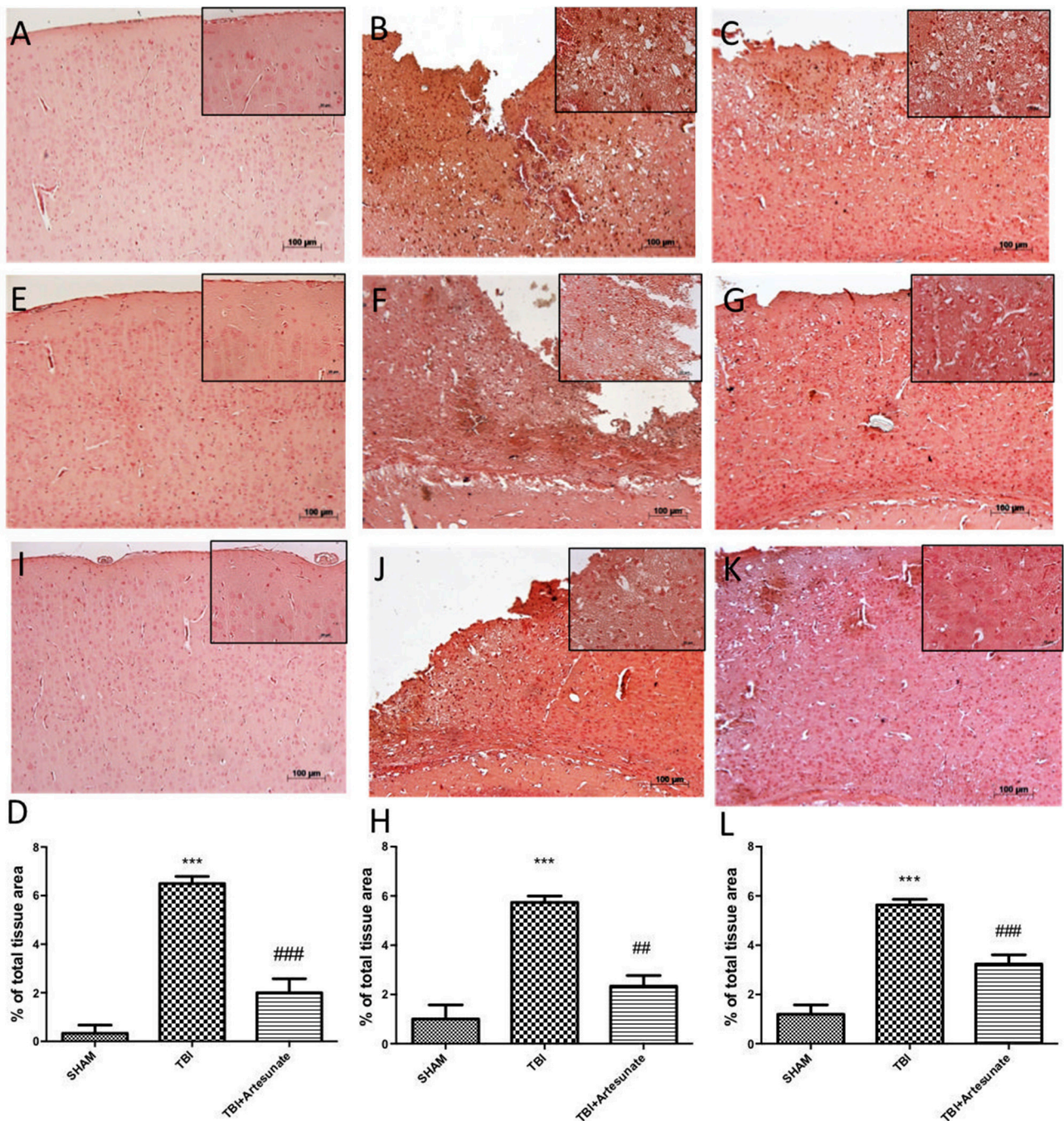


FIGURE 3 | Protective effect of artesunate treatment on cytokines and iNOS expression. (B,F) Showed a significant increase of expression for IL-1 β and TNF- α respectively, 24 h after TBI injury compared with sham group (A,E). When compared with vehicle group, brain section from mice treated with artesunate show a significant reduction in IL-1 β and TNF- α respectively (C,G), see densitometric analysis (D) for IL-1 β , and (H) for TNF- α . The immunohistochemical analysis for iNOS, show that 24 h after TBI injury in the vehicle group there is a significant increase in iNOS expression as show in (J) compared with sham group (I). While as show in (K) treatment with artesunate significantly reduce the iNOS expression compared to vehicle group, see densitometric analysis (L). Data are expressed as Mean $\pm$ SEM from $N = 10$ Mice for each group. *** $P < 0.001$ vs. corresponding sham group. ### $P < 0.01$ vs. respective TBI. ### $P < 0.001$ vs. corresponding TBI. (F-value for IL-1 β = 57.84, $df = 2$, $r^2 = 0.9507$) (F-value for TNF- α = 30.01, $df = 2$, $r^2 = 0.9091$) (F-value for iNOS = 45.29, $df = 2$, $r^2 = 0.883$).

a caspase-recruitment domain (ASC), and the serine protease caspase 1 (Casp1), play a key role in the inflammatory response. Western blot analysis of brain homogenates 24 h

after TBI injury the TBI group shows a significant increase in NLRP3, ASC, and Caspase-1 expression compared to sham group. When compared with vehicle group treatment

with artesunate at Dose of 30 mg/Kg significantly reduce the expressions of inflammasomes components expression NLRP3 (Figures 4A,A1), ASC (Figures 4B,B1) and Caspase-1 (Figures 4C,C1).

Protective Effect of Artesunate Treatment on Apoptosis Process

To test if the treatment with artesunate was able to prevent apoptosis process, brain homogenates, were processed for Western Blot analysis for pro-apoptotic factor Bax and anti-apoptotic factor Bcl-2 expression. Sham-operated mice showed a basal expression for Bcl-2 (Figures 5A,A1,B,B1). Instead the vehicle group showed a significantly reduction in Bcl-2 and significantly increases in Bax expression. Treatments with artesunate a dose of 30 mg/Kg were able to reduce the increase of

Bax expression and to prevent the reduction in Bcl2 expression as show in Figures 5A,A1,B,B1.

Protective Effect of Artesunate Treatment on Astrocytes and Microglia Activation and Cytokine Production

Astrocytes and microglia activation play a critical role in neuroinflammation. When compared to the sham group (Figure 6A, for GFAP and Figure 6E for Iba-1, see yellow arrows), immunofluorescence evaluation of Glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1 (Iba1) revealed a significant increasing in GFAP and Iba-1 positive cell as show in Figures 6B,F respectively. Treatment with artesunate at dose of 30 mg/Kg post TBI injury significantly reduce the number of positive cells for GFAP (Figure 6D) and

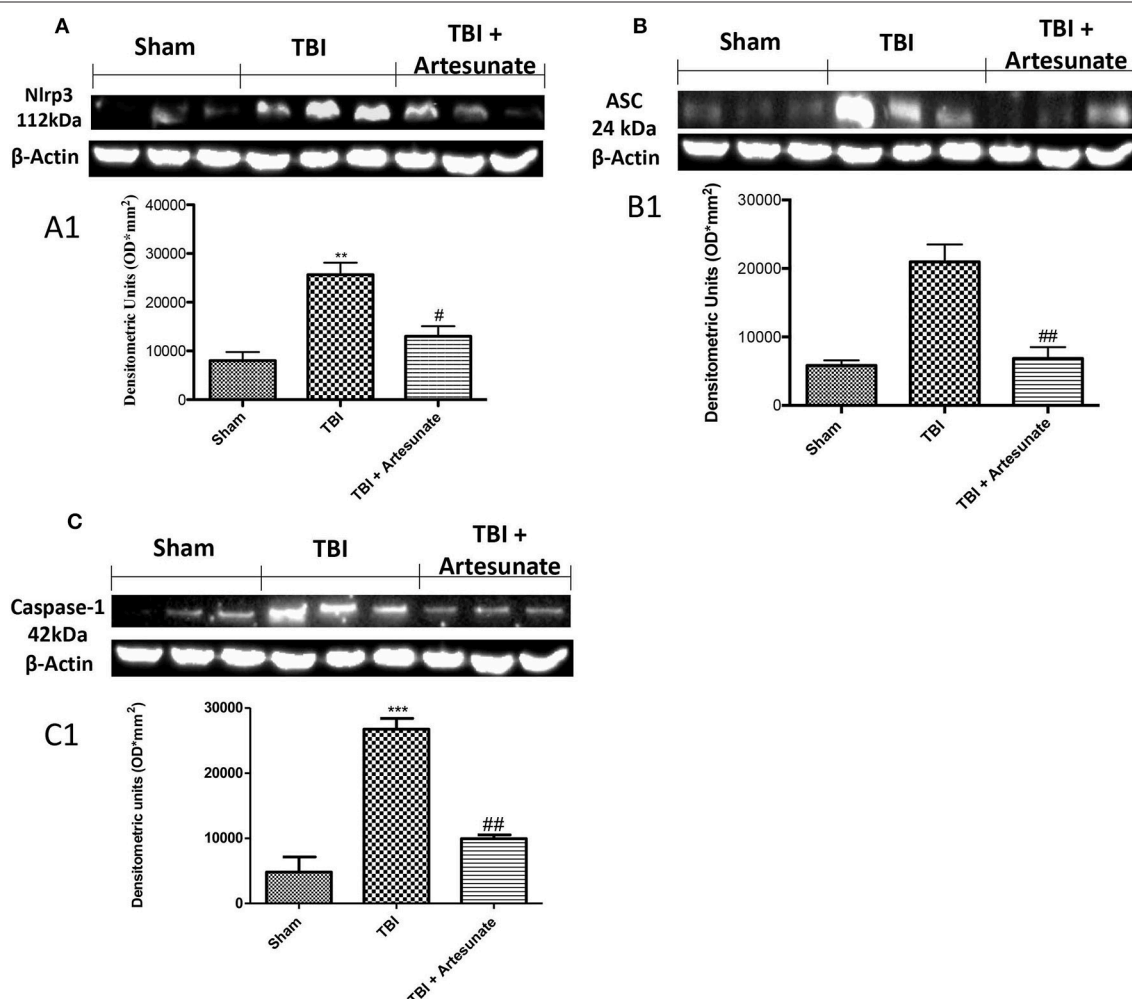


FIGURE 4 | Effect of artesunate on inflammasome components. Western blot analysis show that 24 h after TBI injury in the TBI group there is a significantly increase in expressions of NLRP3 (A), ASC (B), and caspase-1 (C). When compared with TBI group, treatment with artesunate significantly reduce the expression of inflammasome components as show in (A) for NLRP3 see densitometric analysis (A1,B) for ASC see densitometric analysis (B1,C) for Caspase-1 see densitometric analysis (C1). Data are expressed as Mean $\pm$ SEM from $N = 10$ Mice for each group, ** $p < 0.01$ vs. respective sham. *** $p < 0.001$ vs. corresponding sham group; # $p < 0.05$ vs. corresponding TBI. ## $p < 0.01$ vs. corresponding TBI. (F -value for NLRP3 = 18.30. $df = 2$. $r^2 = 0.8591$) (F -value for ASC = 23.14. $df = 2$. $r^2 = 0.8852$) (F -value for Caspase-1 = 47.25. $df = 2$. $r^2 = 0.9403$) (see the Supplementary Figures 3–5 for the triplicate of western blots).

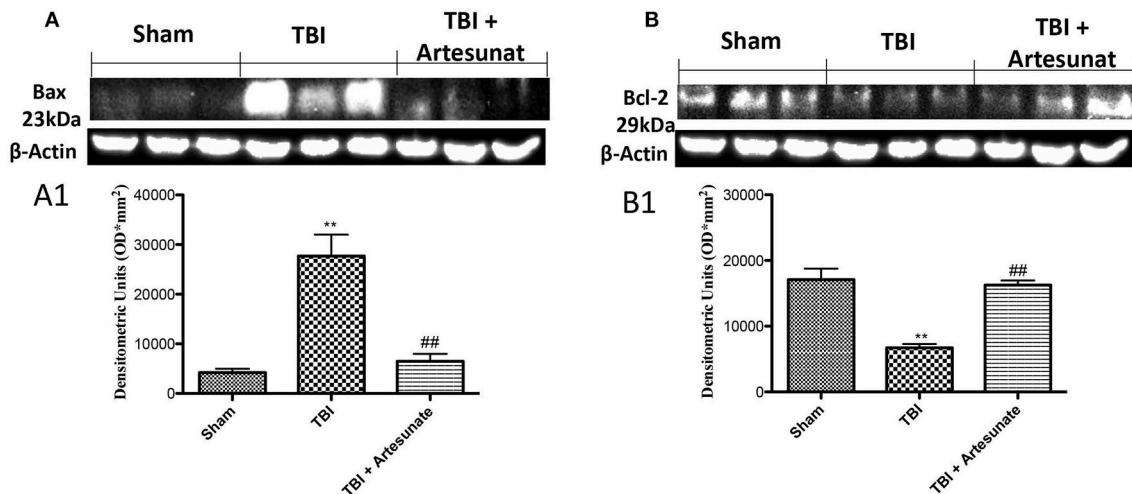


FIGURE 5 | Protective effect of artesunate treatment on apoptosis process. **(A)** Western blot analysis for Bax showed that 24 h after TBI injury, brain from vehicle group showed a significant increase in Bax expression compared to sham group, instead brain from mice that have been treated with artesunate showed a significant reduction in Bax expression compared to vehicle group, see densitometric analysis **(A1)**. **(B)** Showed a basal expression of Bcl-2 in brain homogenates from sham mice, after TBI injury the vehicle group showed a significant reduction in Bcl-2 expressions. Treatment with artesunate was able to prevent the reduction in Bcl-2 expression and reports the levels similar to the sham group, see densitometric analysis **(B1)**. Data are expressed as Mean $\pm$ SEM from $N = 10$ Mice for each group ** $P < 0.01$ vs. respective sham; ## $P < 0.01$ vs. respective TBI. (F -value for Bax = 22.93. $df = 2$. $r^2 = 0.8843$) (F -value for Bcl-2 = 27.64. $df = 2$. $r^2 = 0.9021$) (see the Supplementary Figures 6, 7 for the triplicate of western blots).

Iba-1 (**Figures 6C,G,H**) respectively, compared to the vehicle group.

Moreover, we investigated by double co-localization the expression of IL-1 β with the specific marker for microglia. We observed that IBA-1+/IL-1 β + co-localization was very low in the sham group (**Figure 7A**; densitometric analysis, **Figure 7D**), but significantly elevated following TBI injury (**Figure 7B**; densitometric analysis, **Figure 7D**). Artesunate treatment (30 mg/kg) significantly reduced the TBI-induced expression of IBA-1+/IL-1 β + (**Figure 7C**; densitometric analysis, **Figure 7D**).

Protective Effect of Artesunate Treatment on Neurotrophic Factors

Neurotrophic factors play a key role in neuronal survival and repair processes following damage to the brain. First, by immunohistochemical analysis we evaluated the effects of artesunate on the expression of VEGF, which plays a key role in promoting the repair of damaged tissues. 24 h after the TBI, the vehicle group shows reduced levels of VEGF compared to sham as show in **Figures 8A,B** respectively, instead following the treatment with artesunate (**Figure 8C**) there is a significant increase in the expression of VEGF compared to the vehicle group as show in **Figure 8D**. **Figures 8E,I** showed the basal expression of Brain-derived neurotrophic factor (BDNF) and Glial cell-derived neurotrophic factor (GDNF) respectively, brain section from sham animal. 24 h after TBI injury we found a significantly reduction in BDNF and GDNF expression in brain from vehicle group mice as show in **Figures 8F,J** respectively. While treatment with artesunate at dose of 30 mg/Kg was able to significantly increase the expression of BDNF and GDNF (**Figures 8G,K** respectively) after TBI injury as show in

Figures 8H,L. Subsequently, by immunofluorescence analysis we evaluated the expression of neurotrophic factor Neurotrophin-3 (NT-3). The **Figure 8M** shows the basal expressions of NT-3 in the brain section from sham mice. After the TBI injury, there is a significant reduction in NT-3 expression in the brain section from vehicle group as show in **Figure 8N**. instead the treatment with artesunate significantly prevent the reduction in NT-3 expression induced by TBI, **Figures 8O,P**.

Moreover, we investigated by double co-localization the expression of BDNF and GDNF with Neu-N, a marker for neuronal nuclei. BDNF and GDNF expressions were decreased in animals after TBI injury (**Figures 9B,E** respectively, densitometric analysis, **Figures 9G,H**) compared to sham group (**Figures 9A,D** respectively, densitometric analysis **Figures 9G,H**), while treatment with artesunate significantly increased the release of BDNF and GDNF (**Figures 9C,F** respectively, densitometric analysis **Figures 9G,H**). The yellow arrow indicates the co-localization between BDNF and Neu-N and GDNF and Neu-N.

DISCUSSION

Traumatic brain injury is a leading cause of mortality and is a major public health issue. Currently, most of the drugs available for the treatment of TBI have as a target, a single aspect of the lesion. It is widely recognized that the etiopathology events in the TBI comprise a series of heterogeneous events, which include cellular and molecular mechanisms both in the acute and non-acute phases, thus making it difficult to achieve a single pharmacological treatment. In fact, effective treatment for the TBI should simultaneously attenuate several injury factors.

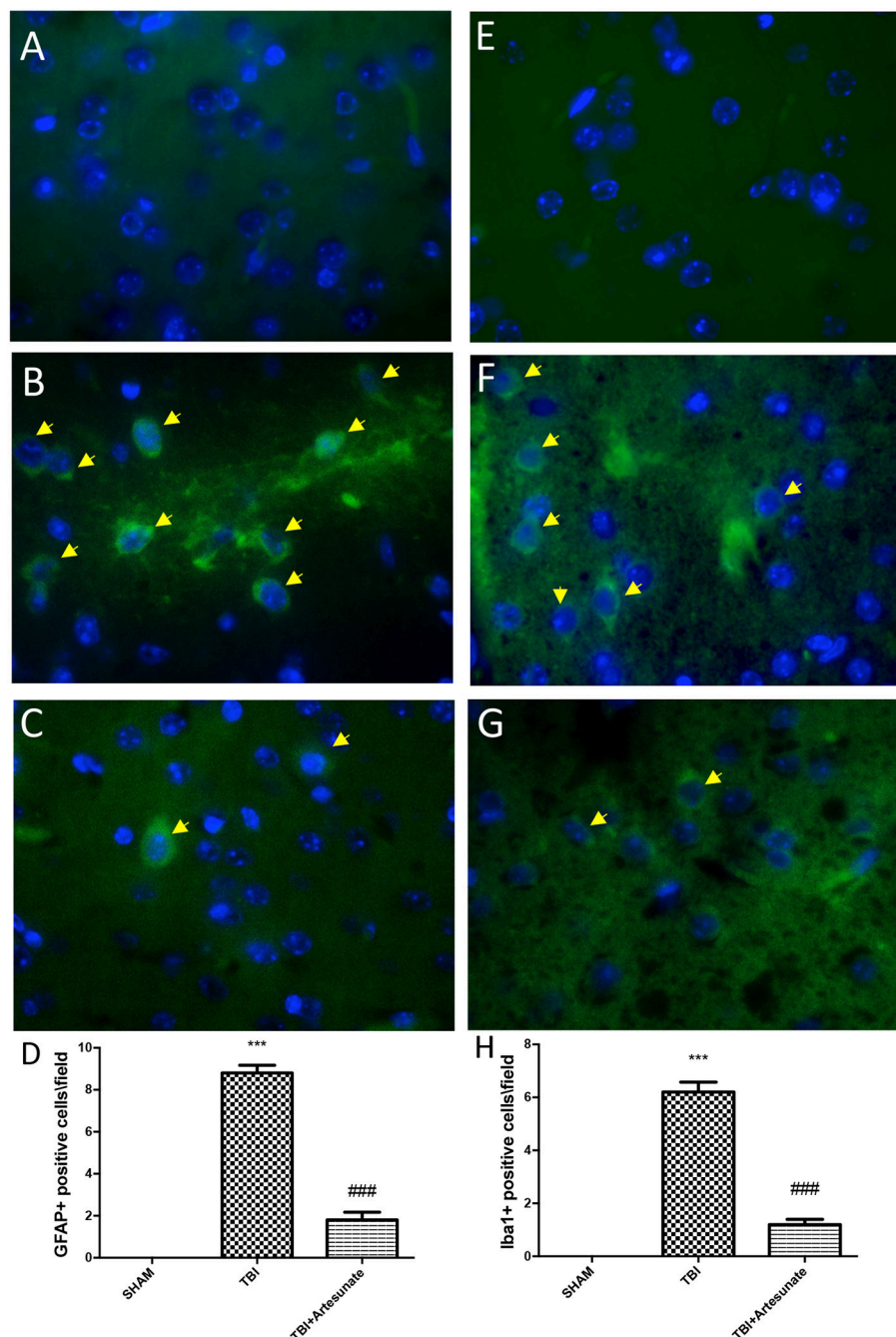


FIGURE 6 | Protective effect of artesunate treatment on astrocytes and microglia activation. (A,E) Showed no immunoreactivity for GFAP and Iba-1 respectively in sham brain tissue. Twenty-four hours after TBI injury in brain tissue there is a significant increase in positive cells for GFAP and Iba-1 as show in (B,F) respectively, see yellow arrows. When compared with TBI group treatment with artesunate significantly reduce the number of positive cell for GFAP and Iba-1 as show in figure (C,G) respectively, see yellow arrows. (D) GFAP densities measured, panels. (H) Iba-1 densities measured. Data are expressed as Mean $\pm$ SEM from $N = 10$ Mice for each group, *** $P < 0.001$ vs. corresponding sham group. ### $P < 0.001$ vs. corresponding TBI. (F -value for GFAP = 231.6. $df = 2$. $r^2 = 0.9747$) (F -value for IBA-1 = 180.2. $df = 2$. $r^2 = 0.9678$).

Thus, attenuate the inflammatory process at the base of the pathological process, and prevent the neurodegeneration and cognitive decline that the TBI involves, is a fundamental step in the treatment of the TBI (15). Agents with more than one

protective effect are attractive as potential therapeutic drugs for neurological disorders. Therefore, in this sense Artesunate appears to be a good candidate for the treatment of central nervous system diseases (11). Artesunate is a derivative obtained

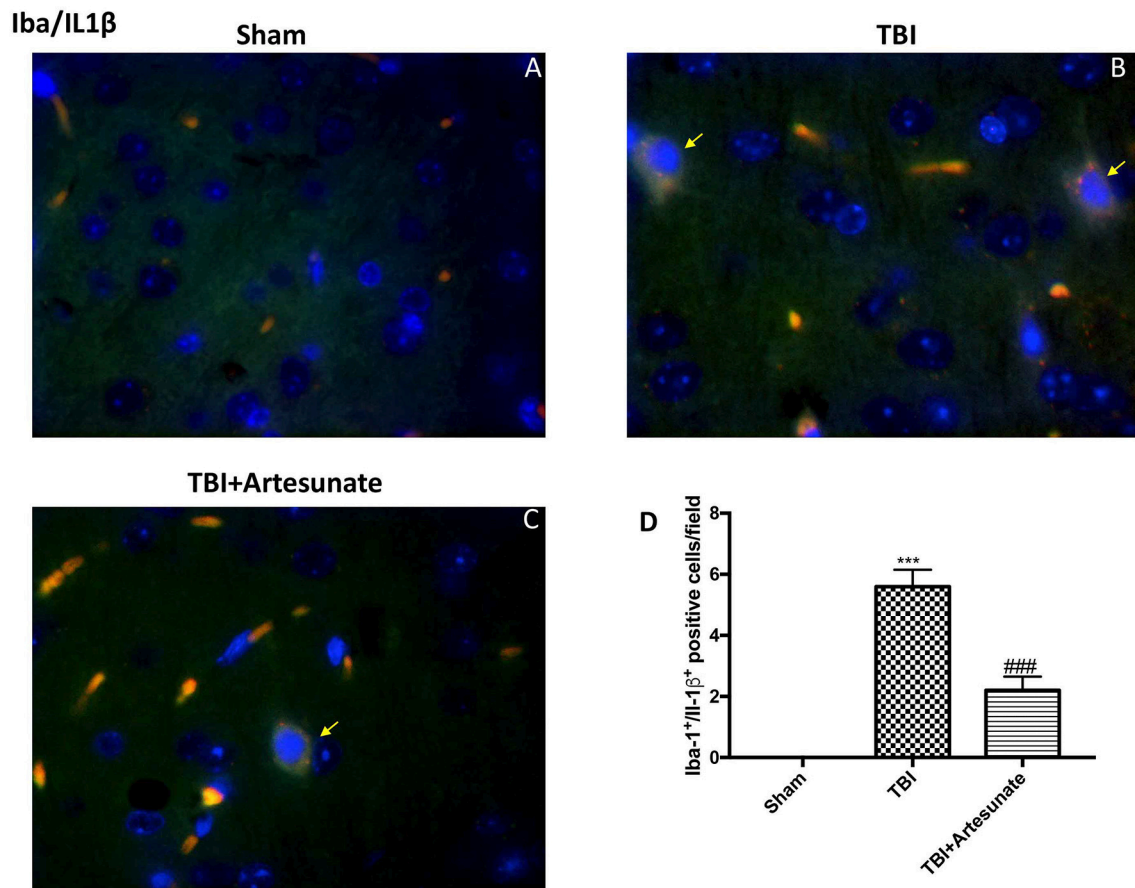


FIGURE 7 | Protective effect of artesunate treatment on microglial cell activation and cytokine production. **(A)** Showed no immunoreactivity for Iba-1+/IL-1β+ in sham group. **(B)** Showed a significant increase in IBA-1+/IL-1β+ co-localization. **(C)** Treatment with artesunate significantly reduced the TBI-induced expression of IBA-1+/IL-1β+. **(D)** Represent densitometric analysis. The yellow arrow indicates the co-localization between IBA-1+/IL-1β+. Data are expressed as Mean ± SEM from $N = 10$ Mice for each group, *** $P < 0.001$ vs. corresponding sham group. ### $P < 0.001$ vs. corresponding TBI. (F -value = 238.8. $df = 2$. $r^2 = 0.9755$).

from a Chinese plant *Artemisia annua*. Currently it is the drug used for the treatment of resistant malaria and for brain malaria, also saw its good safety profile without side effects. Until now, controlled cortical impact (CCI) represents the most frequently used mechanical model to induce TBI, given its accuracy, easy of control, and, most importantly, its ability to produce brain injuries similar to those seen in humans (30). In this study we evaluated the treatment with artesunate at a dose of 30 mg/kg in the modulation of the inflammatory process, as in the activation of $\text{NF-}\kappa\text{B}$, proinflammatory cytokines IL-1 β , TNF- α , and iNOS expression. We also evaluated the protective effects of artesunate in the activation of the NLRP3 inflammasome complex, which plays a key role in the inflammatory process typical of the second phase of the TBI. We also evaluated the protective effects of artesunate in promoting reparative processes (VEGF), and in preventing neurodegeneration through the modulation of neurotrophic factors such as BDNF, GDNF, and NT-3. Histological analysis shows how the artesunate treatment had a significant protective effect in this experimental model of TBI. In fact, we have seen how the group treated with artesunate showed a reduced lesion area, and a minor morphological

modification respect to the vehicle group, following the TBI. Then first, we evaluated the treatment with artesunate in inhibiting the inflammatory process subsequently at TBI. In fact, neuroinflammation is a secondary process that contributes to tissue damage and neurological disorders that the TBI involves. Some research has shown that the presence of neuroinflammation after brain trauma can play a key role in the loss of neurological function (31, 32). The brain trauma causes an alteration of the normal function of the blood brain barrier, thus allowing access to the lesion site, circulating macrophage, neutrophil, and lymphocytes, therefore the accumulation of these cells releases inflammatory mediators that activate the glial and inflammatory cells, thus supporting the inflammatory process (33, 34). The transcription factor nuclear factor kappa B (NF- κB) is a master regulator of inflammation. It also mediates a variety of other cellular processes including cell survival and apoptosis, the activity includes those engaged by neurotrophic factors, neurotransmitters, electrical activity, cytokines, and oxidative stress. Emerging findings support a pivotal role for NF- κB as a mediator of transcription-dependent enduring changes in the structure and function of neuronal circuits (35). NF- κB

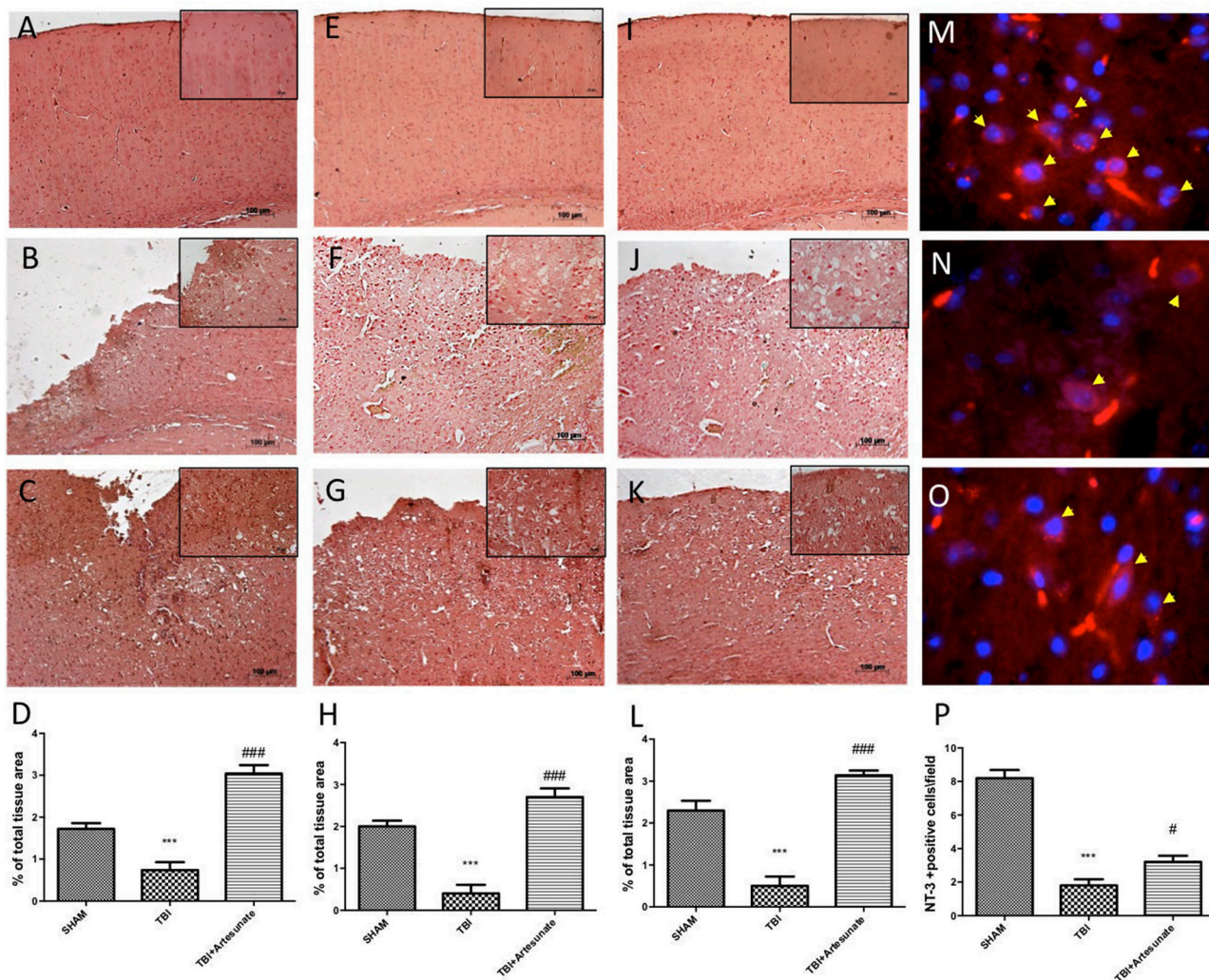
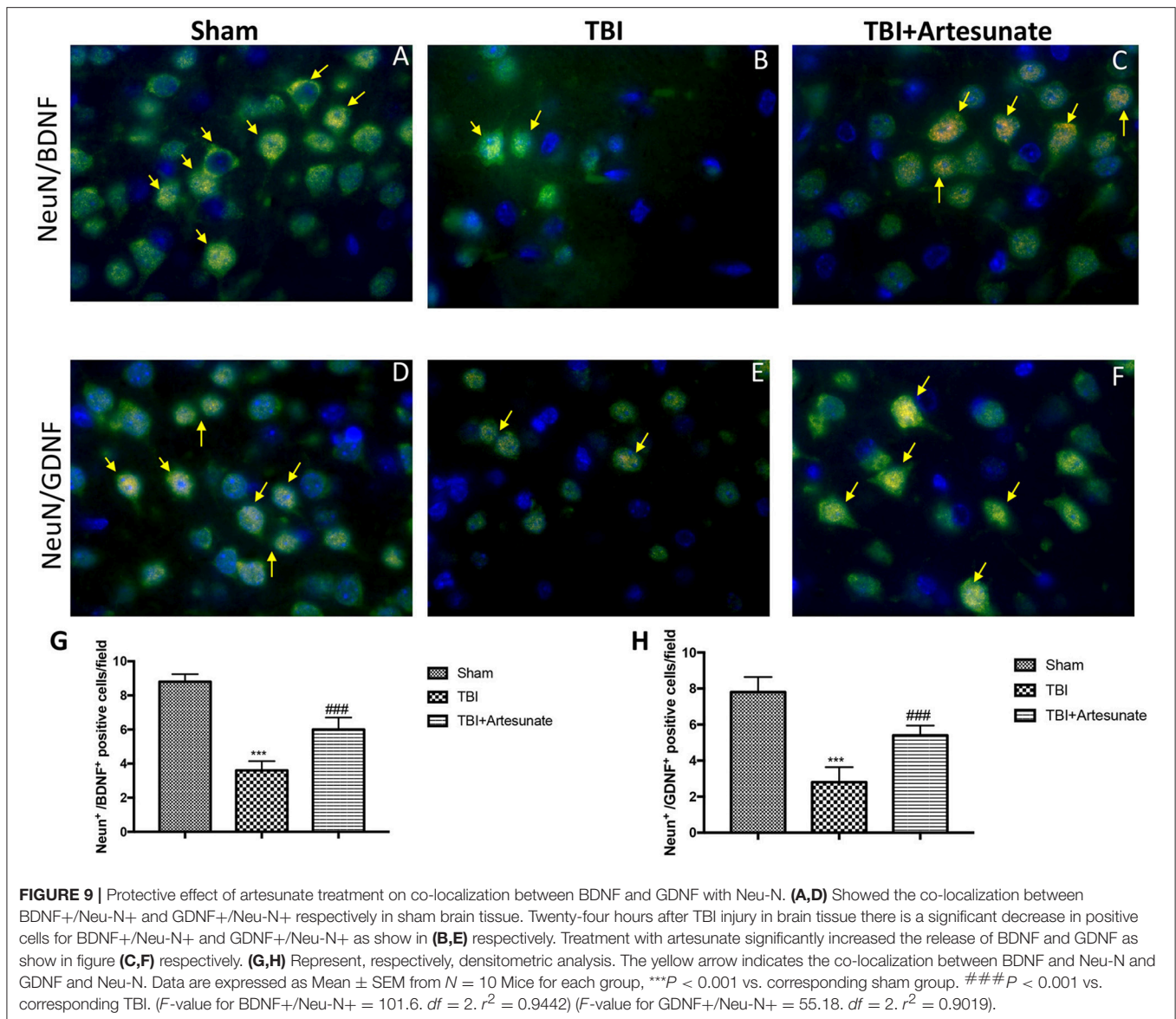


FIGURE 8 | Protective effect of artesunate treatment on neurotrophic factors. (A) Show the immunohistochemical expression of VEGF in brain section from sham group, (B) show a significant reduction in VEGF expression 24 h after TBI injury in brain section from vehicle group, instead (C) show that treatment with artesunate significantly increase the VEGF expression compared with the vehicle group, (D) VEGF density. (E,I) Show basal expression for BDNF and GDNF respectively in brain section from sham mice. 24 h after TBI there is a significant reduction in BDNF and GDNF expression as show in (F,J) respectively, instead (G,K) show that treatment with artesunate is able to significantly increase the expression of BDNF and GDNF respectively (H,L) BDNF and GDNF density. (M) Show the basal NT-3 immunoreactivity (red signal) in brain from sham mice. (N) Brain tissue from TBI group showed a significant reduction in NT-3 levels. (O) When compared with TBI group the brain section from mice that have been treated with artesunate showed a significant increase in NT-3 levels. (P), NT-3 density. Data are expressed as Mean $\pm$ SEM from $N = 10$ Mice for each group, *** $P < 0.001$ vs. corresponding sham group. # $P < 0.05$ vs. corresponding TBI. ### $P < 0.001$ vs. corresponding TBI. (F -value for VEGF = 41.8, $df = 2$, $r^2 = 0.8745$) (F -value for BDNF = 38.97, $df = 2$, $r^2 = 0.8666$) (F -value for GDNF = 46.41, $df = 2$, $r^2 = 0.8855$) (F -value for NT-3 = 65.31, $df = 2$, $r^2 = 0.9159$).

activity is tightly regulated. It is generally bound by the principal inhibitory protein, $\text{I}\kappa\text{-B}\alpha$, and is sequestered in the cytoplasm. $\text{NF-}\kappa\text{B}$ can be activated by cytokines (like $\text{TNF-}\alpha$) or other stimuli including trauma. This requires the degradation of $\text{I}\kappa\text{-B}\alpha$, thereby freeing $\text{NF-}\kappa\text{B}$ to translocate to the nucleus. Moreover, $\text{NF-}\kappa\text{B}$ is important for the regulation of other enzymes such as those involved in the worsening of oxidative stress damage during the TBI. Here in this model of TBI we found that 24 h after TBI injury there was a significant degeneration of $\text{I}\kappa\text{-B}\alpha$ and a consequent increase in $\text{NF-}\kappa\text{B}$ translocation. Instead the treatment with artesunate was able to prevent the degradation

of $\text{I}\kappa\text{-B}\alpha$ and the translocation of $\text{NF-}\kappa\text{B}$. Moreover, the up-regulation of proinflammatory cytokines like $\text{IL-1}\beta$ and $\text{TNF-}\alpha$, oxidative stress, immune cells proteases and toxic metabolites can cause additional tissue damage that subsequently, provokes neuronal cell death and gradual axonal loss over time (36, 37). Our results show that 24 h after the TBI, in the brains of the mice from the vehicle group there was a significant increase in the expression of $\text{IL-1}\beta$ and $\text{TNF-}\alpha$, and that treatment with artesunate significantly reduced the expression of these proinflammatory cytokines after TBI. The iNOS is responsible for the production of reactive nitrogen species, which cause various



types of cell damage including DNA damage (38, 39). Therefore, the increase in the expression of iNOS can weaken the oxidative stress damage during the secondary events to the TBI. In fact an increased expression of iNOS has been found in the areas associated with inflammatory processes after TBI injury (40), where it plays a role in secondary injury from TBI. Therefore, inhibition of iNOS expression has a protective role against TBI damage (41). Our results show an increase in iNOS expression 24 h after the TBI, while the treatment with artesunate at a dose of 30 mg/Kg was able to prevent the increase of iNOS expression. Recently it has been highlighted as nucleotide-binding domain, leucine-rich repeat, pyrin domain containing 3 (NLRP3), a key component of the NLRP3-inflammasome, mediates the inflammatory response, and therefore plays a key role in the secondary injury of the TBI (42, 43). The inflammasome is a multiprotein complex involved in innate immunity and in

a host inflammatory signaling. NLRP3-inflammasome, which also include adaptor proteins such as the apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), and the serine protease caspase 1 (Casp1) (44). the activation of NLRP3 thus leads to the maturation of Caspase-1 which is subsequently responsible for the release of the proinflammatory cytokines IL-1 β and IL-18. These cytokines play a key role in mediating the immune and inflammatory response, therefore NLRP3 activation can modulate neuroinflammation and neurodegenerative processes, as in the TBI (45). In fact, recently it has been seen that inhibition of NLRP3 pathway can help to prevent the pathological events resulting from the TBI (19, 46). In this study, we have seen that, in the vehicle group there is a significant activation of the inflammasome complex, as is evident from the increase in the expression of its components NLRP3 ASC and caspase -1. Instead the treatment

with artesunate was able to significantly reduce the increase in the expression of inflammasomes components, induced by the TBI. Moreover, the major focus of TBI research should be the protection of neurons from apoptotic cell death by reducing the secondary injury of inflammation and oxidative stress (47). The continuous research for new therapeutic strategies for the treatment of TBI, have shown that it is essential to have as its objective the inhibition of the inflammatory process and the reduction of oxidative stress (48). In fact, these conditions are the most responsible for worsening of post-TBI events such as tissue damage and consequent apoptosis, responsible for neuronal death and alterations in neuronal function (49). The apoptotic process, is an important type of programmed cell death activated in the post TBI (50, 51). Therefore, an inhibition of this process is a fundamental step in the treatment of TBI and in preventing the decline of neurological functions. In this study we evaluated whether treatment with artesunate was able to mitigate the induced TBI apoptotic process, by evaluating the expression of two of the factors involved in the regulation of the apoptotic process BAX and Bcl-2. Bax with pro-apoptotic activity is responsible for the development of neuronal death (52), while Bcl-2 has an anti-apoptotic action and therefore protective for neuronal cells (53). Our results show that after 24 h from TBI there was an increase in the expression of bax and a significant decrease of bcl-2 thus highlighting an imbalance toward the apoptotic pathway. Instead the treatment with artesunate has significantly reduced the levels of bax and reported the expression of bcl-2 like the sham group. A typical sign of damage to the central nervous system is the increase in reactive astrocytes as indicated by an increase in GFAP expression (54), moreover a significant increase in GFAP may be due to pathological processes in the central nervous system (55). It has been seen how the neuroinflammation in the TBI sees the involvement of microglia, showed by the increase in the expression of Iba-1 (56). Our results show a significant increase in the expression of GFAP and Iba-1 following TBI, while the treatment with artesunate was able to reduce the increase in GFAP and Iba-1, induced by TBI. The ability of damaged neurons to recover from injury, depends on the expression of genes related to survival and growth. These signals include neurotrophic factors that play a key role in neuronal survival and growth. Therefore, in this experimental model of TBI we examined whether the potential protective effects of artesunate, are also due to its ability to modulate the expression of neurotrophic factors. First of these we evaluated the expression of VEGF, in fact VEGF an angiogenic factor, is also responsible for the neurovascularization which is a fundamental step in the repair of brain tissue and for nerve regeneration (57). Our results show that, 24 h after the TBI, the group treated with artesunate showed a significant

increase in the expression of VEGF compared to the vehicle group, thus indicating that artesunate is grateful to improve the reparative processes following TBI. Neurotrophic factor such as BDNF, NT-3 and GDNF, are known to play a key role in neuronal development and regeneration, in fact it has been seen that these factors promote neuronal survival following a mechanic damage as in the TBI (58, 59). Our results show that treatment with artesunate, in addition to decreasing the inflammatory process typical of the second phase of TBI, can promote neuronal survival and regeneration, a fundamental event in preventing neurodegeneration, and therefore the decline of neuronal functions that the TBI involves. In fact, our results show that the treatment with artesunate is able to significantly increase the expression of the BDNF, GDNF, and NT-3 neurotrophins respect to the vehicle group, following the TBI. Therefore, our results taken together show that the treatment with artesunate has a protective effect against the secondary events after the TBI. In particular we have seen that artesunate is able to inhibit the inflammatory response and neuronal death, moreover the protective effects of artesunate are expressed through the promotion of neurotrophic factors, important for neuronal survival and in the reparative processes of the central nervous system.

DATA AVAILABILITY STATEMENT

All datasets (Generated\Analyzed) for this study are included in the manuscript and the Supplementary Files.

AUTHOR CONTRIBUTIONS

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Selective Brain Cooling Reduces Motor Deficits Induced by Combined Traumatic Brain Injury, Hypoxemia and Hemorrhagic Shock

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Selective brain cooling (SBC) can potentially maximize the neuroprotective benefits of hypothermia for traumatic brain injury (TBI) patients without the complications of whole body cooling. We have previously developed a method that involved extraluminal cooling of common carotid arteries, and demonstrated the feasibility, safety and efficacy for treating isolated TBI in rats. The present study evaluated the neuroprotective effects of 4-h SBC in a rat model of penetrating ballistic-like brain injury (PBBI) combined with hypoxemic and hypotensive insults (polytrauma). Rats were randomly assigned into two groups: PBBI+polytrauma without SBC (PHH) and PBBI+polytrauma with SBC treatment (PHH+SBC). All animals received unilateral PBBI, followed by 30-min hypoxemia (fraction of inspired oxygen = 0.1) and then 30-min hemorrhagic hypotension (mean arterial pressure = 40 mmHg). Fluid resuscitation was given immediately following hypotension. SBC was initiated 15 min after fluid resuscitation and brain temperature was maintained at 32–33°C (core temperature at ~36.5°C) for 4 h under isoflurane anesthesia. The PHH group received the same procedures minus the cooling. At 7, 10, and 21 days post-injury, motor function was assessed using the rotarod task. Cognitive function was assessed using the Morris water maze at 13–17 days post-injury. At 21 days post-injury, blood samples were collected and the animals were transcardially perfused for subsequent histological analyses. SBC transiently augmented cardiovascular function, as indicated by the increase in mean arterial pressure and heart rate during cooling. Significant improvement in motor functions were detected in SBC-treated polytrauma animals at 7, 10, and 21 days post-injury compared to the control group ($p < 0.05$). However, no significant beneficial effects were detected on cognitive measures following SBC treatment in the polytrauma animals. In addition, the blood serum and plasma levels of cytokines interleukin-1 and -10 were comparable between the two groups. Histological results also did not reveal any between-group differences in subacute neurodegeneration and astrocyte/ microglial activation.

In summary, 4-h SBC delivered through extraluminal cooling of the common carotid arteries effectively ameliorated motor deficits induced by PBBI and polytrauma. Improving cognitive function or mitigating subacute neurodegeneration and neuroinflammation might require a different cooling regimen such as extended cooling, a slow rewarming period and a lower temperature.

Keywords: traumatic brain injury, polytrauma, selective brain cooling, neurobehavior, neuroinflammation

INTRODUCTION

Traumatic brain injury (TBI) often presents acute care and treatment challenges. Extending the time window for treating reparable injuries such as hemorrhage or peripheral organ damage, while maintaining cerebral viability, could be a lifesaving strategy. One of the promising approaches is selective brain cooling (SBC), in which the brain temperature is reduced by 2–3°C while maintaining normothermia throughout the rest of the body (1). This approach captures the neuroprotective benefits of hypothermia while limiting any systemic complications attributed to whole body cooling.

Therapeutic hypothermia has been shown to be neuroprotective in experimental TBI (2, 3). Preclinical studies using rodent models of focal or diffuse TBI demonstrated that post-traumatic hypothermia reduced mortality, lesion volume and neuronal damage. It also attenuated axonal damage, blood-brain barrier disruption and neuroinflammation, as well as improved behavioral outcomes following isolated TBI (4–7). Systemic cooling (whole body cooling) which is commonly used in clinical practice for other indications (e.g., cardiac arrest or neonatal encephalopathy), was used to deliver hypothermia in these studies. However, its potential adverse effects such as coagulopathy, hypotension and pneumonia (8, 9) may limit its use in treating TBI casualties with multiple injuries. Only a few studies examined the effects of therapeutic hypothermia in experimental TBI models complicated by secondary insults. Yamamoto et al reported that a 1-h systemic cooling (targeted brain temperature = 30°C) mitigated neuronal damage at 24 h following closed head injury with concomitant hypoxia and hypotension in rats (10). Another study showed that a 4-h local cerebral hypothermia (targeted brain temperature = 33°C) with slow rewarming reduced cortical contusion produced by combined fluid percussion injury and hypoxemic insult (11). On the other hand, a similar cooling paradigm did not improve behavioral deficits or lesion volume in a rat model of controlled cortical impact and hypoxemia (12). Incorporation of secondary insults in TBI models more closely recreates the complex pathophysiology of polytrauma. This multifaceted approach may increase the likelihood of transferring therapeutic interventions such as SBC from bench to bedside (13).

Different strategies have been developed to selectively cool the brain but none have been successfully translated into clinical practice. Ice packs around the head/neck region (1, 12) or blowing cooled air directly onto the skull (7, 11, 14–16) have been used in rodent studies. A recent study used a cooling probe placed over the craniotomized area in mice (17). Nevertheless, heat

loss through conduction or convection might not be sufficient to effectively cool the subcortical or deep brain structures. In addition, the cooling probe cannot be used in the presence of wound or skull fractures. Endovascular infusion of ice-cold fluid has been tested in non-human primate using a micro-catheter placed in the proximal middle cerebral artery. It was shown to be effective in lowering the brain temperature within 10 min to 33.9°C and maintaining for 20 min (18). Yet, a longer cooling duration requires more fluid infusion, which may exacerbate intracerebral hemorrhage in TBI patients.

We have previously established a selective brain cooling method in rats that involved extraluminal cooling of the common carotid arteries using bilateral cooling cuffs. This method effectively reduces the brain temperature (cortical and subcortical) within 30 min without complications (19, 20). In a rat model of cerebral ischemia, reductions in infarct size and peri-infarct depolarization were evident following 90-min of cooling (19). More importantly, protection against intracerebral hemorrhage progression, elevated intracranial pressure, brain edema, impaired blood-brain barrier permeability, lesion size and neurological deficits have been demonstrated following 2-h cooling in a rat model of severe penetrating TBI (20, 21). We further investigated the effects of treatment onset or duration, and found that brain cooling delayed by 2–4 h or extended by 4–8 h still achieved multiple beneficial effects similar to the 2 h of cooling initiated immediately after severe TBI in rats (22). However, it is unclear whether these neuroprotective benefits can be reproduced when TBI is complicated by other insults. The current study focused on evaluating the neuroprotective effects of 4-h brain cooling initiated immediately following injury in a rat model of severe penetrating TBI combined with hypoxemic and hypotensive insults.

METHODS

Subjects

Male adult Sprague-Dawley rats (300–330 g; Charles River Labs, Raleigh, NC, USA) were used in these experiments. All procedures involving animal use were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Walter Reed Army Institute of Research. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication,

2011 edition. Animals were housed individually under a 12 h light/dark cycle in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALACI).

PBBI, HX, and HS

Anesthesia was induced with 3.5% isoflurane delivered in air/oxygen mixture [Fraction of inspired oxygen (FiO_2) = 0.26] and maintained at 1.5% throughout the surgery. In all animals, the right femoral artery and vein were cannulated for mean arterial pressure (MAP) monitoring and fluid resuscitation, respectively. In addition, the tail artery was cannulated for inducing HS by withdrawing blood. Rats were randomly divided into 2 groups that included the control group (PHH; $n = 13$) and the treatment group (PHH+SBC groups; $n = 12$). Both groups received unilateral (right) PBBI that was induced using a simulated ballistic injury device (Mitre Corp., McLean, VA) with a specially designed stainless steel probe (Popper & Sons Inc., Hyde Park, NY). The probe was mounted to a stereotaxic arm at an angle of 50° from the vertical axis and 25° counter-clockwise from the anterior-posterior axis. It was then, manually inserted through the right frontal cortex of the anesthetized rat via a cranial window (+4.5 mm A-P, +2 mm M-L from Bregma) to a distance of 12 mm (from dura). The elastic tubing on the probe was inflated by a rapid (<40 ms) water pressure pulse, forming an elliptical balloon calibrated to 5% of the total rat brain volume to produce an intracerebral temporary cavity. The probe was then gently retracted and the cranial opening was sealed with sterile bone wax. Transient HX was induced 5 min after PBBI by reducing FiO_2 to 0.1 (10% oxygen balanced with 90% nitrogen), resulting in a PaO_2 of <40 mmHg. Normoxia ($\text{FiO}_2 = 0.26$) was restored after 30 min of HX. Five minutes following restoration of normoxia, transient HS was induced by withdrawing blood via the tail arterial catheter using a withdrawal pump (Harvard Apparatus, Holliston, MA) at a constant rate of 0.25 ml/100 g/min to reduce MAP to 30–45 mmHg (monitored via femoral artery catheter connecting to a blood pressure transducer). HS was maintained for 30 min before receiving fluid resuscitation with lactated Ringer's solution (Hospira, Lake Forest, IL) via the femoral vein catheter. The infusion volume was three times the blood volume withdrawn.

Selective Brain Cooling (SBC)

Animals were randomly assigned to one of the two groups: PHH+SBC ($n = 12$) and PHH (without SBC; $n = 13$). Five minutes following fluid resuscitation, SBC was induced by using cooling cuffs around the common carotid artery (CCA) as described previously (19). The cuffs were placed around the exposed segment of each CCA (~0.5 cm below the bifurcation of internal and external carotid artery) and secured by a piece of silk suture. Brain temperature was monitored via a temperature probe inserted into the left cerebral hemisphere. Core body temperature was monitored using a rectal temperature probe (Harvard Apparatus, Holliston, MA) and maintained at $\sim 37^\circ\text{C}$ using a heating blanket. Ice-cold water was pumped into the cuffs continuously to cool the arterial blood as it entered the brain. The target brain temperature was $2\text{--}3^\circ\text{C}$ lower than the

baseline measurement. Spontaneous rewarming was achieved by termination of cold-water circulation. The total cooling time was 4 h. All physiological data (body and brain temperatures, MAP and heart rate) were acquired using PowerLab data acquisition system and analyzed using LabChart software (ADInstruments, Colorado Springs, MO). PHH group underwent the same procedures (same duration of anesthesia) without the cooling.

Rotarod Task

Motor coordination and balance were evaluated on Rotamex-5 rotarod apparatus (Columbus Instruments, OH). Prior to any surgical procedures, rats were trained for 4 days to meet the criterion of maintaining walking balance on the rotarod for >45 s at 3 fixed-speed increments (10, 15, and 20 rpm). Rats did not meet the criterion on the fifth day were excluded from the study. Baseline performance at these three speeds was recorded on the day prior to injury. Two trials were performed at each speed. Rats were tested again at 7, 10, and 21 days post-injury using the same parameters as defined for pre-injury baseline measures. Mean latency to fall off the rotarod at each speed was recorded. This task was performed by an investigator blinded to the groups.

Morris Water Maze (MWM) Task

Cognitive abilities were assessed using a spatial learning paradigm of the MWM (Noldus EthoVision XT, VA). The MWM apparatus consists of a circular basin (75 cm deep; 175 cm diameter) filled with clear water (22°C room temperature) to a depth of 60 cm placed in a dark room with visual cues. A clear, plexiglas platform was submerged to a depth of 2.5 cm from the water surface and placed in the center of the northwest quadrant of the pool. The platform position remained constant throughout all experiments. Rats were placed in the pool at one of the equally spaced starting positions (north, south, east, and west). The starting position was pseudo-randomly determined for each trial within a day, alternating between short- and long-arms in reference to the platform. Each rat was allowed to swim freely to find the hidden platform or until 90 s elapsed. Rats were given 4 trials per day (30 min inter-trial interval) for 5 consecutive days, from 13 to 17 days post-injury. A probe trial (missing platform test) was given on the last day (post-injury day 17) following the last trial of the spatial learning test. Each rat was allowed to swim freely until 60 s elapsed. Mean latency to find the hidden platform and time spent in the target zone searching for the missing platform (probe trial) were recorded. This task was performed by an investigator blinded to the groups.

Histology

On 21 days post-injury (the last day of MWM task), animals were transcardially perfused with phosphate-buffered saline (PBS), followed by 4% cold paraformaldehyde under deep anesthesia. Coronal brain sections (40 μm) were cut from +3.72 to -6.84 mm anteroposterior from Bregma. Four sets of serial sections were collected at 960- μm intervals. All the samples were processed at FD NeuroTechnologies (Ellicott City, MD). The first set was processed for the detection of neurodegeneration with FD NeuroSilverTM Kit II (FD Neurotechnologies, Ellicott City, MD) according to the manufacturer's instructions. The second

and third sets of sections were immunostained for astrocyte [glial fibrillary acid protein (GFAP)] and microglia (ionized calcium-binding adaptor molecule 1) respectively. Briefly, after inactivating the endogenous peroxidase activity with hydrogen peroxidase, sections were incubated free-floating in 0.01 M PBS (pH 7.2) 1% normal donkey serum (Jackson ImmunoResearch, West Grove, PA), 0.3% Triton X-100 (Sigma, St. Louis, MO) and the specific antibodies (rabbit anti-GFAP – 1:15,000, Dako/Agilent Technologies, Santa Clara, CA; rabbit anti-Iba-1 – 1:8,000, Wako Chemicals, Richmond, VA) for 24 h at 4°C. The sections were then incubated for 2 h at room temperature with biotin conjugated secondary antibody and detected by Vectastin elite ABC kit (Vector Lab, Burlingame, CA) and 3',3'-diaminobenzidine (Sigma, St. Louis, MO) as a chromogen. Subsequently, all stained sections were mounted on microscope slides and cover-slipped with Permount (Fisher Scientific, Fair Lawn, NJ). Images of the sections were digitized using an Olympus VS120 Whole Slide Scanning System (Olympus Corporation of the Americas, Waltham, MA) at uniform criteria for sensitivity and exposure time. For GFAP, Iba-1 and silver staining, positive-stained cells were quantified using threshold analysis in the cerebral cortex, hippocampus or corpus callosum

(silver staining only). The threshold value was set to consistently detect maximal positive staining of GFAP or Iba-1 or silver. To ensure objective quantification, the same threshold value was applied to all brain sections for each respective marker. All quantification was performed by an investigator blinded to the groups using Image J (NIH, Version 1.49).

Enzyme-Linked Immunosorbent Assays (ELISA)

Blood was collected 21 days post-injury by cardiac puncture using Z/1.3 clotting tubes (serum; Sarstedt, Newton, NC) or using heparin-coated microcentrifuge tubes (plasma) and allowed to clot at room temperature (serum) or on ice (plasma) for 30 min before centrifugation at 1,200 g for 10 min at 4°C. Serum and plasma were transferred to a storage tube and kept in –80°C until subsequent analyses. Interleukin-1 β (IL-1 β) and –10 (IL-10) sandwich ELISAs were conducted using commercially available kits according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA). Signal intensity was measured in duplicate using a colorimetric plate reader at 450 nm and 550 nm. Target protein concentration was determined from standards.

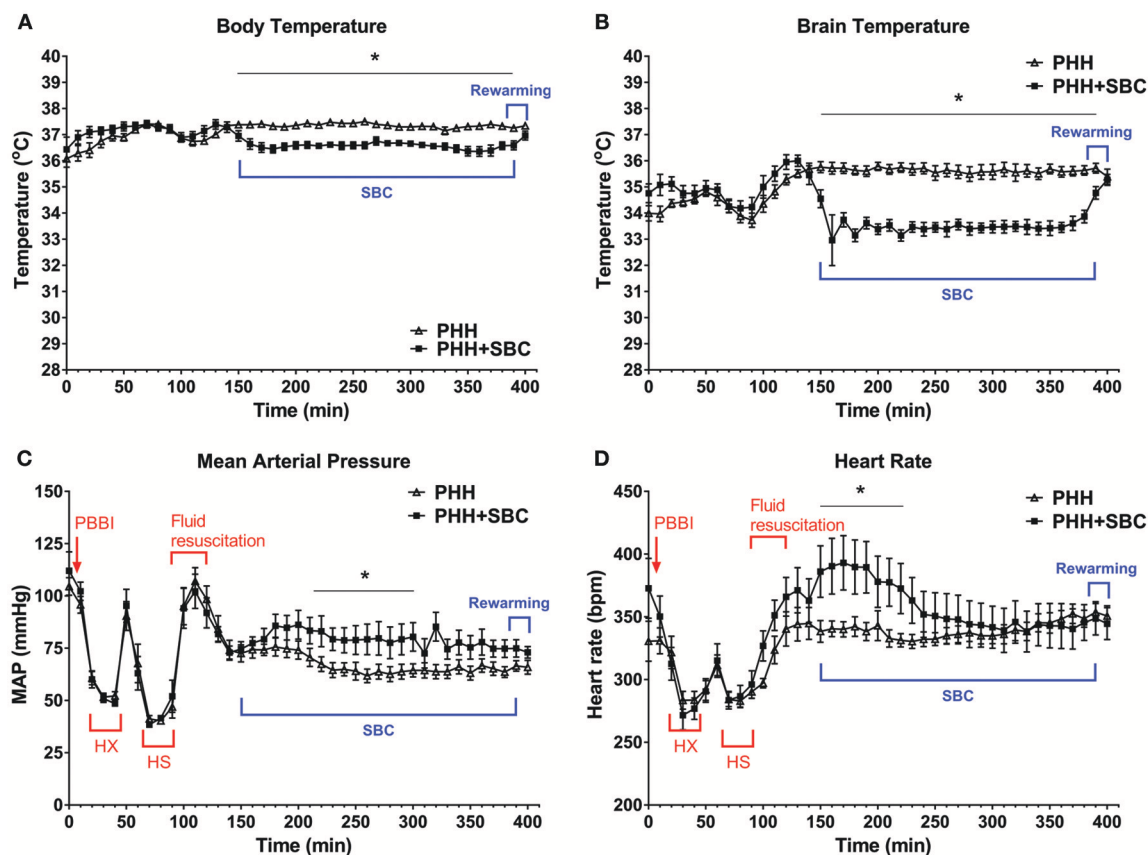


FIGURE 1 | Acute physiological parameters. Body temperature (A), brain temperature (B), mean arterial pressure (C), and heart rate (D) were monitored continuously from pre-injury baseline to the end of rewarming. Data was analyzed as 2-min averages taken at 5-min intervals across the 400-min recording period. For better visualization, data was plotted every 10 min on these graphs. PHH group $n = 13$; PHH+SBC group $n = 12$. * $p < 0.05$ (two-way repeated ANOVA with S-N-K post-hoc test).

Detection and accuracy were confirmed with internal calibrator controls.

Statistical Analysis

Statistical analysis was performed using SAS software v9.1 (SAS Institute, NC) and SigmaPlot 12.0 (Systat Software Inc., CA). Two-way repeated measures analysis of variance (ANOVA) was used to analyze the physiology and behavioral data. Pairwise *post-hoc* analysis was performed with the Student-Newman-Keuls (S-N-K) test. One-way ANOVA was used to compare the data obtained from the probe trial of Morris water maze. Student's *t*-test was used to determine the between-group difference in histology and ELISA data. The significance criterion of all the statistical tests was set at $p < 0.05$. Data are presented as the mean $\pm$ standard error of means (SEM).

RESULTS

General Physiology

Baseline body temperature in both groups was maintained between 36–37°. It dropped about 1° in the SBC group throughout the cooling period (Figure 1A). Brain temperature of the SBC group dropped 2–3° within 30 min, indicating an effective cooling to the targeted temperature. During the spontaneous rewarming period, the brain temperature in the SBC group rose from 33 to 35° within 30 min (Figure 1B).

As shown in Figures 1C,D, MAP and heart rate in both groups dropped significantly during the hypoxemic and hypotensive states but they returned to baseline levels following fluid resuscitation. The MAP then gradually decreased to <80 mmHg and remained low in the control group (PHH) throughout the recording period. In contrast, SBC increased the MAP to about 90 mmHg, which was maintained at a level that was significantly higher than the control group halfway through the cooling period ($p = 0.013$ – 0.047). On the other hand, heart rate in the SBC group increased significantly only during the initial phase of brain cooling ($p = 0.002$ – 0.047 ; two-way repeated ANOVA with S-N-K *post-hoc* test).

Rotarod Task

Prior to injury, all groups showed similar baseline levels of motor performance on the rotarod task (Figure 2). Significant and persistent motor deficits were observed from day 7 to 21 in both groups. However, animals that received SBC performed significantly better (longer latency to fall) than the animals without SBC at the speed of 15 rpm on day 7, 10, and 21 days post-injury ($p = 0.015$, 0.008 , and 0.001 respectively; two-way repeated ANOVA with S-N-K *post-hoc* test).

Morris Water Maze Task

Spatial learning performance was assessed in all groups at 13–17 days post-injury (Figure 3A). The results showed that combined PBBI and HH insults produced significant cognitive deficits as evidenced by the increased latency to platform compared to our previous data obtained from sham control animals. A trend toward improved cognitive function was observed in the SBC group on day 13, yet no significant differences were detected

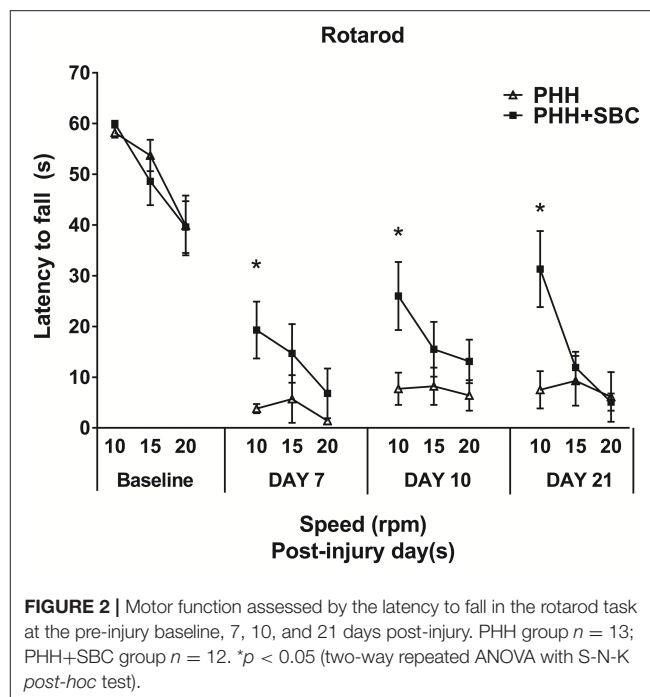


FIGURE 2 | Motor function assessed by the latency to fall in the rotarod task at the pre-injury baseline, 7, 10, and 21 days post-injury. PHH group $n = 13$; PHH+SBC group $n = 12$. * $p < 0.05$ (two-way repeated ANOVA with S-N-K *post-hoc* test).

in either the acquisition or the probe (missing platform) trials (Figure 3B).

Histology

In both groups of animals, PHH resulted in substantial neurodegeneration (15–20% of the corpus callosum area), indicated by the silver staining (Figure 4A). Increased GFAP (Figure 4B) and Iba-1 (Figure 4C) immunoreactivities were detected in the ipsilateral cerebral cortices of both groups, but not the hippocampi. However, none of these markers showed significant differences between the PHH and PHH+SBC groups at 21 days post-injury.

ELISA

As shown in Figure 5, the plasma levels of IL-1 β and IL-10, as well as the serum IL-1 β were comparable between the two groups at 21 days post-injury. In PHH group, the serum level of IL-10 trended higher than that in the PHH+SBC group, but did not reach statistical significance ($p = 0.20$; Student's *t*-test).

DISCUSSION

Our current study showed that 4-h SBC delivered through extraluminal cooling of the common carotid arteries effectively ameliorated motor deficits induced by combined PBBI, hypoxemic and hypotensive insults. The beneficial effects persisted for a prolonged period of time. In addition, SBC transiently augmented cardiovascular function, as indicated by the increase in MAP and HR during cooling. Yet, the treatment did not mitigate cognitive deficits, sub-acute neurodegeneration or neuroinflammation.

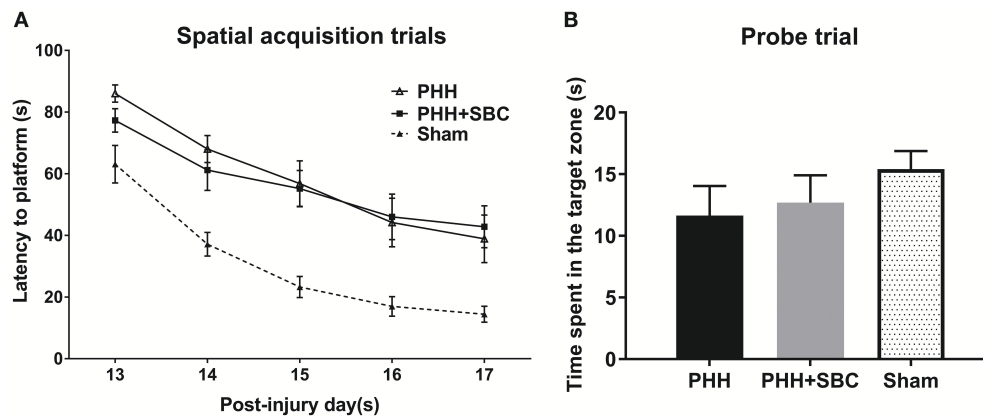


FIGURE 3 | Cognitive function assessed by the latency to platform in the spatial learning task **(A)** and time spent in the target zone in the probe trial **(B)** using MWM at 2 weeks post-injury. The data of sham control group was from our previous study and used as a reference to show the cognitive deficits on the injured animals. PHH group $n = 13$; PHH+SBC group $n = 12$. Sham control group $n = 12$. Two-way repeated ANOVA and one-way ANOVA detected no significant difference between groups.

The efficacy of SBC for improving motor function was consistent with our previous study in which the improvement was sustained through 21 days following 4 or 8-h SBC in animals subjected to isolated PBBI (22). Additional hypoxemic and/or hypotensive insults have been shown to worsen the cerebral hemodynamics (low cerebral blood flow and brain oxygenation) acutely following PBBI (23), as well as the neurobehavior during the subacute phase (24). In the present study, cerebral perfusion was likely increased by the augmented cardiovascular function (transient increase in mean arterial pressure and heart rate) during the cooling period. Our previous study showed that PBBI resulted in an increase of ICP to about 15 mmHg at 1 h post-injury (25). While the MAP in the control group and the treatment group was 60 and 75 mmHg, respectively during the cooling period, the cerebral perfusion pressure (CPP) should be around 45 mmHg in the control group and 60 mmHg in the treatment group. It was suggested that a CPP of 60 mmHg provides adequate perfusion for most TBI patients (26). Lower CPP leads to reduction in cerebral blood flow and is frequently associated with unfavorable outcomes. On the other hand, higher CPP worsens cerebral edema and intracranial hypertension (27). Thus, maintaining cerebral perfusion within an optimal range is critical for minimizing secondary injury following acute brain injury. Without using fluid or vasopressors, SBC using the extraluminal method was able to increase the MAP and possibly CPP during the cooling period in the PBBI animals. This might, in part, contribute to the improved behavioral outcome. More importantly, SBC ameliorated the outcomes worsened by additional insults following TBI, indicative of its strong and robust neuroprotective effects.

No significant improvement in cognitive function (acquisition and retention) was detected in animals treated with 4-h SBC after combined PBBI, hypoxemia and hemorrhagic shock. This is consistent with our previous studies using the isolated PBBI model (20, 22). In fact, extending the SBC duration to 8 h still did not mitigate the cognitive deficit induced by PBBI

alone (22). Other preclinical TBI studies showed mixed results. Improved cognitive outcomes were reported by (6) and (28) after hypothermia treatment, with temperatures lower than the present study (32°C for 2 h in controlled cortical impact (CCI) model and 30°C for 3 h in fluid percussion injury model, respectively). In contrast, no effect on cognitive function was detected in the study using 4-h hypothermia treatment at 32°C following CCI and hypoxemic insult (12). Factors such as types and levels of injury, therapeutic window of the treatment, cooling duration and rewarming rate were suggested to determine the efficacy of the hypothermia treatment paradigm (2, 29). For example, the subgroup analyses of a multicenter clinical trial revealed that therapeutic hypothermia resulted in better outcomes in TBI patients with subdural hematoma (compared with normothermia); whereas, the treatment was detrimental in patients with diffuse brain injury (30). Additionally, clinical and experimental studies demonstrated the neuroprotective benefits of slow and gradual rewarming over rapid rewarming in TBI (11, 31, 32). Although our SBC paradigm has been successful in many aspects (19–22), ameliorating cognitive deficits might require longer cooling duration (>8 h), a slower rewarming, or a lower temperature.

Subacute neurodegeneration and an exacerbated glial response were prominent following PBBI. However, they were not mitigated by the SBC treatment in the current study. Brain cooling targets multiple injury processes including excitotoxicity, apoptosis, and neuroinflammation (3) which occur mostly during the acute phase of TBI. Post-traumatic hypothermia has been shown to attenuate acute inflammatory response (at 4 and 24 h post-TBI) indicated by the altered M1/M2 phenotype balance in microglia, yet such an effect did not last beyond 7 days post-injury (16). Our previous study showed that 4 or 8-h SBC reduced axonal injury, astrocytic and microglial activation at 3 days following isolated PBBI. At 21 days post-injury, however, SBC did not significantly ameliorate neurodegeneration measured by silver staining (22).

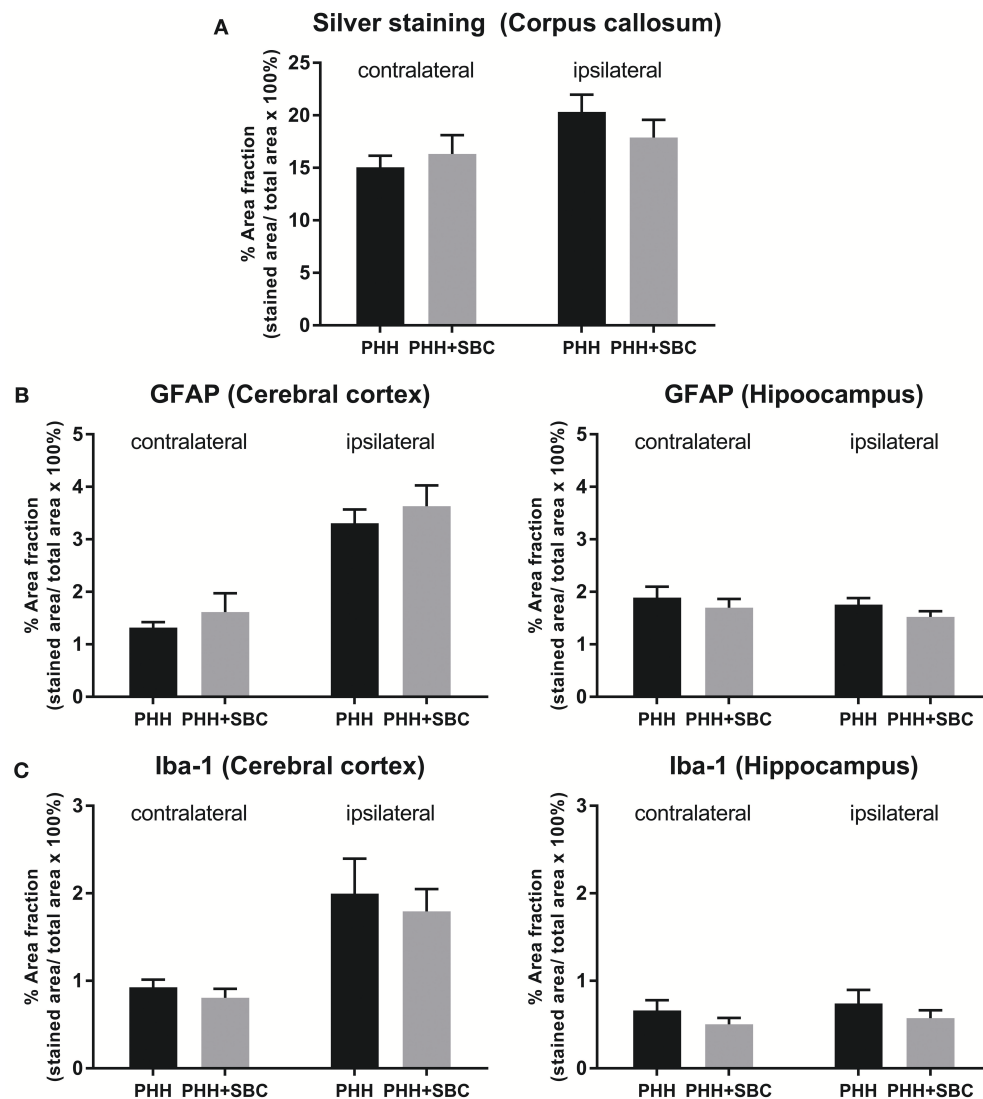
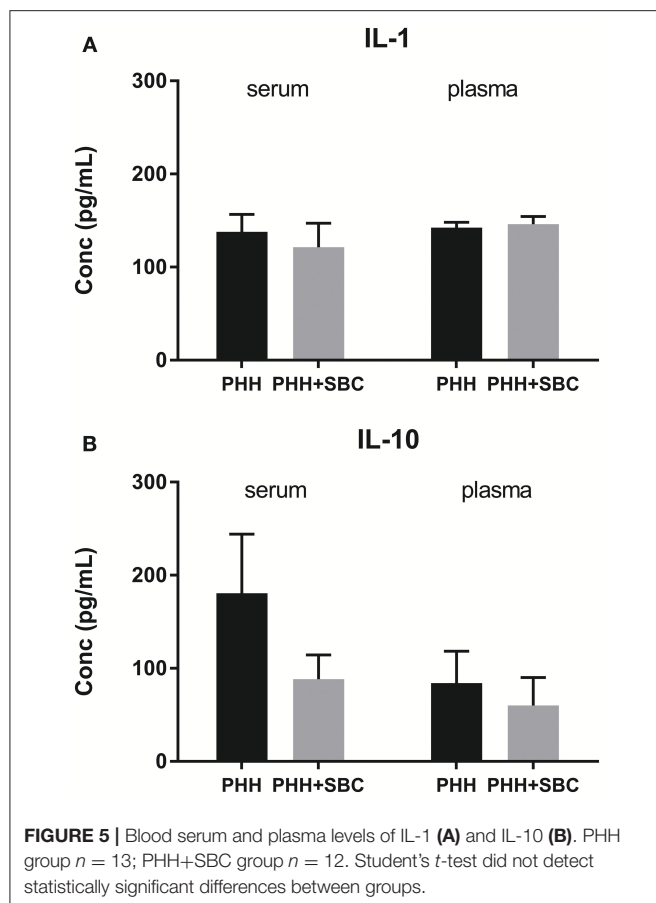


FIGURE 4 | Quantification of neurodegeneration by silver staining in the corpus callosum (A), astrocytic activation by GFAP (B) and microglial activation by Iba-1 (C) in the cerebral cortex and hippocampus of both hemispheres. PHH group $n = 13$; PHH+SBC group $n = 12$. Student's t -test did not detect statistically significant differences between groups.

It is plausible that the effects of SBC on these injury processes do not last more than a few hours or days. The treatment might slow down the abovementioned injury processes in the acute phase but not stop them from progressing in the later phase. Extending the cooling duration beyond the acute phase might improve long-term outcomes. Clinically, TBI patients are often cooled for a period of 24 h, up to several days, after injury with the core temperature targeted at 33–36°C (33). Yet it is both technically and logistically challenging to reproduce these clinical paradigms in the laboratory. Despite the lack of neuroprotective effects on subacute neurodegeneration and neuroinflammation, our data suggested that SBC significantly improved motor function following TBI and polytrauma. Experimental studies revealed the lasting effects of hypothermia on enhancing neurogenesis,

gliogenesis, angiogenesis and neural connectivity (34), that might be associated with improved functional outcomes. These aspects need to be investigated further.

SBC had no effects on the subacute serum/plasma levels of pro-inflammatory cytokine IL-1 β and anti-inflammatory cytokine IL-10. TBI triggers a complex sequence of inflammatory processes that contributes to the pathogenesis. Concurrent secondary insults such as hemorrhagic shock after TBI have been found to shift the cytokine response to a more anti-inflammatory phenotype. These pro-inflammatory and anti-inflammatory responses often occur acutely after TBI and are resolved by 1 or 2 weeks post-injury (35). Preclinical studies suggested that therapeutic hypothermia conveys neuroprotection by suppressing inflammatory processes (7, 36),



whereas clinical studies showed mixed findings. In adult TBI patients, cerebrospinal fluid (CSF) level of IL-1 β was found to be significantly suppressed during hypothermia (37). Such effect, however, was not observed in pediatric TBI (38). Our data showed that SBC did not affect the systemic cytokine levels at the subacute phase of TBI, again suggesting that the effect of SBC did not last beyond the acute phase. Intriguingly, the serum IL-10 level trended higher in the control group compared to the SBC group. Augmented IL-10 response was detected in animals subjected to TBI and hemorrhagic shock (39), as well as TBI patients with polytrauma (40). This might indicate that the injury effect persisted in the control group while it was suppressed by SBC in the treatment group.

There are several limitations in our study. First, the histopathology and cytokines measurement were performed at

only one end point, although multiple mechanisms operating in the early and late post-trauma phases may contribute to the outcomes. While our previous studies have demonstrated a reduced astrocyte and microglial reactivity at 3 days post-injury following a 4-h SBC treatment in rats subjected to isolated PBBI (20, 22), it is unclear whether earlier assessment in the current combined injury model would have revealed more or less robust effects on these outcomes. Another limitation is the rewarming phase. The rate of rewarming following therapeutic hypothermia was suggested to be an important factor in its success or failure. Slow progressive rewarming optimizes the benefits of cooling whereas rapid active rewarming aggravates tissue damage leading to worse outcomes after TBI (31, 33, 41). In the current study, the animals were allowed to rewarm spontaneously, and it typically takes about 30 min for the rat brain to return back to baseline temperature. A slower, controlled rewarming may be needed to maximize the therapeutic benefits of SBC, especially the long-term outcomes.

Overall, the present study has demonstrated a persistent beneficial effect of SBC on motor function in a rat model of combined PBBI, hypoxemic and hypotensive insults. In addition, SBC transiently augmented cardiovascular function which may in part account for motor improvement. Although the treatment did not mitigate cognitive deficits and long-term histopathology, the findings underscore the compelling neuroprotective effect of SBC on motor deficits induced by TBI complicated by hypoxemic and hypotensive insults.

AUTHOR CONTRIBUTIONS

LL wrote the manuscript and designed the study. LL, KC, XY, and BS contribute to data collection, data analysis or interpretation. JG and DS reviewed the study design and data analysis, and edited the manuscript.

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Multi-Center Pre-clinical Consortia to Enhance Translation of Therapies and Biomarkers for Traumatic Brain Injury: Operation Brain Trauma Therapy and Beyond

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Current approaches have failed to yield success in the translation of neuroprotective therapies from the pre-clinical to the clinical arena for traumatic brain injury (TBI). Numerous explanations have been put forth in both the pre-clinical and clinical arenas. Operation Brain Trauma Therapy (OBTB), a pre-clinical therapy and biomarker screening consortium has, to date, evaluated 10 therapies and assessed three serum biomarkers in nearly 1,500 animals across three rat models and a micro pig model of TBI. OBTB provides a unique platform to exploit heterogeneity of TBI and execute the research needed to identify effective injury specific therapies toward precision medicine. It also represents one of the first multi-center pre-clinical consortia for TBI, and through its work has yielded insight into the challenges and opportunities of this approach. In this review, important concepts related to consortium infrastructure, modeling, therapy selection, dosing and target engagement, outcomes, analytical approaches, reproducibility, and standardization will be discussed, with a focus on strategies to embellish and improve the chances for future success. We also address issues spanning the continuum of care. Linking the findings of optimized pre-clinical consortia to novel clinical trial designs has great potential to help address the barriers in translation and produce successes in both therapy and biomarker development across the field of TBI and beyond.

Keywords: biomarker, pre-clinical consortium, neuroprotection, drug screening, reproducibility, theranostic, rigor, target engagement

INTRODUCTION

New approaches are urgently needed to successfully translate therapies and biomarkers from the pre-clinical arena to therapeutic successes in clinical trials in the field of traumatic brain injury (TBI). For therapies, reviews have suggested countless explanations for these failures, usually placing the blame on issues related to clinical trial design, heterogeneity of patients, lack of accurate injury phenotyping, inadequate outcome assessment tools, and/or sub-optimal drug dosing, among other concerns (1–3). Clinical research in TBI has begun to take on the translational challenge and propose innovative approaches to address a number of the potential roadblocks to therapy development. For example, the emergence of several large multi-center clinical consortia in the field of TBI incorporating novel trial designs such as comparative effectiveness (4–6) and the development of phenotype-directed trials (7, 8), among others, are exciting developments. Challenges and limitations to pre-clinical study design could also underlie some of the failures in translation. Given many negative or inconclusive clinical trials and the well-recognized anatomical and pathogenetic heterogeneity of TBI phenotypes, it seemed logical to consider strategic alliances and collaborations capable of tackling these challenges through assembly of a multi-center pre-clinical consortium. To that end, a multi-center pre-clinical therapy and biomarker screening consortium, Operation Brain Trauma Therapy (OBTB) was developed, supported by the United States Department of Defense (DoD). Ten manuscripts, to date, have been published by OBTB including primary findings on individual therapies, reports on serum biomarkers, and reviews and overviews (9–18).

By using multiple pre-clinical models in a multi-center design, OBTB established two major goals for TBI therapy development and advancement, (1) to identify the most promising therapies—those with robust beneficial effects across models which might be successful across all TBI phenotypes in a conventional randomized controlled trial (RCT) in humans, and (2) to identify therapies that show model dependence which could help guide precision medicine based on therapeutic trials in patients with specific anatomical TBI phenotypes.

We also superimposed the assessment of serum biomarkers of brain injury, specifically two in current clinical development/testing (i.e., glial acidic fibrillary protein [GFAP] and ubiquitin carboxy-terminal hydrolase-1 [UCH-L1]), in an attempt to generate robust and rigorous pre-clinical evidence for their use as surrogate endpoints for predicting clinical outcomes and therapeutic benefit (i.e., testing their theranostic value). A second biomarker-related goal of OBTB is to create repositories of blood samples and brain tissue to facilitate opportunities for legacy research in order to test novel TBI biomarker candidates. OBTB's galvanizing efforts and accomplishments support the role and utility of pre-clinical consortia in TBI and suggests that OBTB has only scratched the surface of the potential of this approach. Several reviews and updates on the findings of OBTB have been published (10, 17, 18). In this review, we build on the lessons learned from the work of OBTB and focus on how its approach might be further harnessed to optimize

future development of consortium-based therapy and biomarker screening to facilitate future translational successes.

CONSIDERATIONS FOR DESIGNING THE INFRASTRUCTURE OF PRE-CLINICAL TBI CONSORTIA

Screening Models

Owing to the great heterogeneity of clinical TBI, a number of animal models mimicking the different aspects of human TBI have been developed. In OBTB, we chose to use three rodent TBI models, namely parasagittal fluid percussion injury (FPI), controlled cortical impact (CCI), and penetrating ballistic-like brain injury (PBBi), covering a spectrum of injury that included contusion, diffuse injury, and penetrating injury, respectively. These three models also represented the principle rat models being used in each of the three screening centers. Moreover, midline FPI in the micro pig was selected as the large animal model to test therapies, as it represented both a gyrencephalic TBI animal model and produced a mild diffuse injury, not captured in the rat models. Nevertheless, a number of additional modeling strategies could be adopted in an attempt to embellish OBTB, alter its scope, and/or craft a novel consortium targeting different facets of TBI and/or addressing different goals. For example, to more comprehensively address therapies across the full spectrum of severe TBI, consideration should be given to incorporating secondary insults such as hypoxemia, hemorrhage, and/or polytrauma, given their important contributions to unfavorable outcome (19, 20). Similarly, it might be of value to include one or more of the established mild TBI models into the OBTB design—or into one or more separate new TBI consortia. Such an approach might provide unique insight as to what secondary injury mechanisms may cross the spectrum of injury severity as a potential therapeutic target. As the DoD was instrumental at the inception of OBTB, the inclusion of a blast TBI model would represent another valuable addition to a new DoD funded TBI consortium. Another modeling strategy to consider would be to include more than one injury severity level in each model, an approach that is rarely taken even in individual laboratories. As injury severity can vary widely even within similar clinical TBI phenotypes, that approach might represent a valuable experimental framework to evaluate the performance of candidate therapies.

A focused pre-clinical consortium approach also lends itself to studying mild TBI and/or repetitive mild TBI. OBTB has directed all of its effort on assessing therapeutic effects on outcomes assessed over about 1 month after injury. Given the interest in long-term outcomes, chronic traumatic encephalopathy (CTE), and links between TBI and neurodegenerative diseases, studies assessing outcomes of much longer durations are needed. Studies to 1-year outcome have been carried out in both FPI and CCI (21, 22). Further discussion of the issue of outcome duration will follow in the section on therapeutic testing. Finally, OBTB, or other multi-center TBI consortia, could readily incorporate additional behavioral outcomes to study the link between TBI and posttraumatic stress disorder and its treatment.

Other aspects of modeling deserve consideration for incorporation into future multi-center TBI consortia. OBTT allowed each site to use their model as it was employed in current operation, without major modifications. This included approaches to anesthesia, analgesia, surgery, and most aspects of behavioral testing and histology. This approach was taken to ensure that the consortium was not bogged down in model development and could promptly launch therapy testing. Model development can produce challenges to successful transition of consortium development to therapeutic testing, as was seen in the pioneering work of the Multicenter Animal Spinal Cord Injury Studies (MASCIS) consortium (23). Among additional modeling issues that merit consideration are sex and age. In OBTT, young adult male rats were selected for the cross-model screening, given the preponderance of young adult males in TBI-related combat casualty care, germane to the goals of the DoD, which is funding the work. The choice of male rats was also influenced by the fact that most published studies testing therapies in pre-clinical models of TBI were carried out in young adult male rats, which was important for therapy selection and dosing in OBTT. However, differing approaches might be desirable depending on the target population. Moreover, research interpreting the effects of drugs in the context of sex and its impact on pathobiology represents another important step forward informing future clinical trial design.

Efficacy Endpoints in Therapy Screening

In OBTT, considerable thought was placed into designing an approach to compare therapeutic efficacy across models. For the rat studies, a 66 point scoring matrix was developed that weighted each model equally (a maximum of 22 points in each model), and addressed conventional outcomes already used at each site. Outcomes manageable in a screening approach were selected including assessments of motor testing, cognitive testing, and histology (10, 11).

- Motor testing: Since approaches to motor testing differed across centers, each site was allowed to use their established motor function tests.
- Cognitive testing: Morris water maze (MWM) testing was used to evaluate cognitive outcome at each site—given that it was already routinely applied to each rat model at each center. However, there were differences in the details of the approach to MWM testing at each site that were, once again, not altered. Given the importance of cognition to producing favorable outcomes in clinical trials, it was weighted the highest with a maximum of 10 of the 22 points in each model being allocated to MWM testing.
- Histology: Lesion volume and hemispheric (CCI and PBBI) or cortical (FPI) tissue loss were the histological outcomes scored for the rat studies in OBTT.

These outcome measures were selected given their extensive track record in the field of TBI and routine use at each site. Nevertheless, a host of additional cognitive and behavioral outcome assessment tools have been used in TBI and are available including fear conditioning, novel object recognition, open field testing, elevated plus maze, and forced swim testing among

others (24). Given the extensive track record that the MWM has had in the field of TBI, and its routine use at each site, it was the logical choice. Nevertheless, a battery of cognitive outcome tasks might also provide a greater opportunity to detect beneficial effects given that robust beneficial effects of therapies on cognitive outcome have been surprisingly limited in the initial work of OBTT. A battery of cognitive outcome tests might also better reflect the functional recovery seen in humans—since the Glasgow Outcome Scale, the currently used outcome tool for clinical trials in severe TBI, is a general assessment tool—and its analog in rodent models remains undefined. Thus, beyond the screening approaches currently used by OBTT, alternative behavioral outcome tasks may be desirable, in future consortium designs.

Drug screening in our rat models might also benefit from the assessment of additional histological outcomes. Examples include assessment of hippocampal or cortical neuronal death—using either conventional histological approaches, or Fluoro-jade or NEU-N staining (20, 25), or assessing axonal injury using markers such as amyloid precursor protein, which are established outcomes in our micro pig model (26). Beyond simply using additional neuropathological readouts, more sophisticated approaches such as linking neuropathology to behavior, might be necessary to reveal beneficial effects for some therapies. For example, in work by Zhao et al. (27) assessing the efficacy of the cholinesterase inhibitor galantamine in CCI, preservation of GABAergic neurons in the dentate hilus was noted and specifically linked to improvements in hippocampally-mediated memory processing using contextual fear testing. Trade-offs resulting from the use of general screening vs. target-specific outcomes thus represent a challenge to designing a therapy screening consortium.

Analytical Approaches

In OBTT, conventional approaches to data analysis were taken using the same statistical software (SPSS) at each site. Standard statistical tests were applied to the data in each model, points for the effects (positive or negative) were tabulated in our outcome matrix (22 points for each model), and the results across models were summed to generate an overall score for each therapy (10, 11). This also allowed cross model comparisons of efficacy. We also used a pooled analysis of four pre-defined outcomes that were used at each site, namely, (1) average MWM latency, (2) percent time in target quadrant in the probe trial, (3) contusion volume and (4) total tissue loss in the injured hemisphere (CCI and PBBI) or cortex (FPI). This allowed for a direct comparison of models across four shared outcomes, providing a useful tool to monitor the stability of each model from study to study and show their reliability and reproducibility.

An additional innovative analytical approach that has not been taken thus far by OBTT, but has been successful in other studies, is a topological data analysis (TDA). This “big data” analytic approach was recently applied to an archived database from the MASCIS consortium (28). TDA of the MASCIS database was used to examine the impact of various factors associated with outcome in experimental spinal cord injury to reveal that peri-operative arterial hypertension was highly

predictive of unfavorable outcome. It is thus appealing for applications in the design of future pre-clinical TBI consortia.

Finally, one of the surprising findings in OBTT has been the fact that so few of the drugs tested have shown robust efficacy, either across models or in individual models. Only two of the 10 drugs tested showed clear beneficial effects. Levetiracetam was beneficial in both FPI and CCI (17) while glibenclamide, in preliminary analysis, showed benefit specifically in CCI (18). Given these findings and the fact that our goal has been to search for therapies with the greatest likelihood of clinical success, an alternative analytical approach that may also be explored is to use a P -value of 0.1 rather than 0.05 as the threshold for defining an effect. The rationale for this approach centers on the fact that in a screening consortium the sample size is not statistically powered for each outcome, and with novel therapies there may not even be information on anticipated effect size. This may preclude definitive sample size calculations. Also, the optimal dose and route of administration for a given drug may differ across models. Comparing one or two doses of a drug using a single treatment protocol across models in screening thus limits the ability to optimize a given therapy in each model, potentially warranting application of a lower threshold for identifying therapeutic efficacy or priority. The trade-off to a lower statistical bar would be the potential for identifying therapies more likely to fail. Also, we do not believe that a large number of additional beneficial effects would emerge adding a P -value window from 0.05 to 0.1. Other innovative analytical approaches may also be informative mirroring the novel approaches to clinical trial design—such as adaptive trial design—that are now being used (29).

Reproducibility in Screening vs. a Robust Effect That Defies the Noise

Although much has been written about problems with clinical trial design as a cause for translational failure in TBI, concerns over reproducibility of pre-clinical studies may also play a role. This topic has been discussed in detail in the field of cancer research, where concerns over the inability to reproduce numerous pre-clinical reports in high impact journals spawned the term “reproducibility crisis” (30, 31). The purpose of OBTT was not to serve as a tool to evaluate reproducibility of published pre-clinical investigations; however, given that the published pre-clinical work importantly guided therapy selection and dosing, and that efforts toward achieving a high level of rigor were substantial, its findings by default provide some insight into the issue of reproducibility. Use of common data elements can help to maximize the chance of reproducing published findings (24), however, extremely subtle methodological differences between protocols can greatly affect findings (32). In OBTT even if the model and dosing selected was identical to that used in published reports, many other discrepant factors may have influenced the findings including issues such as differences in anesthesia, animal strain, vendor, age, diet, surgical approach, brain temperature, details of the injury, and others. Lithgow et al. (32) in a recent commentary stated that it is a rare project that specifies methods with a high level of precision and that standardization may be counterproductive—suggesting that it may be better to focus

on highly robust results that persist across a wide range of conditions than to chase fragile findings that occur only within narrow parameters. Such an approach mirrors that taken by OBTT, where rather than testing reproducibility, therapeutic efficacy across multiple established models is sought. Given that both anatomical TBI phenotypes and injury severity vary greatly within clinical trials, such an approach seems justified for therapy screening.

Monitoring Consortium Stability and Performance

Appropriately designed multi-center pre-clinical consortia allow for rigorous protocolized comparisons of therapies and biomarkers across multiple models and also provide unique insight into the pathophysiology of TBI through direct model comparisons. Beyond simply testing of therapies and biomarkers and comparing models, the consortium approach also allows monitoring of model stability and performance. To optimize comparison of multiple therapies tested in multiple models, it is essential that model stability be monitored, given that in a consortium like OBTT, years are required to carry out demanding therapeutic *in vivo* studies. Model stability, defined as a given model's ability to produce the same magnitude of injury response over time, can be influenced by staff changes, mechanical wear on the injury device, and other factors in the laboratory environment such as alterations that impact the microbiome, temperature, lighting, or other factors. Pooled analysis of four key TBI outcomes across models for each therapy tested by OBTT not only allows for an objective comparison of the therapies, but also allows for an assessment of temporal stability of each model by comparing outcomes in the TBI vehicle group for each model in each study. This ensures that a stable and appropriate therapeutic target is generated and allows minor discrepancies to be addressed if slight changes in model severity are seen over time.

The Unspoken Challenge: Transparent Reporting and Publishing Negative Results

OBTT represents a new rigorous paradigm-changing approach to identify neuroprotective therapies for clinical TBI. As such, the bar for performing the studies, reporting the data and presenting the results has been appropriately raised. The OBTT investigators are committed to standards such as use of a manual of standard operating procedure and publication of all findings regardless of the outcome (11–14). It may be noteworthy that the disappointing results seen by OBTT across models for treatment with erythropoietin (EPO) were also seen in subsequent clinical trials (33, 34). However, our goal is not to be prescriptive or proscriptive, recognizing the many limitations inherent in therapy screening strategies, especially across models. Our goal is simply to carry out high quality, rigorous, and timely screening studies of therapeutic efficacy across multiple models to advance therapies to successful clinical trials either across TBI phenotypes, or in a precision-based clinical trial.

Considerations for Advancement to a Gyrencephalic TBI Model

In OBTT, therapies and biomarkers showing promise are advanced to testing in a large animal, gyrencephalic, pre-clinical TBI model, namely midline FPI in micro pigs. Taking an approach that includes a second tier of therapy screening in a higher order animal model is logical, given the likelihood of gaining additional translational insight into a given therapy by carrying out studies in multiple species, including one with a gyrencephalic brain. This approach also addresses the practical issue regarding the high cost of carrying out initial therapy screening in large animal models. An approach such as the one taken by OBTT, has been outlined and updated in the RIGOR guidelines for stroke (35). Using a gyrencephalic animal may be of even greater importance in TBI than stroke given the key role of traumatic axonal injury in contributing to pathological progression and subsequent outcomes after TBI (36). The fact that in TBI neuropathology in long-term sequelae such as CTE, is prominent within the sulci, where mechanical strain and strain rate are hypothesized to be greatest in the gyrencephalic human brain, also reflects the usefulness of assessing gyrencephalic animal models of TBI prior to clinical translation (37). A large animal model can also facilitate the use of physiological monitoring, such as assessment of intracranial pressure or partial pressure of brain tissue oxygen (mirroring clinical care), and more extensive blood sampling. Large animal models may, in some cases, also require dosing paradigms that may more closely reflect the human condition and the immune system in rodents differs importantly from human (38, 39). Although other gyrencephalic species have been used on a sporadic basis, potentially due to the neuroanatomical and immunological similarities to humans, studies in pigs or micro pigs, have been used in the majority of large animal TBI investigations (40). Use of computational modeling of the key factors effecting drug response (i.e., allometric scaling) across more than one species is the more accurate method for estimating human equivalent dosing. Although there is added expense in the assessment of multiple species, such methods improve the accuracy of estimation of key factors of drug disposition (41, 42). Recent, preliminary studies have suggested that the ferret may represent a lower-order gyrencephalic species that deserves consideration (43). Indeed, the original development of the CCI model was carried out in ferrets (44). However, work to date in ferrets has been limited with regard to two key facets of therapy screening. First, behavioral outcome characterization in ferrets has been exploratory in nature, even in studies using the CCI model (43). Second, there is little work evaluating potential therapies in ferret TBI models, therefore, substituting our pre-clinical rat studies with ferrets would be impractical. Also, as there is limited support to either substantiate or refute potential therapeutic efficacy in either humans or large animal models following the detection of benefit in rodent preclinical models, the approach taken by OBTT of carrying out initial screening in rat models then advancing the therapies with promising findings in those rat models to pigs or other large animals is logical. However, a recent publication in *Nature*, suggests that for drugs targeting hippocampal neurogenesis, studies in rodents may be misleading,

since the mechanism affected in rodents is not present in the human hippocampus (45). Finally, parallel studies in rodent and large animal models might represent a reasonable alternative screening strategy (46), however that approach does not address the prohibitive cost associated with the substantial numbers of large animals required for large animal screening.

HOW DO WE SELECT THE BEST POSSIBLE THERAPIES TO ADVANCE FROM THE BENCH TO THE BEDSIDE?

In clinical trials across the field of acute brain injury including TBI, stroke, and global ischemia from cardiac arrest, and other conditions, there has been a consistent lack of successful RCTs testing novel pharmacological agents. Issues such as heterogeneity of the insult mechanism and severity, age, gender, and insensitive outcome assessment tools in humans have been implicated as reasons for these failures. However, recent highly successful studies in stroke assessing the efficacy of clot retrieval provide insight into considerations for the design and goals of pre-clinical consortia across the field of acute brain injury. Multiple RCTs of clot retrieval have reported highly significant benefit in stroke, with huge effect sizes >30% (47, 48). Indeed, some of the trials have been stopped early because therapeutic efficacy was shown with fewer patients than anticipated (47, 48). This suggests that a key to overcome the inherent “noise” in studies of TBI is to have a therapy with a large effect size. It is unclear whether or not any pharmacological approach in TBI can produce an effect size that matches the impact of rapid reperfusion (resulting from clot retrieval) vs. no reperfusion in stroke. However, it suggests that in TBI, a rigorous consortium-based approach using multiple models to identify highly robust therapies may be essential to achieving that goal. There are many aspects of therapy selection that merit discussion and careful consideration for a consortium approach moving forward.

Literature Based vs. High Throughput Screening Based Therapy Selection

In OBTT, a literature-based approach was used for therapy selection for testing in screening across the rat models. After a comprehensive literature review and consideration of recommendations from the OBTT investigators, advisory board, and programs in the DoD, a table of potential therapies with a description of the published studies in pre-clinical models of TBI was provided to the site principal investigators in OBTT and a secret ballot vote was taken to rank the therapies. This was followed by a discussion and final ranking of those therapies each year at an OBTT investigators meeting that was held at the annual meeting of the National Neurotrauma Society. Generally 3-4 therapies were selected for testing each year. This approach allowed the consortium to leverage the published literature, which for many of the therapies was fairly extensive. Nevertheless, it is not fully systematic and is challenged by the many differences between published studies in dosing and treatment protocols, species, outcomes, and other parameters—making it difficult to rank therapies objectively in either a

quantitative or qualitative manner. An alternative strategy, or one that may be able to be coupled to a literature based approach is to consider the use of drug screening first in an established *in vitro* screening TBI model, such as stretch injury in neuron or neuron/glia cultures (49–51). In addition to standard approaches targeting neuronal death, novel *in vitro* approaches, to more closely mimic the *in vivo* environment in neuron/glia stretch models have suggested exciting profiles that can highlight axonal injury without appreciable neuronal death (52). More sophisticated systems biology models, such as 3D cell culture and “organ on a chip” approaches are used in cancer biology and liver disease to screen therapies (53–55). A high throughput screen was reported using induced pluripotent stem cells as a source of neurons in a model system to screen therapies against tauopathies in Alzheimer’s disease (56). Similarly, neuronal stretch in a 96 well plate format has also been reported (57). Obviously, *in vitro* screening approaches are limited in their ability to incorporate clinically relevant features of TBI *in vivo* such as alterations in perfusion, ICP, inflammation, and other extra-cerebral factors, such as the microbiome, but they have the potential to screen and compare thousands of agents, including both those with strong literature support along with highly novel therapies. More advanced high throughput *in vivo* screening for leukemia has been carried out in zebrafish and, although exploratory as a tool for TBI, several reports of TBI modeling in zebrafish have been published (58, 59). Similarly, invertebrate TBI models such as in *drosophila* could be considered (60). The concept of incorporating an *in vitro* or other higher throughput screening strategy merits consideration. A paradigm illustrating options for therapy selection is shown in **Figure 1**.

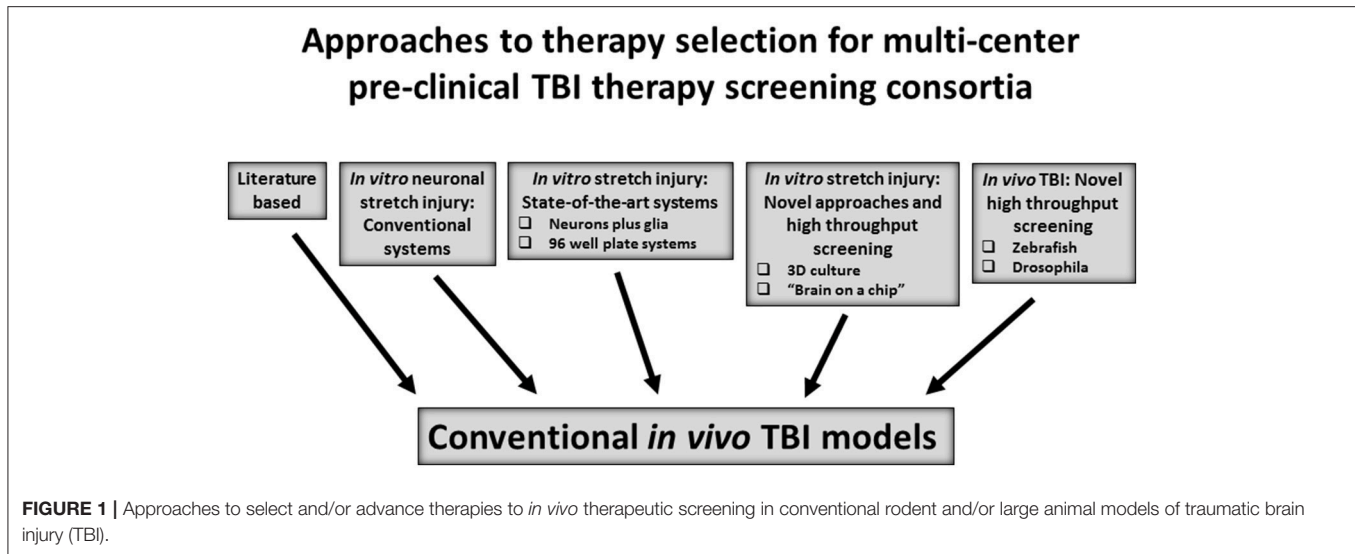
NEW HORIZONS FOR TESTING THERAPIES AND BIOMARKERS BY PRE-CLINICAL CONSORTIA: DEFINING THE RIGHT THERAPEUTIC TARGETS AND MONITORING TARGET ENGAGEMENT

A Multi-Model Consortium-Based Screening Approach May Be Essential to Successful Therapy Development for a Traditional RCT

In selecting therapies for screening by OBTT, a powerful influence has been the pressing need for new neuroprotective agents that can be rapidly translated to clinical trials. Therapies with pre-clinical literature support in one or more established models and that represented “low hanging fruit” i.e., drugs that are already FDA approved for other uses, were considered prime candidates. If successful, they could be rapidly brought to clinical trials, given that general drug safety was established. Often these drugs have effects (many of which represent “off target” effects of the drug’s originally intended use) that produce neuroprotection in pre-clinical studies and might translate to benefit in human TBI. Many of these therapies have pleiotropic beneficial effects, such as targeting inflammation, mitochondrial failure, neuronal death, oxidative stress, or regeneration. Given

the multifaceted secondary injury response to TBI, drugs with many potential therapeutic targets are alluring. However, they can present critical challenges for therapy development—in both the multi-model consortium setting and in clinical trials. For therapies that target multiple mechanisms, it may be unclear what mechanisms are critical to their neuroprotective effects. EPO and progesterone are examples of agents with pluripotent effects that have produced success in pre-clinical studies but have not translated successfully to humans (33, 34, 61). Neuroprotection by a given therapy may also be mediated by a different spectrum of effects in different models. The amount of blood-brain barrier (BBB) permeability, which can affect drug penetration into the brain, also differs across TBI models and varies as a function of injury severity, brain region, and time after injury even within a given model. A similar case exists for cerebral perfusion, which can be compromised to different degrees after injury in different models and in different brain regions in the same model (62–64). Thus, the amount and distribution of a given therapy in the injured brain after systemic administration may differ in each model. Layered upon this, the mechanism (or mechanisms) being targeted may vary in importance across models, and across brain regions and injury severity levels even in a given model. This can create major challenges for primary screening of therapies across TBI models, unless the drug being tested is one that has a high degree of BBB permeability, is highly potent, and/or has low toxicity—such that the necessary brain exposure can be achieved. It may be more than coincidence that the two drugs tested by OBTT with the greatest efficacy, levetiracetam and glibenclamide both have excellent BBB penetration and limited systemic toxicity. Although the issues of model and injury severity impacting both the mechanistic targets and drug delivery to brain might seem to represent a limitation for the pre-clinical consortium approach, these same challenges are seen in traditional RCTs in severe TBI, which feature heterogeneity of anatomical and pathological phenotypes, injury mechanism, injury severity, BBB permeability, perfusion, edema, and axonal injury, among other factors. Thus, if the goal is that of therapy development for a traditional clinical trial in severe TBI, for example enrolling patients with a Glasgow coma scale (GCS) score of 3 to 8, the multi-model pre-clinical consortium approach would seem to be appropriate. What approaches might best address treatment protocol design given these challenges?

In primary screening strategies, mechanism-based studies are generally not the goal, rather, clues as to potential efficacy, either in a single model or multiple models are sought to prompt more complete exploration of promising candidates. In OBTT a single literature based protocol, usually testing two doses and a vehicle group and sham, was evaluated across rat models. That approach allowed for therapies to be compared when administered in an identical manner across models. Although this allowed for rigorous comparisons, it is clear from the discussion above, that the dose and treatment regimen might be optimal for one model but suboptimal for another, depending on the studies in the literature on which dosing was based. In contrast, one might argue that, if a wide dose range was studied, and the therapy was reasonably non-toxic even at high doses, potential efficacy for use in a conventional RCT in TBI might be well defined. The strategy



that we implemented, however, may have its greatest potential to identify therapies with efficacy in specific TBI phenotypes. The biggest challenge to therapy screening in multiple models is selecting the doses and treatment protocols. Many other strategies to dose and treatment protocol selection could be used for multi-model consortium based therapy screening in TBI. For example, in studies targeting the pre-clinical development of therapies for pediatric TBI, Kilbaugh et al. (44) used rodent and piglet models concurrently focusing on a single therapy (cyclosporin A), studying a range of doses, and selecting effects on mitochondria as the target mechanism. Cyclosporin A, in OBTT showed considerable model dependence in effects, ranging from modest benefit in the FPI model, no benefit in CCI, and toxicity in PBBI (13)—suggesting that dose, timing, and duration of treatment could be very challenging to optimize in human TBI, unless a specific TBI phenotype and injury severity level were targeted (65). Cyclosporin A indeed has considerable toxicity with use in humans (66). Rather than using a literature-based approach for drug dosing, studies could be carried out to generate serum, plasma, or CSF exposures to better target one or more putative mechanisms. However, a thorough assessment of pharmacokinetics and pharmacodynamics, including studies in brain, are generally beyond the scope of primary therapy screening by a consortium. Indeed, assessment of drug or brain tissue levels was surprisingly rare even in the studies in the literature upon which the treatment protocols were based in OBTT. Nevertheless, recognizing the failure to detect robust benefit for 4 of the initial 5 drugs tested, OBTT chose to directly address this for therapies 6–10. We measured serum drug levels in separate cohorts of treated rats in 3 of those therapies (i.e., glibenclamide, AER-271, and minocycline) to optimize the treatment protocols. Levels were not measured for amantadine, owing to the substantial pre-clinical literature base for its testing in rats and for VA64—given that it is a polymer. The best approach to dosing and protocol selection for drug testing in TBI by a pre-clinical consortium merits great consideration. Additional considerations are discussed below.

Serum Biomarker Assessments of Efficacy and Target Engagement

OBTT has provided considerable insight into an additional strategy to monitor and optimize dosing and treatment protocols, and to promptly evaluate efficacy in screening via the use of target engagement biomarkers. In screening, although definitive mechanism-based studies are not the goal, a rapid assessment of either overall potential efficacy or some evidence that the mechanistic target for a given therapy is being modulated is desirable. Throughout the screening carried out in rat models, serum biomarker levels were serially assessed with the goals of (1) comparing the biomarker profile across models and (2) exploring the theranostic potential of biomarkers in therapy screening (16). The results of OBTT's primary screening in rat models support the use of the serum biomarkers GFAP and UCH-L1 as TBI diagnostics. They were useful across models and assessments at 4 or 24 h after injury corroborated injury severity, correlated with cognitive deficits assessed between 13 and 21 days after injury, and predicted ultimate lesion volume and brain tissue loss, assessed at 21 days after injury (16). The associations were strongest in CCI. The data generated by OBTT were submitted and reviewed by the FDA and viewed as an important preclinical component of the total submission package for clinical development. GFAP and UCH-L1 were recently approved for clinical use. More important to the development of novel theranostics for rapid *in vivo* drug screening in TBI, GFAP showed promise in predicting therapeutic efficacy, notably predicting contusion volume and/or tissue loss (14–16). For example, levetiracetam's effect on hemispheric tissue loss at 21 days after CCI was predicted by 24 h serum GFAP levels. Thus, serum biomarkers have the potential to serve as early post injury indicators of therapeutic efficacy. This approach is already being used by others (67). GFAP has also been shown to be valuable in identifying and monitoring adverse effects associated with drugs tested by OBTT (13, 14). Thus, GFAP has the potential to address the need for sensitive preclinical safety biomarkers and be implemented in clinical trials and regulatory pathways

for therapy testing. Given the efforts by the DoD to develop serum GFAP and UCH-L1 for use in combat casualty care at the time OBTT was launched, it was logical to begin with those two biomarkers in the work of OBTT. Other markers such as neuron specific enolase and S100 β , along with novel markers (discussed later) merit study within the pre-clinical consortium framework. Serum biomarkers could serve in an additional capacity, germane to therapy screening in individual models in multi-model consortia, namely, as readouts of successful target engagement. For example, OBTT recently reported that serum levels of phospho-neurofilament-H (pNF-H), a marker of axonal injury, were also reduced by treatment with levetiracetam (68). This suggests that pNF-H may represent an example of a target-engagement biomarker to rapidly assess therapies specifically targeting axonal injury and/or contribute to understanding of how therapies primarily targeting other mechanisms impact axonal injury. Additional serum target engagement biomarkers could also prove useful as early readouts for therapy screening. For example, the cardiolipin lipid profile of brain mitochondria is unique, and serum levels of brain specific cardiolipins at 24 h after TBI could be used to screen therapies targeting brain mitochondrial injury (69). Other target engagement serum biomarkers merit exploration for TBI therapy screening such as those monitoring inflammation, BBB disruption, or synaptic injury, among others. Beyond using serum, it is also possible that magnetic resonance imaging could be used to screen for target engagement efficacy, such as for drugs targeting inflammation (70), although issues of cost and throughput could be challenging. In any case, evidence of mechanistic efficacy to complement conventional outcomes could greatly enhance therapy screening in a multi-model pre-clinical approach.

NEW HORIZONS FOR TESTING THERAPIES AND BIOMARKERS BY PRE-CLINICAL CONSORTIA: PHENOTYPE BASED THERAPIES

Treatments for TBI may need to be phenotype specific. This concept has been discussed frequently for severe TBI, where experts in the field often use the example of multiple patients with highly different pathologies on admission computed tomographic scans are all being administered the same therapy in RCTs (71). Phenotype based multi-center therapy screening may need to be linked to phenotype based clinical trials. One could envisage that this could be efficient on multiple fronts, including (1) directing therapy selection for screening based on the specific pathophysiologic mechanisms of the TBI phenotype, (2) guiding dosing and treatment protocol selection based on the time course of the key secondary injury mechanisms in that phenotype and the required drug exposure to alter that mechanism, and (3) selecting the most clinically relevant outcomes in the pre-clinical models based on the phenotype. For example, a new therapy to reduce ICP might be able to be efficiently developed in a consortium by targeting brain edema that develops in a specific TBI phenotype such as contusion. That approach would still not resolve the contribution of genetics,

epigenetics, or extra-cerebral confounders (72, 73), but could address many challenging issues in consortium based screening and clinical RCTs. Phenotype-based therapies are particularly important to mild TBI, where divergent behavioral sequelae such as cognitive dysfunction, PTSD, sleep disorders, headache, and depression, among others are the therapeutic targets (74). Thus a phenotype-based screening approach is likely essential in mild and mild repetitive TBI. This approach could also be informative to serum biomarker development in TBI, since pre-clinical models with specific phenotypes might be able to help unravel the contribution of various insults in patients with complex pathologies.

NEW HORIZONS FOR TESTING THERAPIES BY PRE-CLINICAL CONSORTIA: ARE DRUGS THE ANSWER?

With the exception of levetiracetam and glibenclamide, the limited efficacy of the initial therapies tested by OBTT (nicotinamide, EPO, cyclosporin A, simvastatin, kollidon VA64, amantadine, minocycline, and E64d) has been surprising. Given the demands that showing efficacy across multiple models placed on a rigorous screening approach, this may not be surprising. But given the failure of multiple RCTs of drugs such as EPO (33, 34, 61), it may be that our approach is optimal for developing therapies to be tested in conventional RCTs of acute therapies in severe TBI. However, beyond the approach discussed above for phenotype based drug screening, it may be that for rigorous multi-model therapy testing, alternatives to drugs are needed. Combination therapy may also be necessary. However, since clear efficacy of individual agents has been difficult to confirm, the selection of drug combinations is challenging. Strategies such as combining a therapy showing efficacy on behavior with one that improves histology could be optimal. Combining drugs that target divergent or similar mechanisms, seeking additive or synergistic effects, might also be optimal. However, the approach to dosing in combination therapy requires considerable expertise (75). Many trials of combination therapy in pre-clinical models of TBI have failed or shown that benefit of one agent is negated by a combination approach (76). Given the need for a robust therapy, that penetrates the brain, and has limited toxicity, it may be that approaches beyond drug therapy are needed, such as cellular therapies (77), nanoparticles (78), or manipulation of the microbiome (79). A detailed discussion of innovative therapies for TBI, however, is beyond the scope of this review (36, 80, 81).

NEW HORIZONS FOR TESTING THERAPIES AND BIOMARKERS BY PRE-CLINICAL CONSORTIA: BEYOND ACUTE THERAPIES

OBTT has focused on the development of acute therapies for severe TBI. However, there are other exciting possibilities for pre-clinical therapy screening using a multi-center, multi-model or phenotype based approach. The most obvious potential

opportunity for pre-clinical consortium development is in the study of mild and/or mild repetitive TBI. A host of mild TBI models are available and several therapies have shown benefit

in these models (82–85). Similarly, given the importance of long-term outcomes and the link and common mechanisms underlying TBI and neurodegenerative diseases, it would be

Approaches to pre-clinical consortium composition targeting key TBI and treatment scenarios in rodent and/or gyrencephalic species

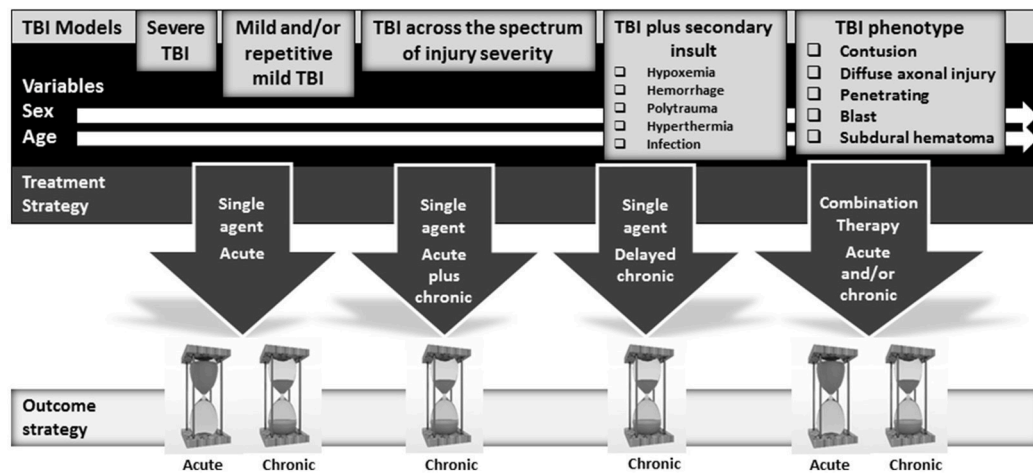


FIGURE 2 | Potential compositions of pre-clinical consortia for screening therapies for the treatment of traumatic brain injury (TBI) including (1) models targeting mild or severe TBI, secondary insults, and TBI phenotypes, (2) treatment strategies, and (3) evaluation of early or long-term outcomes.

Potential approach to consortium design for therapy development in TBI: From therapy selection to RCT design

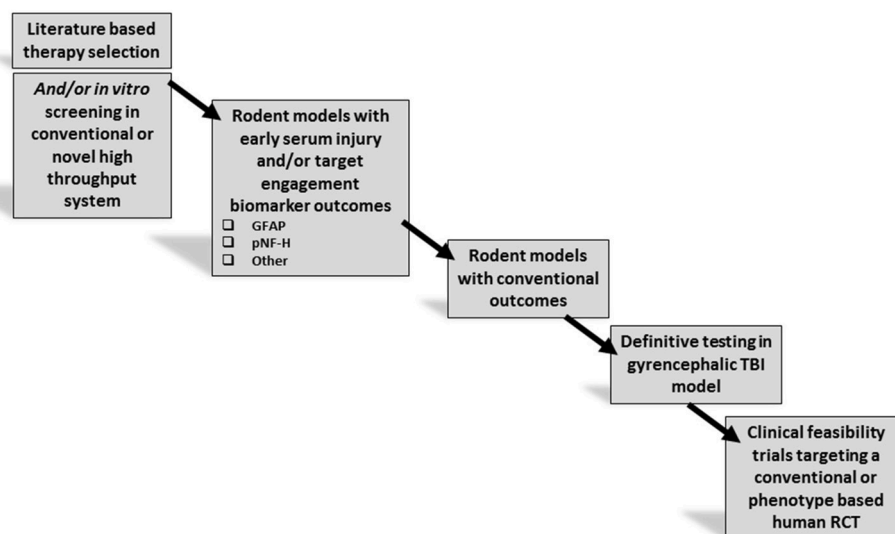


FIGURE 3 | Overall strategy for therapy development using target engagement biomarkers to aid in screening prior to advancing to definitive studies of long term outcome.

exciting to create long-term outcome oriented consortia capable of testing therapies to mitigate or prevent neurodegeneration. Seminal reports in the CCI and FPI models on 1-year chronic outcomes revealed dramatic targets including progressive tissue loss and persistent cognitive deficits (21, 22), setting the stage for similar studies in mild and repetitive mild TBI models. The approach to therapy testing in this setting could include (1) acute treatment, (2) delayed chronic treatment, and (3) acute plus chronic therapy. Some work on TBI in individual centers has begun to use these types of approaches (86). Long term studies would represent perfect opportunities to evaluate the impact of enriched environment with and without pharmacotherapy, mimicking clinical TBI rehabilitation (87). Conventional outcomes and those germane to chronic neurodegeneration, plasticity, and chronic neuro-inflammation, should be included. Consortia addressing long-term outcome therapy testing could also guide biomarker development, given the need for biomarkers linking acute injury with chronic TBI pathologies and neurodegenerative disease, an area that is only beginning to be explored (88). Potential approaches to pre-clinical consortium composition targeting key TBI and treatment scenarios in rodent and/or gyrencephalic species are shown in **Figure 2**.

NEW HORIZONS FOR THERAPY AND BIOMARKER DEVELOPMENT BY PRE-CLINICAL CONSORTIA: PARTNERING WITH PHARMA

Despite legal, ethical, and financial implications, public-private partnerships that allow the pooling of expertise, resources and funding, as well as providing cross-fertilization, are gaining momentum and strongly encouraged by government agencies including NIH (89). Collaborative projects involving industry and academics represent a unique conceptual framework and a promising cost-effective opportunity-risk and reward sharing approach. They may represent a logical avenue to consider in future therapy testing. For example, given the putative key role of cerebral edema in secondary injury after severe TBI, OBTT tested the aquaporin-4 antagonist AER-271 (Aeromics Inc.), a novel proprietary drug, in primary screening in rats (as therapy number 8 tested), navigating the necessary legal and administrative issues required for such a multi-center, DoD-funded partnership with the pharma (18).

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CONCLUSIONS

Linking the Findings of Pre-clinical Consortia to Optimized Clinical Trial Design

As pre-clinical drug development is evolving, and the novel strategies proposed in this review are advanced, clinical trials are also experiencing a number of advances in design. In severe TBI, comparative effectiveness approaches are being carried out in large numbers of patients in both adults and children (4–6). Novel clinical trial design, such as adaptive designs, where computer-driven randomization algorithms allow for the study of multiple therapies simultaneously and with greatly reduced sample sizes (29, 90) and large clinical studies based on big data approaches, are gaining utility in TBI (91, 92). Finally, in mild TBI, exciting new phenotype-based approaches are underway, including approaches such as TRACK TBI and TEAM TBI (74, 93). The intersection between novel pre-clinical consortia and emerging advanced clinical investigations has potential for breakthroughs in TBI therapy across the spectrum of injury severity. A synopsis paradigm outlining an overall potential approach to consortium design for therapy development in TBI is provided in **Figure 3**.

AUTHOR CONTRIBUTIONS

PK wrote the original draft, assembled and incorporated comments from the co-authors and crafted the final draft. All of the other co-authors contributed to manuscript review and revision.

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Changes in Posttraumatic Brain Edema in Craniectomy-Selective Brain Hypothermia Model Are Associated With Modulation of Aquaporin-4 Level

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Both hypothermia and decompressive craniectomy have been considered as a treatment for traumatic brain injury. In previous experiments we established a murine model of decompressive craniectomy and we presented attenuated edema formation due to focal brain cooling. Since edema development is regulated via function of water channel proteins, our hypothesis was that the effects of decompressive craniectomy and of hypothermia are associated with a change in aquaporin-4 (AQP4) concentration. Male CD-1 mice were assigned into following groups ($n = 5$): sham, decompressive craniectomy, trauma, trauma followed by decompressive craniectomy and trauma + decompressive craniectomy followed by focal hypothermia. After 24 h, magnetic resonance imaging with volumetric evaluation of edema and contusion were performed, followed by ELISA analysis of AQP4 concentration in brain homogenates. Additional histopathological analysis of AQP4 immunoreactivity has been performed at more remote time point of 28d. Correlation analysis revealed a relationship between AQP4 level and both volume of edema ($r^2 = 0.45$, $p < 0.01$, **) and contusion ($r^2 = 0.41$, $p < 0.01$, **) 24 h after injury. Aggregated analysis of AQP4 level (mean $\pm$ SEM) presented increased AQP4 concentration in animals subjected to trauma and decompressive craniectomy (52.1 ± 5.2 pg/mL, $p = 0.01$; *), but not to trauma, decompressive craniectomy and hypothermia (45.3 ± 3.6 pg/mL, $p > 0.05$; ns) as compared with animals subjected to decompressive craniectomy only (32.8 ± 2.4 pg/mL). However, semiquantitative histopathological analysis at remote time point revealed no significant difference in AQP4 immunoreactivity across the experimental groups. This suggests that AQP4 is

involved in early stages of brain edema formation after surgical decompression. The protective effect of selective brain cooling may be related to change in AQP4 response after decompressive craniectomy. The therapeutic potential of this interaction should be further explored.

Keywords: traumatic brain injury, decompressive craniectomy, brain edema, hypothermia, aquaporin-4

INTRODUCTION

Traumatic brain injury (TBI) remains one of the main causes of death and disability in developed countries (1–3). What determines a patient's outcome following TBI is not only the degree of primary injury, occurring during trauma by mechanical force application to the head. As it was proven, the following series of pathophysiological changes known as secondary injury plays a crucial role in determining post traumatic recovery (4–6). As a consequence of secondary brain damage, edema, and a consequent raise of intracranial pressure (ICP) may develop (7). If this condition remains resistant to standard care, raised ICP may be the major contributing factor for the fatal outcome (8–13).

Among second-tier therapy options in neurotrauma, two methods recently evoked the researchers' particular interest. Firstly, decompressive craniectomy (i.e., partial surgical removal of skull bone) could be demonstrated as a method of efficiently relieving increased intracranial hypertension, reducing brain edema formation and improving neurological outcome after head trauma in several animal experiments (14–16). However, in clinical setting the beneficial effect is limited: One of two recent multicenter randomized controlled clinical trials on decompressive craniectomy (RescueICP) reported that surgical treatment decreased mortality after TBI, however at the cost of increased number of severely disabled patients up to 12 months after trauma (17, 18). More so, the previous of the randomized craniectomy trials (DECRA) suggested a deleterious impact of surgical decompression on neurologic outcome (17, 18). This conclusion could be supported by various experimental studies (including our own analyses), reporting increased structural damage and poorer functional recovery in animals treated by surgical decompression after head injury or subarachnoid hemorrhage (19–22).

The other of these mentioned second-tier therapies, cerebral hypothermia, was hoped to be an efficient method to attenuate secondary brain damage mediated by its ICP-reducing and neuroprotective properties, evident both in animal

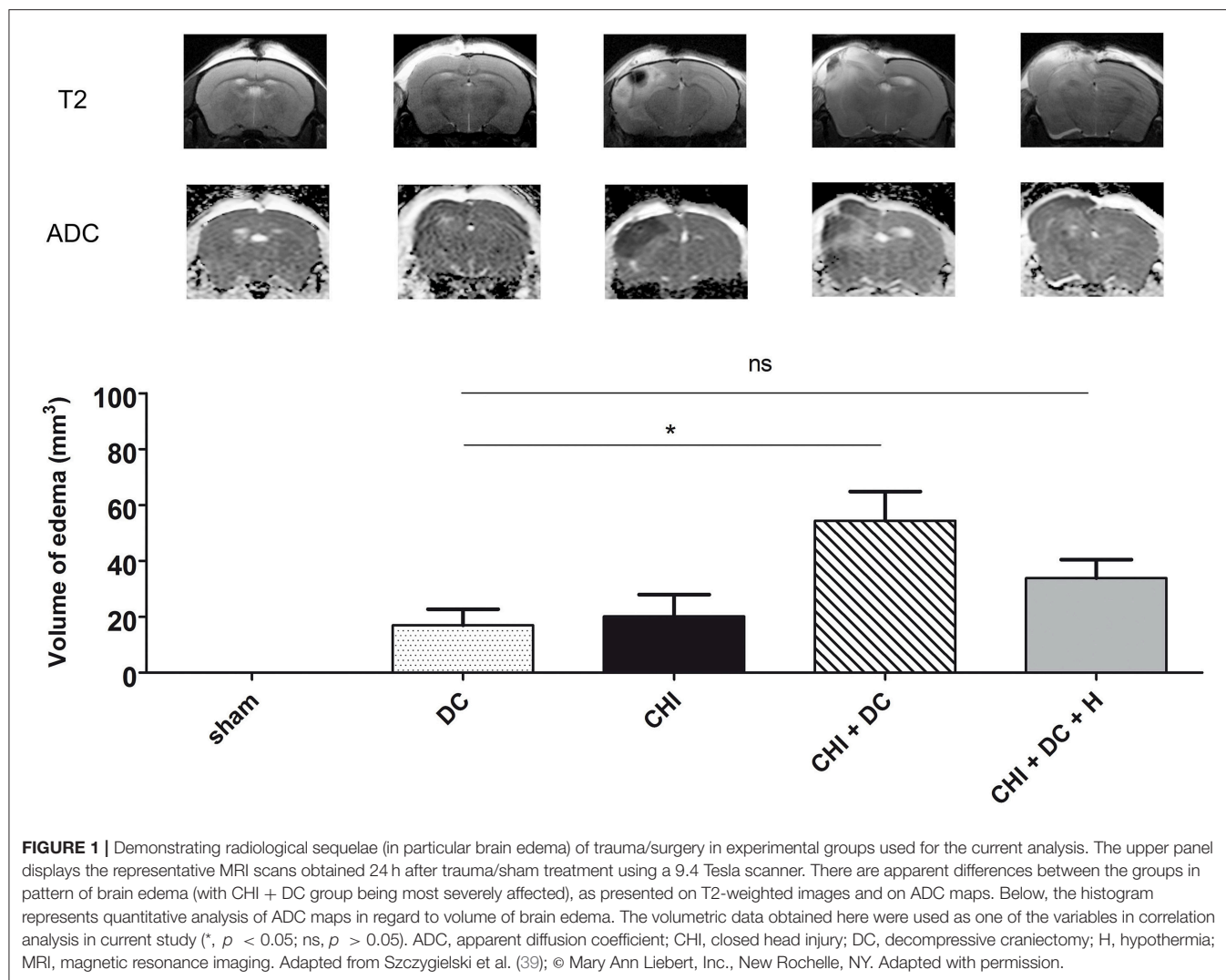
experiments (23–27) and single-center clinical settings (24, 28–32). Unfortunately, hope for the efficacy of systemic hypothermia in improving patients' long term outcome was refuted in large multicenter clinical trials (33–36), mostly due to severe systemic side effects including electrolyte derangement, coagulopathy, and infectious complications (34, 37, 38). Therefore, whole body cooling has been abandoned as a standard therapy for TBI. To achieve reported neuroprotective effects of hypothermia without risk of previously mentioned systemic side effects, selective, or focal brain cooling got into focus. Some previous studies (conducted also by our group, see **Figure 1**) were able to report a limitation of brain edema formation due to focal application of hypothermia (20, 39–45).

Obviously, reducing of brain edema is the main target in management of raised intracranial pressure. Canonical work published by Klatzo et al. distinguishes between vasogenic brain edema, resulting from damage to the blood-brain-barrier with subsequent extracellular water accumulation and between cytotoxic brain edema, where water excess gathers in the intracellular compartment of neurones and astrocytes (46). Later on, Marmarou and associates refined these definitions, pointing out that energy depletion, necessary for active maintenance of ion-water homeostasis is the main pathomechanism in cytotoxic brain swelling (7, 47–51).

This dichotomy is represented also in radiological studies, visualizing both brain edema types by implementing magnetic resonance imaging (MRI). For example, estimation of brain edema character may be provided *in vivo* by measuring of water particles diffusion in tissue and demonstrated as difference of intensity in apparent diffusion coefficient maps (ADC). Using this technique, a heterogeneous (both vasogenic and cytotoxic) character of posttraumatic edema has been documented (52, 53). Importantly, the proportion of both edema types changes within the posttraumatic course, with cytotoxic edema (demonstrated as hypointense ADC areas) being the predominant form of swelling during acute phase (54–56). This MRI-based observation was made also in experiments based on closed head injury (CHI) model (57–60) used in our laboratory (22).

Posttraumatic brain edema formation in its both forms is governed by many molecular interplayers. One of these, aquaporin-4 (AQP4), deserves particular attention. AQP-4 is a water channel protein that is present on astroglial foot processes, near to cerebral capillaries or CSF spaces (61). Numerous studies report a crucial role of AQP4 in development and resolution of brain edema of any origin, e.g., of ischemic (62–64), hemorrhagic (65–68), infectious (69, 70), and traumatic one (71–75). During the time course of brain edema formation following primary injury, the task of AQP4 changes significantly

Abbreviations: AQP4, aquaporin 4; ADC, apparent diffusion coefficient; BBB, blood brain barrier; CCI, controlled cortical impact; cDNA, complementary deoxyribonucleic acid; CHI, closed head injury; DC, decompressive craniectomy; DWI, diffusion weighted image; ELISA, enzyme-linked immunosorbent assay; FPI, fluid percussion injury; H, hypothermia; IC, immunohistochemistry; ICP, intracranial pressure; ICU, intensive care unit; IF, immunofluorescence; IR, immunoreactivity; MRI, magnetic resonance imaging; mRNA, messenger ribonucleic acid; RARE, rapid acquisition with refocused echoes; RNA, ribonucleic acid; RT-PCR, real-time polymerase chain reaction; TBI, traumatic brain injury; TSE, turbo spin echo; WB, Western blot.



and depends strongly on underlying edema subtype (vasogenic vs. cytotoxic), differing by injury character (71, 74–77). For the analysis of the AQP4 role in cerebral edema development, above cited distinction between two forms of cerebral edema (cytotoxic vs. vasogenic) is of great importance: The role of AQP4 differs diametrically between vasogenic and cytotoxic brain swelling (74, 78, 79), with AQP4 being usually increased in brain injury models demonstrating mostly vasogenic edema type (77, 80, 81). Furthermore, the most solid body of evidence is provided by experiments using AQP4 knockout mice. In ischemic stroke models, where mainly cytotoxic brain edema is represented, AQP4-deficient animals presented with reduced edema formation and improved functional outcome, both in models creating permanent and transient ischemia (62–64). Thus, an AQP4-mediated deleterious effect on blood-brain-barrier water permeability is indicated and a protective mechanism to reduce increase of cytotoxic edema formation through AQP4-downregulation can be suggested. In contrast, in vasogenic edema, the role of AQP4 channels seems to be

beneficial by facilitating the reabsorption of excessive fluid and thus the clearance of brain edema. Accordingly, animals lacking AQP4 presented with a greater amount of brain edema compared to wild-type littermates in model of central nervous system bacterial infection (69, 70) as well as in brain tumor model and cold brain lesion model (82), both being characterized by predominantly vasogenic brain edema.

Numerous pharmacological interventions targeted toward the reduction of posttraumatic edema formation exert their effect by AQP4 modulation (83–88). Importantly, impact on AQP4 expression / level could also be reported by experimental groups using decompressive craniectomy (16) or hypothermia (89, 90) as solitary treatment modes.

In a series of previous experiments, we elaborated a decompressive craniectomy mouse model based on the well-established paradigm of closed head injury (CHI) (22, 91, 92). Using this model, we were also able to successfully implement a combined treatment, composed of surgical decompression and subsequent focal cooling of the contused

area. Accordingly, deleterious sequelae of combined trauma—decompressive craniectomy treatment were less prominent, if selective deep cooling of injury epicenter has been performed (20, 39). However, the molecular background of these phenomena remained unclear. Thus, for the purpose of current study we hypothesized, that in our model, the structural changes (in particular brain edema) and biochemical changes (AQP4 level) share the same pattern across the experimental groups. We also presumed, that AQP4 level correlates with extent of brain edema. In order to explore this hypothesis, we initiated a biochemical analysis of the brain tissue obtained in previously conducted experiments in order to show whether the reported detrimental impact of decompressive craniectomy as well as alleviating effect of selective brain hypothermia are associated with the influence on aquaporin-4 expression. Our second aim is to analyze the correlation between biochemical sequelae (AQP-4 level) and radiological features (edema/contusion) of TBI.

METHODS

Animals and Trauma Model

All animal experiments were performed with approval by the local ethical board (28/2006, Saarland Ethical Commission), in line with the laws for animal protection, including Directive 2010/63/EU and by following all institutional and national guidelines for the care and use of laboratory animals.

Male wild-type, CD-1 mice of 9–12 weeks of age, without previous surgical or drug treatment, weighting 35.49 ± 0.59 g were acquired from the Charles River Germany GmbH & Co and kept in local Animal Facility of Institute for Clinical and Experimental Surgery.

Before starting the experimental procedure, mice were randomly assigned in one of the following experimental groups: 1. sham-operated (sham); 2. closed head injury alone (CHI); 3. decompressive craniectomy alone (DC); 4. CHI followed by DC at 1 h post-TBI (CHI+DC); 5. CHI+DC and selective hypothermia maintained for 1 h (CHI+DC+H) ($n = 5$ animals from each group, suitable for analysis as described further).

For the surgical part of the experiment, isoflurane anesthesia protocol has been established basing on recommendations of several Animal Welfare Agencies (93) and under assent of local Representative of Animal Welfare Board, Saarland University. According to protocol, spontaneously breathing mice were kept under general anesthesia by isoflurane inhalation (Forane[®], Baxter, administered via Isoflurane Vapor[®] 19.1 device, Dräger; initial dose 3% in 97% O₂, maintenance 0.8–1.5%, in 99.2–98.5% O₂).

For groups 2, 4, and 5, experimental TBI was induced using a weight drop device [adapted from Chen et al. (91)]. Briefly, the animals were placed on a heating pad with an additional heat lamp used if necessary. Target core and head temperatures were measured by a rectal probe and a needle temperature probe placed in the right temporal muscle, respectively and maintained at $37 \pm 0.5^\circ\text{C}$ during the whole experiment. Following a midline longitudinal head skin incision the skull was exposed, the head was placed manually on the base of the weight drop device

(Laboratory Tools Workshop, Department of Pharmacology, School of Pharmacy, The Hebrew University of Jerusalem, Israel). A 75 g weight was dropped from the height of 30 cm on a silicone cone resting on the exposed skull, resulting in focal brain injury to the left hemisphere. For groups 1 and 3 (sham and decompressive craniectomy alone), the same procedure was performed without weight dropping. In the CHI+DC and CHI+DC+H groups, an unilateral DC was performed 1 h after trauma as described previously (22). In brief, a bone flap was created in the parietal and temporal bone using a dental drill. The temporal bone was then removed down to the skull base and dura was opened above occipital lobe using microscissors and microforceps. Subsequently, skin was closed using 6-0 polypropylene sutures (Premilene[®], Aesculap AG).

In DC group, the same procedure was performed on non-traumatized brain/skull 1 h following sham injury.

In hypothermia group (CHI+DC+H), additional selective brain cooling was applied using a carbon dioxide-driven cryosurgery device as described in detail previously (39). For selective, controlled cooling of the traumatized area, a modified cryosurgery apparatus was used. In hypothermia group a 3 mm cooling probe with thermocoupling (Erbokryo AE, ERBE Elektromedizin GmbH) was placed on the skin covering the decompressed area and chilled to 4°C . Utmost care was taken in order to avoid compression of the underlying brain in the process of cooling. After reaching target temperature, consecutive cooling was maintained for 1 h.

After 3 h, and after assuring the adequate whole body temperature ($\geq 37^\circ\text{C}$) rectal and temporal temperature probes were removed and anesthesia was withdrawn. Animals were put back into their cages and allowed to recover including passive rewarming in an environment with controlled room temperature without additional heating devices.

Magnetic Resonance Imaging

Twenty-four hours after CHI or sham treatment, animals ($n = 5/\text{group}$) were enrolled in imaging experiments. For MRI, anesthesia was induced after placing the animals in an airtight box, by applying a 3.0/97.0% mixture of isoflurane and O₂ to the spontaneously breathing animals. Anesthesia was maintained by application of a 2.0/97.5% to 0.8/99.2% mixture of isoflurane and O₂ via a nose cone integrated into the animal frame. Respiration rates were recorded via a pneumatic cushion (Graseby infant respiration sensor, Smith Medical Germany, Grasbrunn, Germany), while cardiac rates were collected via electrodes for neonatal humans (3M Red Dot 2269T neonatal monitoring electrode, 3M Germany, Düsseldorf, Germany), both with a dedicated animal monitoring system with integrated external computer and special software (PC-SAM32, SA Instruments Inc., Stony Brook, NY, USA). Temperature was maintained at 37°C by placing the animals on a special tray with an integrated heating system.

MR images were acquired using a system developed for rodent imaging, with a static magnetic field strength of 9.4 T (Bruker BioSpec Avance III 9.4/20 with ParaVision 5.1 operating software), equipped with an actively shielded gradient (G_{max} 675 mT/m, Gradient Rise Time 114.8 μs). An actively detuned

single channel volume coil with an inner diameter of 70 mm, a maximum peak pulse power of 1,000 W and a maximum single pulse energy of 5 Ws served as transmitter (in transmit-only mode). For receiving MRI signals, an actively decoupled pretuned phased array surface coil with 2x2 elements designed for imaging of the mouse brain was placed over the skull and centered over the brain midline. After placing the animal in the isocenter of the magnet, a FLASH localizer sequence was performed (Field of View $3.84 \times 3.84 \text{ cm}^2$, Matrix Size = 256×256 , Slice Thickness 1 mm, Interslice Distance 0.5 mm, TR/TE = 100/20 ms, Number of Excitations = 2, Duration 25 s 600 ms) generating a set of five subsequent slices in axial, sagittal, and coronal orientation. The symmetry axis of the brain was identified, evaluating the position of the inner and outer parts of the ear and various lobes of the cerebellum and cerebrum. A 3D FISP sequence (Field of View $1.76 \times 1.50 \times 1.73 \text{ cm}^3$, Matrix Size = $236 \times 200 \times 23$, resulting Slice Thickness 0.75 mm, Interslice Distance 0.0 mm, TR/TE = 8.0/4.0 ms, Number of Excitations = 3, Duration 1 m 25 s 423 ms) in axial orientation was then used to verify correct positioning with symmetric imaging of the brain, and slice geometry data was loaded into standard T1 and T2 weighted MRI sequences and an Echo Planar Imaging technique.

T1 weighted imaging for morphological analysis and planning of T2 weighted and DWI experiments was performed with a

Multi Slice Multi Echo technique (Field of View $1.76 \times 1.50 \text{ cm}^2$, Matrix Size = 234×200 , Slice Thickness 0.75 mm, Interslice Distance 0.0 mm, Number of Slices = 23, TR/TE = 1,000/10 ms, Number of Excitations = 4, Duration 13 m 20 s), generating a set of images covering the whole brain.

Matching axial images for identification and quantification of possible hemorrhage were acquired with a Turbo Spin Echo (TSE) sequence (Field of View $1.76 \times 1.50 \text{ cm}^2$, Matrix Size = 234×200 , Slice Thickness 0.75 mm, Interslice Distance 0.0 mm, Number of Slices = 23, TR/TE = 2,500/30 ms, Number of Excitations = 5, Duration 5 m 12 s).

For accurate quantification of brain tissue inflicted by edema, axial diffusion-weighted echo planar imaging was performed with the following parameters: Field of View $1.92 \times 1.92 \text{ cm}^2$, Matrix Size = 192×192 , Slice Thickness 0.75 mm, Interslice Distance 0.0 mm, Number of Slices = 7, TR/TE = 2,000/18.2 ms, Number of Excitations = 1, Duration 48 s, B Values of 6.45 s mm^{-2} and 786.74, 789.19, and 789.19 s mm^{-2} in sagittal, axial, and coronal direction.

Edema and hemorrhage were identified in ADC maps calculated from the DWI data and in TSE images, respectively. Matching Regions of Interest (ROI) were manually created with the Paravision 5.1 ROI tool (example presented in **Figures 2a–d**). Resulting size measurements (in pixels and mm^2) were exported

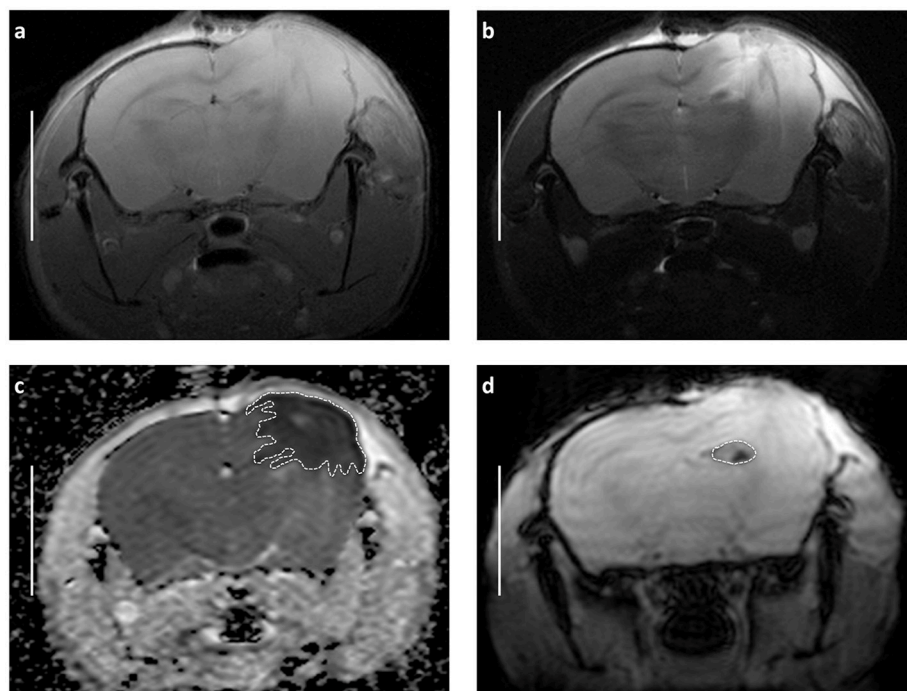


FIGURE 2 | Demonstrating main radiological features of decompressive craniectomy in closed head injury model and approach for volumetric assessment of MRI images basing on representative scans obtained 24 h after trauma and craniectomy (CHI + DC group). **(a)** T1-weighted image **(b)** T2-weighted image **(c)** Apparent diffusion coefficient (ADC) weighted map. The dashed line defines the area of edema as marked manually by an independent observer. The area measurement on series of slices was followed by calculating of edema volume by defined slice thickness. Note the size and character of swollen region (marked hypointensive area underlying the craniectomy window). These features suggest domination of cytotoxic brain edema involving vast cortical areas after surgical decompression. **(d)** The same MRI layer presented in T-RARE sequence. Here, the area of hemorrhage is outlined by dashed line. Again, the calculation of contusion volume is performed based on the areas of contusion on single slides and on the slide thickness. Bar = 5 mm.

via a specially adapted macro, and the total volume of the different lesions was calculated from the areas on the single maps and the thickness of the scan slices after importing the data into Microsoft Excel 2003[®] for Windows XP[®] and thereafter into GraphPad Prism[®] 5.0 for further analysis (see Statistics).

Biochemical Analysis (ELISA)

Twenty-four hours after trauma or sham injury, animals were sacrificed using *in situ* freezing with liquid nitrogen while under inhalative anesthesia (sublethal concentration of isoflurane 3.5–4% in O₂). Snap frozen brains were dissected from surrounding tissue and brain stem and cerebellum were discarded. Thereafter cerebrum was dissected and the region of interest (ROI) was separated from the remaining brain tissue (ROI was defined as the brain tissue located $-0.1/+0.1$ cm from the point of maximal injury (virtual in trauma groups or hypothetical in experimental groups without trauma), seen in the brain coronal slice presenting CA1 and CA3 hippocampal areas). For this purpose, frozen brain specimens were cut in the coronal plane using gross section setting of cryotome (Leica, working temperature: -20°C , slice thickness $50\text{ }\mu\text{m}$). Between four gross sections, one regular thin slice ($12\text{ }\mu\text{m}$) was obtained, stained with haematoxylin-eosin and analyzed under light microscope (Olympus, magnification 40x and 100x) for comparison with stereotactic mouse brain atlas (94) in order to confirm the proper cutting plane and ensure anatomical reference for ROI. The gross sections were diligently collected, and parts representing ipsi- and contralateral hemisphere were separated. In that manner, four separate samples from each animal brain ($n = 5/\text{group}$), as referring to the site and distance from epicenter of (hypothetical) injury (ipsi- vs. contralateral x ROI vs. remnant tissue) were obtained. Thereafter, specimens were stored at -80°C until final processing. For analysis of AQP-4 level in brain tissue, ELISA method was used. Frozen samples were lyophilized overnight. Dried tissue was then homogenized (FastPrep24, MP Biomedical) and resuspended in 1:10 PBS (DPBS, Dulbecco). Protein concentration was measured (Quant-It Assay, LifeTechnologies) and concentrations adjusted to 20 mg/mL. Aquaporin-4 concentrations were measured in a 10-fold dilution with a mouse Aquaporin 4-ELISA Kit (Hoezel Diagnostika, Germany; Reference number 90582Mu) according to the manufacturer's protocol. All samples were measured in duplicate on a Genios (Tecan, Germany) plate reader at 450 nm and concentrations were calculated with the Magellan software (Tecan, Germany).

Histological Analysis (Immunohistochemistry)

In order to gain more detailed information about spatial distribution and time course of AQP4 expression, additional subset of animals has been used and histological analysis of AQP4 immunoreactivity was performed 28d after initial treatment (see also **Supplementary Materials and Methods**).

Statistical Analysis

Values of AQP4 concentration were recorded as pg/mL (of origin homogenizate). For each experimental animal, data set

of four values has been obtained (AQP4 concentration in: 1. ipsilateral ROI; 2. ipsilateral remote area; 3. contralateral ROI; 4. contralateral remote area). Aggregating of data matched according to anatomical descriptors (lateralization: ipsi- vs. contralateral; longitudinal proximity: ROI vs. remote area of the brain) was performed and assessed supplementary to the analysis of the distinct data sets (values matched according to both anatomical descriptors). To avoid a pseudo replication bias, the aggregated data have been averaged, so that the one animal contributed only one value to the analysis. Both separate and aggregated parameters were expressed as mean $\pm$ SEM for each experimental group. For assuring the Gaussian distribution character of sampled data, Shapiro-Wilk test retrieving *p*-value as validation of normality for single group was performed. For data sets with confirmed Gaussian distribution of values, one-way ANOVA was implemented, otherwise Kruskal-Wallis test followed by Dunn's multiple comparison test was used for single analysis step.

To analyze the correlation between size of structural damage (volume of edema or volume of contusion) and between biochemical marker (tissue level of AQP4), the matching data from the single animals were analyzed (zero-value outliers of sham groups being excluded) according to Pearson correlation coefficient and a subsequent linear regression analysis method was performed.

For all parts of assessment (analysis of variance, correlation analysis), significance was set at $p < 0.05$ and statistical software GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com as well as IBM SPSS Statistics for Windows, Version 22.0 IBM Corp. Released 2013. Armonk, NY: IBM Corp. was used. In order to verify the validity of the analysis and to assess the risk of type II error in small cohort study, G*Power software, Version 3.1.9.2. was used for *post-hoc* assessment of the statistic power for both ANOVA and correlation analysis (95, 96).

RESULTS

AQP4 Concentration

Analysis of AQP4 concentration revealed no significant difference between experimental groups / areas, if concentrations were calculated separately for ROI vs. remaining tissue in ipsi- vs. contralateral hemisphere (**Figure 3**).

However, analysis of aggregated values of AQP4 concentration (mean $\pm$ SEM) presented a statistically significant increase in AQP4 level in animals subjected to decompressive craniectomy after trauma compared to decompressive craniectomy alone (DC: 32.8 ± 2.4 pg/mL vs. CHI + DC: 52.1 ± 5.2 pg/mL, $p = 0.01$; *). Notably, if additional hypothermia after surgical decompression was applied, this effect could not be documented (DC vs. CHI + DC + H: 45.3 ± 5.6 pg/mL, $p > 0.05$; ns) (**Figure 4**), although direct comparison between the groups CHI + DC vs. CHI + DC + H presented no statistical significance ($p > 0.05$; ns); (*post-hoc* statistical power analysis for ANOVA: power of 0.98 by effect size $f = 1.01$).

More detailed analysis revealed, that the effect of decompressive craniectomy, increasing AQP4 level in global

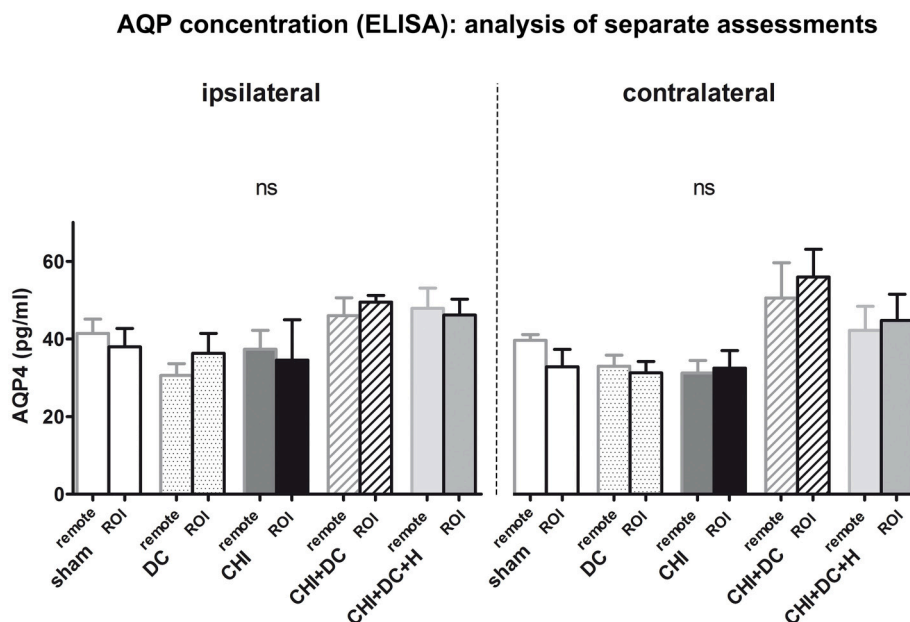


FIGURE 3 | Histogram showing concentration of AQP4 24 h after trauma/sham injury according to ELISA assessment. The graph represents separate analysis of data i.e., the values are discriminated as to the localization of the sample (ROI vs. remote parts of the brain and ipsi- vs. contralateral hemisphere). Both the response to injury (AQP4 level change) and the differences between single treatment groups seem to be more prominent in contralateral hemisphere (containing more viable tissue), although according to the ANOVA analysis, no statistically significant differences can be registered (ns, $p > 0.05$ for comparison of groups, matched in distance or laterality in regard to injury epicenter). CHI, closed head injury; DC, decompressive craniectomy; H, hypothermia; ROI, region of interest.

aggregated calculation, resulted from an increase of AQP4 level in non-traumatized hemispheres, since the difference between decompressive craniectomy animals (DC) and CHI+DC group as well as between trauma animals and decompressive craniectomy group was statistically significant in aggregated analysis of AQP4 concentration parameters in contralateral but not in ipsilateral hemispheres (for ipsi: DC: 33.4 ± 3.1 pg/mL, vs. CHI + DC: 48.5 ± 2.6 pg/mL, $p > 0.05$; ns; CHI: 35.9 ± 6.1 pg/mL vs. CHI + DC, $p > 0.05$; ns); (*post-hoc* statistical power analysis for ANOVA: power of 0.70 by effect size $f = 0.65$); for contra: DC: 32.1 ± 2.3 pg/mL vs. CHI + DC: 55.8 ± 7.8 pg/mL, $p < 0.01$; **, CHI: 31.8 ± 2.8 pg/mL vs. CHI + DC $p < 0.01$; **); (*post-hoc* statistical power analysis for ANOVA: power of 0.97 by effect size $f = 0.97$); (Figures 5A,B).

Correlation of AQP4 Concentration With Radiological Sequelae of TBI

The results of volumetric analysis of edema and contusion [as described previously by our group (39) and demonstrated by Figure 1] provided one of the variables for subsequent correlation analysis. As second variable, results of ELISA AQP4 assessment were adapted.

A linear correlation analysis demonstrated a correlation between edema volume measured in ipsilateral hemisphere and between concentration of AQP4 assessed in the ROI contralateral to the injury site ($r^2 = 0.45$; $p = 0.002$; **); (*post-hoc* statistical power analysis for correlation: power of 0.98 by effect size $\rho = 0.67$); (Figure 6). Also, contusion volume was correlated

with the AQP4 level in the corresponding region (ROI) of the contralateral hemisphere ($r^2 = 0.41$; $p = 0.004$; **); (*post-hoc* statistical power analysis for correlation: power of 0.97 by effect size $\rho = 0.64$); (Figure 7).

Histological Analysis

Qualitative analysis of histopathological material yielded observation similar to previous anatomical description of AQP4 immunoreactivity in mice (97, 98) without any statistical difference between groups (see also Supplementary Results and Figure S1).

DISCUSSION

In our previous experiments on the effects of decompressive craniectomy and hypothermia in a murine CHI model, we were able to demonstrate an increase of brain edema formation and neurological impairment due to the combined effect of mechanical trauma and surgical decompression. We also observed a mitigation of this deleterious effect of surgical decompression by consequent focal cooling of the traumatized brain area via the created craniectomy window (20, 22, 39). Our current results demonstrate the potential molecular background of these phenomena: In the same set of experimental animals these processes are associated with the change in AQP4 level affecting remote brain areas rather than trauma epicenter at the analyzed time point 24 h posttrauma.

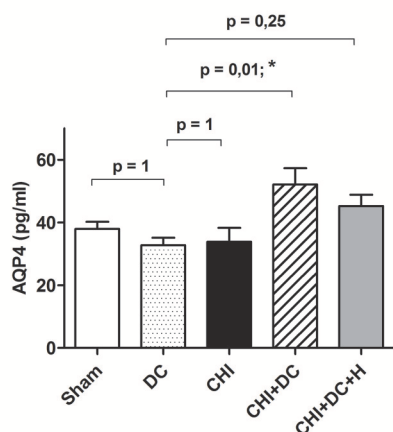
AQP4 concentration (ELISA): global averaged analysis

FIGURE 4 | Histogram showing concentration of AQP4 24 h after trauma/sham injury according to ELISA assessment. The graph represents analysis of global pooled data i.e., the values obtained separately at the level of ROI (ipsi- and contralateral) as well as outside the ROI (ipsi- and contralateral) have been pooled and averaged. According to the ANOVA analysis, the global AQP4 level was significantly higher in the animals subjected to trauma and craniectomy as compared to craniectomy-only group, serving as reference group (CHI+DC vs. DC, $p = 0.01$, *). This effect vanished, when additional hypothermia has been applied (CHI+DC+H vs. DC, ns, $p > 0.05$). CHI, closed head injury; DC, decompressive craniectomy; H, hypothermia; ROI, region of interest.

The main result of our current analysis is the increase of AQP4 concentration in animals subjected to both trauma and decompressive craniectomy. That is completely opposing the observation of Tomura et al. who reported significant increase of AQP4 expression level affecting at 48 h only animals not subjected to surgical decompression, while the use of decompressive craniectomy reduced both brain water content and AQP4 protein expression in rat model of fluid percussion injury (FPI) (16). To explain this discrepancy, the differences between animal TBI models with regards to their pathophysiological characteristics should be discussed in the light of general principles of brain edema formation (as presented in Introduction). First, FPI model is characterized by diffuse injury pattern (reaching brain stem or even cerebellum) (99–102). In contrast, trauma-decompression model used in our study represents a rather focal injury, where trauma epicenter (including contusional changes) evokes pathophysiological response of the surrounding tissue (22, 39). Second, in FPI model, presence of vasogenic brain edema already at early stages of the posttraumatic course have been described (99–102). Opposite, in CHI the initial posttraumatic edema is predominantly cytotoxic, as presented in previous reports (57–60) as well as in former radiological assessments performed by our group (22). As learned from the AQP4 knockout animal experiments (69, 70), the molecular response by AQP4 expression in posttraumatic brain edema depends on the predominant edema form (vasogenic vs. cytotoxic) and this rule is to be extrapolated into traumatic

brain injury models. An overview of selected studies on AQP4 implementing different models of experimental TBI is demonstrated in Table 1.

In studies using controlled cortical impact model (characterized by predominant cytotoxic edema formation) (48, 110, 111) decreased posttraumatic AQP4 level in brain tissue has been revealed (104, 105, 108) although Taya and associates could report an increase in AQP4 expression in the early stages after CCI (71). Moreover, interference with AQP4 expression (107) or function (84, 86, 87, 105) by pharmacological intervention resulted in decreased posttraumatic brain edema formation and improved neurological outcome in studies using CCI trauma model. In contrast, in fluid percussion injury model, where vasogenic mechanism of swelling plays an important role (53, 56) due to BBB breakdown (112–114), trauma resulted in a surge of AQP4 concentration (16, 106) and/or in increased AQP4 gene expression (109).

In the light of this data on relationship between AQP4 function and form of brain edema, a certain mismatch in our set of results needs to be admitted: The previous description of CHI model, as well as our own radiological results suggests that cytotoxic edema prevails in our experimental setting. At the same time, the observed changes in AQP4 level follow the pattern characteristic of vasogenic edema type, at least in the trauma + decompressive craniectomy group, as characterized by raise in AQP4 concentration. In detail: According to primary description, CHI model is characterized by the domination of cytotoxic edema, at least in the early phase (1–24 h after injury). This has been presented in previous MRI studies performed in experiments using CHI (57–60). Also our own observation, showing predominance of hypointense areas of ADC maps (to be identified as regions of cytotoxic edema) is sound with the previous evidence (22). On the other hand, the observed pattern of AQP4 changes (especially correlation analysis) suggests participation of vasogenic edema in early sequence of events following surgical decompression. In particular, previous publications describing stroke-related brain swelling, were able to report a negative correlation of edema volume and AQP4 level or expression in the phase of cytotoxic edema formation, while the development of vasogenic edema was closely linked with AQP4 increase, the latter seen also in our analysis (77, 80, 81). Hypothetically, a raise in AQP4 concentration in vasogenic edema formation may represent the attempt of functional brain tissue to counteract the rapid increase of extracellular fluid (115). This role of AQP4 is confirmed by previous experiments analyzing inflammatory edema (115) which shares some characteristics with perifocal edema accompanying intracerebral hematoma (115, 116). Notably, in the presented trauma-decompression model, a substantial amount of tissue injury results from hemorrhagic transformation of the traumatized cortex with secondary perifocal edema formation (22, 39). A similar involvement of AQP4 has also been documented in edema formation surrounding the clot in animal models of intracerebral hemorrhage (65, 116–118) with spontaneous and traumatic hematomas sharing main features of perifocal brain swelling (119, 120). These previous observations match our current

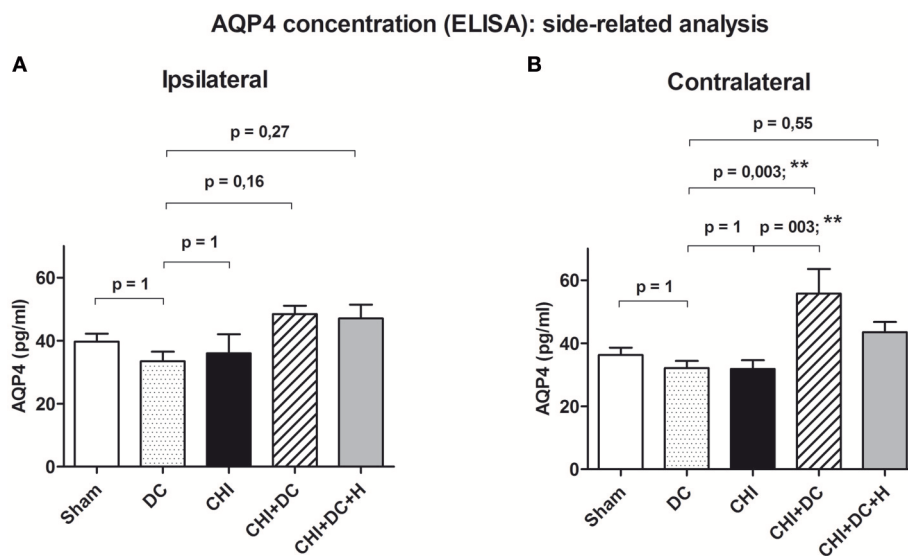


FIGURE 5 | Histogram presenting concentration of AQP4 24 h after trauma/sham injury according to ELISA assessment. This part of analysis involves the data pooled and averaged separately for the contralateral hemisphere (at the level of ROI plus outside the ROI) and for the ipsilateral one (again, at the level of ROI plus outside the ROI). **(A)** According to ANOVA, the differences between AQP4 level values, averaged for ipsilateral hemispheres were not significantly different between the treatment groups. **(B)** Analogous analysis in contralateral hemispheres revealed significant raise in AQP4 concentration in animals subjected to trauma and decompressive craniectomy as compared to non-traumatized reference group as well as to trauma-only group (CHI+DC vs. DC, $p < 0.01$, **; CHI+DC vs. CHI, $p < 0.01$, **). Similar to global pooled analysis, this effect could not be seen in group where hypothermia was added to the treatment (CHI+DC+H vs. DC, $p > 0.05$, ns). CHI, closed head injury; DC, decompressive craniectomy; H, hypothermia; ROI, region of interest.

Correlation analysis
AQP4 concentration in Region of Interest vs. volume of edema

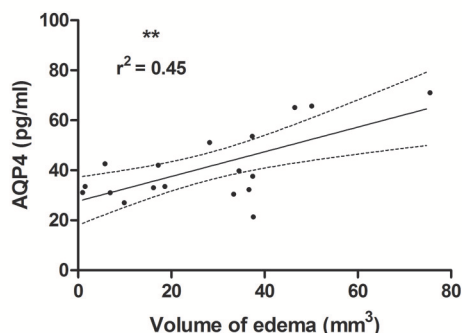


FIGURE 6 | Histogram presenting analysis of correlation between volume of edema 24 h after trauma/sham injury and AQP concentration, assessed in contralateral hemisphere at the level of injury (ROI contralateral). There was a correlation between increased volume of brain edema and elevated concentration of AQP4 according to ELISA ($r^2 = 0.45$; $p = 0.003$; **).

Correlation analysis
AQP4 concentration in Region of Interest vs. volume of contusion

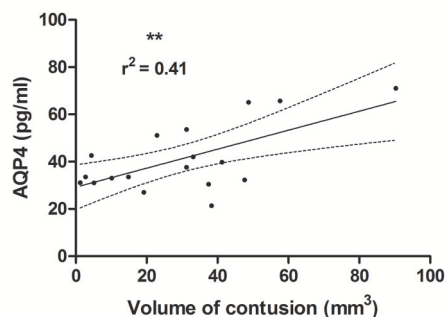


FIGURE 7 | Histogram presenting analysis of correlation between volume of contusion 24 h after trauma/sham injury and AQP concentration, assessed in contralateral hemisphere at the level of injury (ROI contralateral). Similar to analysis of edema size, a correlation between increased volume of contusion and elevated concentration of AQP4 according to ELISA ($r^2 = 0.41$; $p = 0.004$; **) could be demonstrated.

analysis documenting a close correlation between AQP4 level and the size of contusional changes.

Certainly, the method of radiological analysis (MRI ADC mapping) provides only restricted information about edema type. However, not only the character of AQP4 reactive changes allows us to speculate that vasogenic edema was

involved in pathophysiology of brain edema after decompressive craniectomy in our model. One mechanistic link is the character of pressure changes usually following surgical decompression. If the raised intracranial pressure is relieved by surgical opening of the cranial vault, the pressure gradient between blood vessels and brain parenchyma rapidly changes (21), possibly

promoting vasogenic edema formation due to hydrostatic driving force (121, 122). Also changes in cerebral perfusion associated with deranged cerebrovascular autoregulation as seen in post-craniectomy patients (123–125) may lead to hyperemia and thus, to the enhanced vasogenic brain edema formation (19, 46). Notably hyperperfusion could be well correlated with the degree of brain edema amount measured on CT scans in clinical settings (126). Possibly this aspect provides a valid explanation for above mentioned discrepancy between our results and those provided by Tomura et al. In our model (other than in the study of Tomura and associates), extensive brain edema development after decompression was not restricted by meticulous control of blood pressure and subsequent hydrostatic gradient (16), leading to early, massive brain swelling (demonstrated by increased water content and external brain herniation, as previously reported) (20, 22). Possibly this uncontrolled brain edema development seen already 6 h posttrauma (20) lead to impaired neurological recovery and increased neuronal loss (22). These observations vary from reports of other groups reporting neuroprotective effects of experimental surgical decompression after TBI (14, 15, 127). Significant differences of the TBI model used (diffuse vs. focal trauma pattern) as well as differing injury severity may be quoted as reasons for the diverse conclusions of animal studies on decompressive craniectomy.

Another important conclusion from our results is that the influence on AQP4 concentration caused by treatment modality differs according to the traumatized brain region [albeit one cannot exclude a bias, relying on difference in ANOVA power, as calculated *post-hoc* for analysis of contra- and ipsilateral hemispheres (128, 129)]. The laterality of posttraumatic changes in AQP4-level deserves particular attention. The different impact of head injury on AQP expression ipsi- and contralateral to trauma site has been analyzed in previous animal studies. In a rat TBI weight drop model, reduced AQP4 mRNA expression has been reported in the ipsilateral but not in the contralateral hemisphere (103). Also following controlled cortical impact in the rat, intensity and time course of AQP4 concentration differed between ipsi- and contralateral hemispheres and a decrease of AQP level in the lesion core parallel with an increase in the penumbra zone was described (104). Furthermore, the problem of inhomogenous AQP4 expression across the traumatized brain has been closely approached in several studies. A clear difference between trauma epicenter (AQP4 reduction) and penumbra zone (AQP4 increase) could have been stated (103, 105, 130). A decrease in AQP4 level, that has been attributed to the necrotic transformation of the core of contused area (105, 131) fits well to our observation: As reported previously, a vast area of ipsilateral cortex in our model was affected by necrotic changes and hemorrhagic contusion, most prominent in the trauma + craniectomy group (22). The following upregulation of AQP4 level may be blunted by the severe loss of AQP4 expressing cells, which is more abundant in the ipsilateral hemisphere, while the contralateral viable tissue, remote to the injury epicenter is able to execute this compensatory mechanism in less restricted way. This hypothesis is further supported by studies targeted strictly on the contralateral brain tissue, since early (<24 h) AQP4

overexpression in a rat model of severe TBI could be seen in the cortex contralateral to injury site (132). Also Zhang and associates describe the difference between ipsi- and contralateral hemispheres considering molecular response and emphasize delayed dynamics of AQP4 peak in areas contralateral to injury (75).

Finally, we would like to discuss the influence of focal brain cooling on AQP4 expression pattern. According to our results, we suggest that hypothermia potentially ameliorates the posttraumatic edema course, which reflects in reactive changes of AQP expression. Several analyzes previously investigated the impact of hypothermia on AQP4 expression / function. Results of cell culture studies were not conclusive: Fujita et al. reported a reduction of AQP4 expression in cultured astrocytes subjected to temporary hypoxia, followed by a secondary raise in AQP4 mRNA expression when subjected to mild hypothermia. In the normothermic condition, induced AQP4 depletion was sustained (133). In an astrocyte culture setting of Lo Pizzo et al. hypothermia led to a reduced AQP4 level (133). In contrast, Salman et al. report an increased presence of membrane AQP4 channels in cortical astrocytic cultures under hypothermic condition without a change in global AQP4 protein expression (134). However, cell culture models do not necessarily recollect intricacy and dynamics of AQP4-related posttraumatic changes in the mammalian brain. Therefore, several groups analyzed the influence of hypothermia on AQP4 expression/function *in vivo*, using rodent models of hypoxia / ischemia. In these models, AQP4 level increase caused by ischemia (135) or ischemia-reperfusion injury (136) could be attenuated by reducing cerebral temperature, albeit this effect seemed to be strongly dependent on rewarming conditions (137). Results, most relevant for our current analysis could have been provided by Gao et al. (138). In this work, focal brain cooling reduced both AQP4 expression and concentration following a stereotactic injection of thrombin, simulating deep intracerebral hematoma in rat. This observation suits well our results, considering the fact that the protective effect of hypothermia is mediated by a limitation of contusional changes (39, 42) and that contusion volume correlates well with AQP4 level in our current analysis. Thus, selective brain hypothermia may reduce not only the size of contusional hematoma but may also diminish its negative impact on surrounding tissue. Several mechanisms of action need to be discussed. First, direct impact of reduced temperature on the AQP4 gene expression in affected cells may be postulated (138). This however does not explain the remote influence of focal hypothermia. This effect relies rather on modulation of inflammatory mechanisms postulated by Kurisu et al. (136): It has been proven, that both localized hematoma and focal injury may trigger the inflammatory reaction and blood brain barrier (BBB) disturbance even in distant parts of the brain (139–141). Thus, the remote effect of injury may be limited, if the course of focal events (inflammation or BBB-breakdown) is arrested by hypothermia (142). Nevertheless, this hypothesis implies that the observed changes of AQP4 level need to be interpreted as only secondary indicator of injury severity. Regardless the underlying mechanism, our study is (according to our best knowledge) the first report to describe the effect of focal cooling

TABLE 1 | Summary of reported AQP4-related changes in different animal models of TBI.

Study	Animal	Trauma model	Additional intervention	AQP4 changes	Method of assessment
Ke et al. (103)	Rat	Weight drop	None	↓	RT-PCR AQP4- mRNA
Kiening et al. (104)	Rat	CCI	None	↓	WB
Zhao et al. (105)	Rat	CCI	TBI + Sulforaphane (vs. TBI)	↓ ↑	IF
Taya et al. (71)	Rat	CCI	None	↑	WB
Tomura et al. (16)	Rat	FPI	TBI + Decompressive craniectomy (vs. TBI)	↑ ↓	IC
Quintard et al. (106)		FPI	TBI + MLC901 (vs. TBI)	↔ (7d) ↑ (7d)	IF, WB
Fukuda et al., (107)	Rat	CCI	None	↑	IF
Gatto et al. (84)	Rat	CCI	TBI + rhEPO (vs. TBI)	↑ ↓	WB
Zhang et al. (75)	Rat	CCI	None	↑ (>24 h)	WB
Blixt et al. (108)	Rat	CCI/Weight drop	None	↓	IF/WB
Jin et al. (109)	Rat	FPI	TBI + Levetiracetam (vs. TBI)	↑ ↓	WB, RT-PCR AQP-mRNA /cDNA
Zhang et al. (86)	Rat	CCI	TBI + Astaxanthin (vs. TBI)	↑ ↓	RT-PCR AQP-mRNA, WB
Blixt et al. (87)	Rat	CCI/Weight drop	TBI + Erythropoietin (vs. TBI)	↓ ↑	IF
Szczygielski et al. (this paper)	Mouse	CHI	TBI + Decompressive craniectomy (vs. TBI)	↔ ↑	ELISA

Description of animal models: CCI, controlled cortical impact; CHI, closed head injury; FPI, fluid percussion injury. Description of assessment methods: cDNA, complementary deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; IC, immunohistochemistry; IF, immunofluorescence; mRNA, messenger ribonucleic acid; RT-PCR, real-time polymerase chain reaction; WB, Western blot.

of the traumatic lesion core on AQP4 level in an experimental setting.

Another advantage of our study we would like to highlight is the animal species used. We deliberately focused on a murine model of closed head injury model as the basis for our craniectomy experiments, even if performing this procedure in small rodents requires experimenters' particular manual dexterity. This effort is gratified by the future possibility to convey this experimental design into genetically modified animal models, usually being mouse breed. This step could be of particular value for the further development of AQP4 targeted treatment strategies, as has been stressed by Yao et al. In this work, drugs or maneuvers applied following TBI in order to alter AQP4 expression have been judged critically as since AQP4 knockout mice subjected to CCI displayed only a mild improvement of neurological function and lesion volume compared to wildtype littermates as well as only a transient beneficial effect of AQP4 knockout on brain water content (74). Again, the above-mentioned heterogeneous character of cerebral edema formation following TBI as well as the kinetics of cytotoxic and vasogenic edema contribution over the posttraumatic course have to be considered. A premature extrapolation of experimental results into clinical context may be counter-productive, if the predominantly underlying edema subtype in the considered posttraumatic phase is not respected (74). Thus, the role of AQP4 in the different stages of posttraumatic course should be analyzed in animal models tightly resembling clinical setting. The advantage of our experimental

paradigm is the sequence of moderate to severe TBI followed by early craniectomy performed on non-trephined skull, similar to clinical scenario, including trauma severity warranting indication for surgical decompression (22). By performing decompression on an intact skull, the potentially confounding disadvantage of skull trepanation prior to injury can be avoided (143–146). In order to respect the above-mentioned “true sham” effect in experiments requiring skull trepanation, we deliberately chose the decompression-without-trauma (DC) group as primary reference group for statistical evaluation. In our previous studies, we were able to validate this strategy (22, 39), again showing most profound changes in the group subjected to both trauma and subsequent craniectomy.

Certainly, our study is not free of several drawbacks. First, the analysis is limited to the time point 24h postinjury, as predetermined by the setup of our previous experiments (22, 39). According to previous reports, focused on the radiological time course of brain edema development, the early course of posttraumatic changes is characterized by cytotoxic edema type, while vasogenic swelling peaks not before 3 days after primary insult (52, 102, 115, 116). According to this, current preliminary analysis possibly does not display the full diversity of brain edema formation under different combinations of treatment. Certainly, we have tried to compensate this gap by presenting the preliminary data from long-term analysis, implementing AQP4 immunohistochemistry staining (see **Supplementary Data**). However, the results reported here did not reveal any significant difference between treatment

groups. This observation is in concordance with Fukuda et al. who demonstrated mitigated brain edema formation and reduced AQP4 expression (due to administration of small interfering RNA) 3 days after trauma, but without any change in AQP4 immunoreactivity as assessed 60 days post injury (107). Second, since the current results are based on offshoot analysis of brain tissue material obtained previously, the choice of method for AQP4 analysis was limited. Certainly, immunostaining method or microdissection of the anatomical structures would provide more detailed information about spatial AQP4 expression. However, even using our microtome-based, rough method for separation of different areas of the traumatized brain we were able to demonstrate inhomogeneity in molecular AQP4-based response at single time point of edema build-up phase. Finally, as predefined by the setup of the source experiment, current biochemical analysis could be performed in very limited number of animals. This is the possible cause of inhomogeneous statistical power across single analysis steps (ANOVA for ipsilateral AQP4 level with power < 0.8, while in other reported parts of analysis the risk of the type II error was quite low with the *post-hoc* power value of 0.97–0.98). The low number of animals resulted also probably in differences between single treatment groups becoming apparent first after aggregating of AQP4 concentration values. This, however, is in concordance with the previous observations, reporting no significant effect of trauma on AQP4 expression up to 48 h post injury (16). Also Yao et al. analyzing the impact of AQP4 knockout after TBI stated the influence of AQP4 depletion on posttraumatic course to be minimal (74). This leads to expect only a scarce difference in AQP4 level, which justifies form of analysis (aggregation of the single data). Nevertheless, we overcame the temptation of plainly multiplying the data set and analyzing repeated measures as independent values. Instead, we chose to average the aggregated data, which is a simple, yet effective method to reduce the flaw resulting from pseudoreplication of individual records (147).

In conclusion, the increase of brain edema formation following decompressive craniectomy in a murine model of severe CHI is accompanied by an increase in AQP4 level. This elevation seems to be reactive and most probably represents an attempt to resolve extracellular water, possibly resulting from a disturbed hydrostatic gradient following mechanical decompression. Due to the focal injury character caused by the weight drop model, the molecular changes differ across the various regions of traumatized brain. Nevertheless, the extent of this posttraumatic response seems to be governed by the core lesion volume. Due to our results, selective application of focal hypothermia at the injury epicenter is associated with less prominent AQP4

response even in remote areas of the brain. Certainly, this effect may be secondary. However, basing on our preliminary animal experiments we recommend further analysis of this phenomenon in order to explore the therapeutic potential of i.e., pharmacological influence on AQP4 expression/function as treatment strategy supplementary to decompressive craniectomy and/or hypothermia.

In spite of these promising data, therapeutic implications of the AQP4-modulating effect on cerebral swelling should at this stage be taken with caution, since our results were provided using a limited number of experimental animals. More importantly, cerebral edema formation occurring after trauma and subsequent decompressive craniectomy (both in animal models and in clinical settings) is apparently represented by a dynamic mixture of cytotoxic and vasogenic brain edema with a beneficial or deleterious property of AQP4 strongly depending on ratio of these two constituents.

AUTHOR CONTRIBUTIONS

JS, K-HS, AEM, KS, and JO contributed conception and design of the study. JS, CG, CS, and VH conducted the animal experiments. JS, CG, MK, CS, and K-HS performed the biochemical analysis. AM and VH performed the radiological assessment. JS, AM, MK, and K-HS performed the statistical analysis. JS wrote the first draft of the manuscript. AM and VH wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fneur.2018.00799/full#supplementary-material>

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Transplantation of Embryonic Neural Stem Cells and Differentiated Cells in a Controlled Cortical Impact (CCI) Model of Adult Mouse Somatosensory Cortex

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Traumatic brain injury (TBI) is a major cause of death worldwide. Depending on the severity of the injury, TBI can reflect a broad range of consequences such as speech impairment, memory disturbances, and premature death. In this study, embryonic neural stem cells (ENSC) were isolated from E14 mouse embryos and cultured to produce neurospheres which were induced to generate differentiated cells (DC). As a cell replacement treatment option, we aimed to transplant ENSC or DC into the adult injured C57BL/6 mouse cortex controlled cortical impact (CCI) model, 7 days post-trauma, in comparison to saline injection (control). The effect of grafted cells on neuroinflammation and neurogenesis was investigated at 1 and 4 weeks post-transplantation. Results showed that microglia were activated following mild CCI, but not enhanced after engraftment of ENSC or DC. Indeed, ipsilateral lesioned somatosensory area expressed high levels of Iba-1+ microglia within the different groups after 1 and 4 weeks. On the other hand, treatment with ENSC or DC demonstrated a significant reduction in astrogliosis. The levels of GFAP expressing astrocytes started decreasing early (1 week) in the ENSC group and then were similarly low at 4 weeks in both ENSC and DC. Moreover, neurogenesis was significantly enhanced in ENSC and DC groups. Indeed, a significant increase in the number of DCX expressing progenitor cells was observed at 1 week in the ENSC group, and in DC and ENSC groups at 4 weeks. Furthermore, the number of mature neuronal cells (NeuN+) significantly increased in DC group at 4 weeks whereas they decreased in ENSC group at 1 week. Therefore, injection of ENSC or DC post-CCI caused decreased astrogliosis and suggested an increased neurogenesis via inducing neural progenitor proliferation and expression rather than neuronal maturation. Thus, ENSC may play a role in replacing lost cells and brain repair following TBI by improving neurogenesis and reducing neuroinflammation, reflecting an optimal environment for transplanted and newly born cells.

Keywords: TBI, NSC, neurosphere, astrocyte, neuron, lesion, transplantation, cortex

INTRODUCTION

The complexity of the cerebral cortex in terms of cell specificity and projections is revealed by the difficulty to repair damaged areas caused by injuries or diseases within the central nervous system (CNS). Indeed, the loss of cortical neurons is a common characteristic to many neuropathological conditions such as lesions (trauma, stroke) or neurodegenerative diseases (amyotrophic lateral sclerosis, Huntington's disease) and is associated with irreversible functional deficits and cell death (1, 2). Traumatic brain injury (TBI) is a worldwide silent epidemic problem leading to the disability and sometimes the death of patients (3). Annually, one out of 200 people suffers from TBI (4) and an estimated 1.5 million people lose their lives due to TBI (5). Mild TBI, which accounts for ~75% of all head injuries (6), is a concussion that causes neurobiological modifications in the CNS (7), short-term confusion, cognitive impairments, and transient unconsciousness (8). According to pathology, TBI causes primary injury due to mechanical forces at the time of the incident followed by a secondary injury. The latter is characterized by cytotoxicity, cerebral edema, oxidative stress, apoptosis, mitochondrial dysfunction, and inflammation (9–11). After brain injury, the blood-brain barrier (BBB) is disrupted allowing the migration of immune cells to the site of injury associated with long-lasting neuronal dysfunction and cell death (12).

Many studies have shown spontaneous functional recovery after TBI (13); however, it remains limited (14). Animal models mimicking patients TBI have been established to better understand TBI pathophysiology and to develop potential treatments (15–19). Several preclinical studies have targeted secondary injury events in animal TBI models to test the therapeutic efficacy of drugs such as calcium channel blockers, inhibitors of lipid peroxidation, excitatory amino acid inhibitors and others (20). Except for few limited treatments relieving specific symptoms, especially in acute animal models, available TBI therapies were either ineffective on patients or didn't reach clinical trials (21–24). The role of stem cells in tissue reconstruction, neuroprotection and trophic support to the host tissue makes them a potential therapeutic option (25). Recently, neural transplantation as a prospective therapy for TBI has emerged using mesenchymal stem cells (MSCs) (26), neural stem cells (NSCs) (27–30), induced pluripotent stem cells (iPSCs) (31) and embryonic neural stem cells (ENSCs) (32).

In response to TBI, the capacities of axonal regrowth and spontaneous regeneration of the CNS becomes limited. ENSCs are the optimal cell source for neural transplantation owing to their unlimited capacity of self-renewal and plasticity. When transplanted into damaged brain areas, these cells are capable of differentiation, migration, and innervation (32). A recent study confirmed that transplantation of pre-differentiated human

ENSCs increased angiogenesis and reduced astrogliosis along with improving long-term motor function (33, 34). Another study established that transplantation of differentiated human ENSCs into severely injured controlled cortical impact (CCI) rat brains resulted in neuronal survival at the lesioned area, reduction in astrogliosis and enhancement in angiogenesis (33). Moreover, it was shown that transplantation of embryonic cortical neurons, immediately after injury in the adult motor cortex, allows the anatomical reconstruction of injured motor pathways and the development of projections to appropriate cortical and subcortical host targets (35, 36). Furthermore, it was demonstrated that cortical progenitors are capable to develop into specific neuronal phenotypes and to express specific transcription factors according to their developmental program (37). Neuronal transplantation appears as a promising therapeutic strategy to replace neurons and damaged pathways. Its effectiveness depends on the capacity of grafted cells to develop appropriate cellular populations and to integrate into host preexisting neuronal circuitry. Understanding the spatiotemporal development of different cell populations within the graft, and their axonal outgrowth, is of crucial importance.

In fact, TBI can lead to both sensorimotor and cognitive deficits (38). It could be hypothesized that many of the deficits observed after TBI, whether cognitive or sensory, are aggravated by damages in sensory processing (39). Multiple studies used different models of trauma or ischemia in the somatosensory cortex and aimed to determine the reproducibility of these models for behavioral and functional consequences after the injury (40, 41). In the present study, we aimed to establish a mCCI model targeting the primary somatosensory cortical area followed by studying the consequences of transplantation of embryonic neural stem cells (ENSCs) or differentiated cells (DCs) on inflammation and neurogenesis.

In this study, following mCCI, we hypothesized that cell therapy by transplantation of ENSCs or differentiated cells (DCs) can reduce neuroinflammation and promote neurogenesis in the adult injured cortex. Therefore, a mCCI was performed in the cortical somatosensory area of adult C57BL/6 mice followed by transplantation, 7 days post-injury, with either an injection of ENSCs, DCs, or saline. Using specific markers, the capacity of transplanted cells to improve injured cortical area, replace lost cells and decrease neuroinflammation was then investigated after CCI. Furthermore, a time-course analysis was performed at 1 and 4 weeks post-transplantation comparing the response to the transplantation of ENSCs and DCs after mCCI.

METHODS

Animals

This study was approved by the Institutional Animal Care and Utilization Committee (IACUC) of the American University of Beirut (AUB). All animal experimental procedures and housing were carried out in accordance with the guidelines of the Agriculture Ministry and the European Communities Council Directive (2010/63/EU). All experiments were conducted in compliance with current Good Clinical Practice standards and in accordance with relevant guidelines and regulations and the

Abbreviations: ENSC, embryonic neural stem cells; DC, differentiated cells; BBB, blood-brain barrier; CCI, controlled cortical impact; IF, immunofluorescence; GE, Ganglionic eminences; GFAP, glial fibrillary acidic protein; DCX, doublecortin; NeuN, neuronal nuclei; PBS, Phosphate-buffered saline; Iba-1, ionized Ca binding adaptor molecule; H&E, Hematoxylin and Eosin; SVZ, subventricular zone; DG, dentate gyrus.

principles set forth under the Declaration of Helsinki (42). All efforts were made to reduce the number of animals used and their suffering. Female mice were coupled with males for one night before removal of the male. Females were then tested for vaginal plug and positive mice were considered pregnant. A total of 56 mice were used in this study: 8 C57BL/6 pregnant mice at E14 were used as donors of ENSCs and DCs, 16 mice were used as controls (TBI/injected with saline) and divided into 2 time points (1 and 4 weeks, $n = 8$ each), 16 mice were transplanted with ENSCs after TBI and divided into 2 time points (1 and 4 weeks, $n = 8$ each), and 16 mice were transplanted with DCs after TBI and divided into 2 time points (1 and 4 weeks, $n = 8$ each).

Establishment of the Experimental CCI *in-vivo* Model

A CCI device (Leica Angle Two System, Leica microsystem, USA) was used to produce TBI in mice as described previously (43). Briefly, adult (6–8 weeks old) C57BL/6 mice (~ 20 g, $n = 48$, Jaxon laboratories, Maine, USA) were anesthetized with a mixture of xylazine (90 mg/kg, Panpharma) and ketamine (10 mg/kg, Interchemie), injected intra-peritoneally. The animal was held in the instrument U frame with the head at the closed end of the “U.” Animals were supported in a stereotactic frame in a prone position and secured by ear and incisor bars. To secure the animal during surgery, the ear bars, nose clamp, and incisor bar were attached to the U frame. Thereafter, a midline scalp incision was made and the skin was retracted from the skull surface. Using the software associated with the device, a target site between bregma and lambda was set, where craniotomy was made. A unilateral (ipsilateral to the site of impact) craniotomy (2 mm diameter) was performed adjacent to the central suture, midway between bregma and lambda (ML: 1.88 mm, AP: -0.57 mm, DV: -1.58 mm). To produce mild CCI in the somatosensory area, an impact was induced by an impactor of 1 mm diameter with an impact velocity of 4 m/sec, a depth of 1 mm and a dwell time of 150 ms, after which the surgical site was sutured. CCI was always performed at 7 days prior to cell injection. ENSCs were injected after 14 days of cell culture (P2) whereas DCs were injected 21 ± 3 days thereafter.

Experimental Design

A total of 48 mice, divided into 6 groups of 8 mice each, were used in this study. After 7 days of CCI lesion establishment, two groups of 8 mice each received a single injection of either saline, embryonic stem cells (ENSC, 1.5×10^5 cells) or differentiated cells (DCs, 1.5×10^5 cells). Injection of cells or saline was performed using a Hamilton syringe in a total volume of 3 μ l. One group of mice was sacrificed after 7 days of injection (1 week) while the second group was left for 28 days (4 weeks). A total of 4 mice from each group and time point were used for immunofluorescence (IF). Ipsilateral and contralateral cortical or hippocampal brain tissue samples were then harvested and kept at -80°C for further analysis.

Harvesting ENSCs From E14 Embryos

ENSCs were isolated from E14 embryos after anesthesia of pregnant females. Briefly, heads were separated from the embryos

at the level of cervical spinal cord, a horizontal cut made at the level of the eyes and then midline from the forehead toward the back of the head. Brains were removed by pushing the edges of the cut section, held steady and then a cut was performed through the cortex of each hemisphere, originating from the olfactory bulbs to the back of the hemisphere. Ganglionic eminences (GE) containing the ENSCs were then exposed and micro-dissected.

Neurosphere Assay

The harvested GE tissue is dissociated by gentle pipetting in 3 ml complete media (F12-DMEM, Sigma) supplemented with 3% B27 (50X), 0.5% N2 (100X), 1% ABAM, 1% glutamax and 1% BSA (all from Gibco). The mixture was then centrifuged for 5 min at 110 g and the pellet was resuspended in 1 ml complete media. After counting the cells using a hemacytometer, a total of 200,000 cells were seeded in T25 flasks containing 5 ml of complete media supplemented with 10 μ l EGF (20 ng/ml) and incubated at 37°C and 5% CO_2 . At this stage, the obtained cells (Passage 0/Day 0, P0/D0) would grow into primary neurospheres, which takes 6–7 days to observe in the flask. After 3 days (D3) of culture, 1–2 ml complete media supplemented with 2 μ l EGF (20 ng/ml) were added. At D 6–7, neurospheres were observed and were ready for the first passage. The suspensions of neurospheres were then collected, centrifuged at 110 g for 5 min. A total of 1 ml trypsin-EDTA (0.05%) was then added on the neurosphere pellet for about 2–3 min at 37°C and then its activity was stopped using 2 ml DMEM. The suspension of dissociated cells was then centrifuged at 110 g for 5 min, re-suspended in 1 ml complete media, and counted using a hemacytometer. A total of 4×10^5 cells were then distributed in T25 flasks containing 5 ml of complete media supplemented with 10 μ l EGF (20 ng/ml), and incubated at 37°C and 5% CO_2 . At this stage, the obtained cells (P1/D7) would grow into secondary neurospheres. This time point (D7) is the day of performing CCI. Three days after the first passage (D10), 1–2 ml complete media supplemented with 2 μ l EGF (20 ng/ml) were added to each flask. Seven days after their passage (D14), secondary neurospheres were ready for injection.

Neuroblast Assay (NBA)

To produce differentiated cells (DCs), the neuroblast assay was used which is divided into 2 parts: the proliferation stage and the differentiation stage. During the proliferation stage (3–4 days), cells obtained from secondary neurospheres (P2) were plated at a concentration of 4×10^5 cells/ml in the above ENSC complete media supplemented with 20 ng/ml EGF and 5% heat-inactivated FCS (Sigma). Culture flasks were then incubated in a 37°C humidified incubator with 5% CO_2 . The obtained cells proliferated and grew in a monolayer attached to the substrate. During the differentiation stage (3–4 days), when cells became 90% confluent, the media of each flask was switched to ENSC complete media supplemented with 5% FCS, without any growth factors. Culture flasks were again incubated in a 37°C humidified incubator with 5% CO_2 . Neuronal progenitors appeared on top of an astrocytic monolayer. These neuronal progenitors proliferated and produced colonies of immature neuronal cells.

Immunocytochemistry (ICC) of Neurospheres

Secondary neurospheres were fixed with 4% PFA for 20 min and then washed twice in PBS (1X) for 5 min. Cells were then resuspended and washed in PBST (0.5% Triton-X in PBS 1X) 3 times for 10 min each. Thereafter, neurospheres were blocked (3% BSA+ 10% HS in PBST) for 20 min at room temperature and then rinsed again with PBST 3 times for 10 min each. Cells were then incubated for 2 h with primary antibodies against glial fibrillary acidic protein GFAP (rabbit anti-mouse polyclonal antibody PAb, Abcam, 1/1,000) and Nestin (goat anti-mouse polyclonal antibody, Abcam, 1/3,000), washed 3 times in PBST for 10 min each and incubated with appropriate secondary antibodies (goat anti-rabbit, Cruz Fluor 594, 1/1,000 and rabbit anti-goat, Alexa Fluor 488, Abcam, 1/500) for 1 h at room temperature. The nuclei were counter stained with Hoechst for 10 min and washed 3 times in PBST for 10 min each. Finally, stained neurospheres were mounted on microscopic slides and revealed by a fluorescent microscope (Axio Observer Inverted Microscope, Carl Zeiss, Germany).

Immunocytochemistry (ICC) of Differentiated Cells (DCs)

Cells in NBA cultures were validated by a double immunostaining assay using glial and neuronal specific markers. Cells were first fixed with 4% PFA for 25 min and then washed twice in PBS (1X) for 5 min. DCs were permeabilized in PBST (0.1% Triton-X in PBS 1X) 3 times for 10 min each then blocked (5% FBS in PBST 1X) for 30 min at room temperature. DCs were then rinsed with PBST 3 times for 10 min each, followed by subsequent midnight incubation with primary antibodies against GFAP (mouse anti-GFAP, Abcam, 1/1,000), Tuj1 (rabbit anti-mouse PAb to beta-Tubulin III, Abcam, 1/2,000) and Neuronal Nuclei NeuN (rabbit anti-mouse PAb to NeuN, Abcam, 1/1,000). Appropriate secondary antibodies (donkey anti-mouse PAb, Alexa-fluor 488, 1/1,000 and goat anti-rabbit, Alexa Fluor 488, 1/1,000, Abcam) were added for 1 h at RT followed by washing the cells in PBST (3x, 10 min each). Hoechst was then added for 5 min to counter stain the DCs nuclei which were then washed in PBST (2x for 10 min each). Finally, DCs were mounted on microscopic slides and revealed by a fluorescent microscope (Axio Observer Inverted Microscope, Carl Zeiss, Germany).

Transplantation

After cell culture, ENSCs and DCs were ready to be injected in mouse brains at the somatosensory area injury site. After cell counting, a total of 1.5×10^5 cells were prepared in 3 μ L DMEM in a Hamilton syringe for injection into each mouse. Adult (6–8 weeks old) C57BL/6 mice ($n = 48$, Jaxon laboratories, Maine, USA) were used as recipients after establishing TBI lesion. The origin of cortical donor cells was from embryonic day 14 C57BL/6 mice ($n = 8$) embryos. Briefly, the mouse was anesthetized, placed in the stereotaxic frame and the CCI machine impactor was replaced with the Hamilton syringe containing the 3 μ L suspension of cells. A midline incision was made to expose the skull, then Bregma and Lambda points were located manually

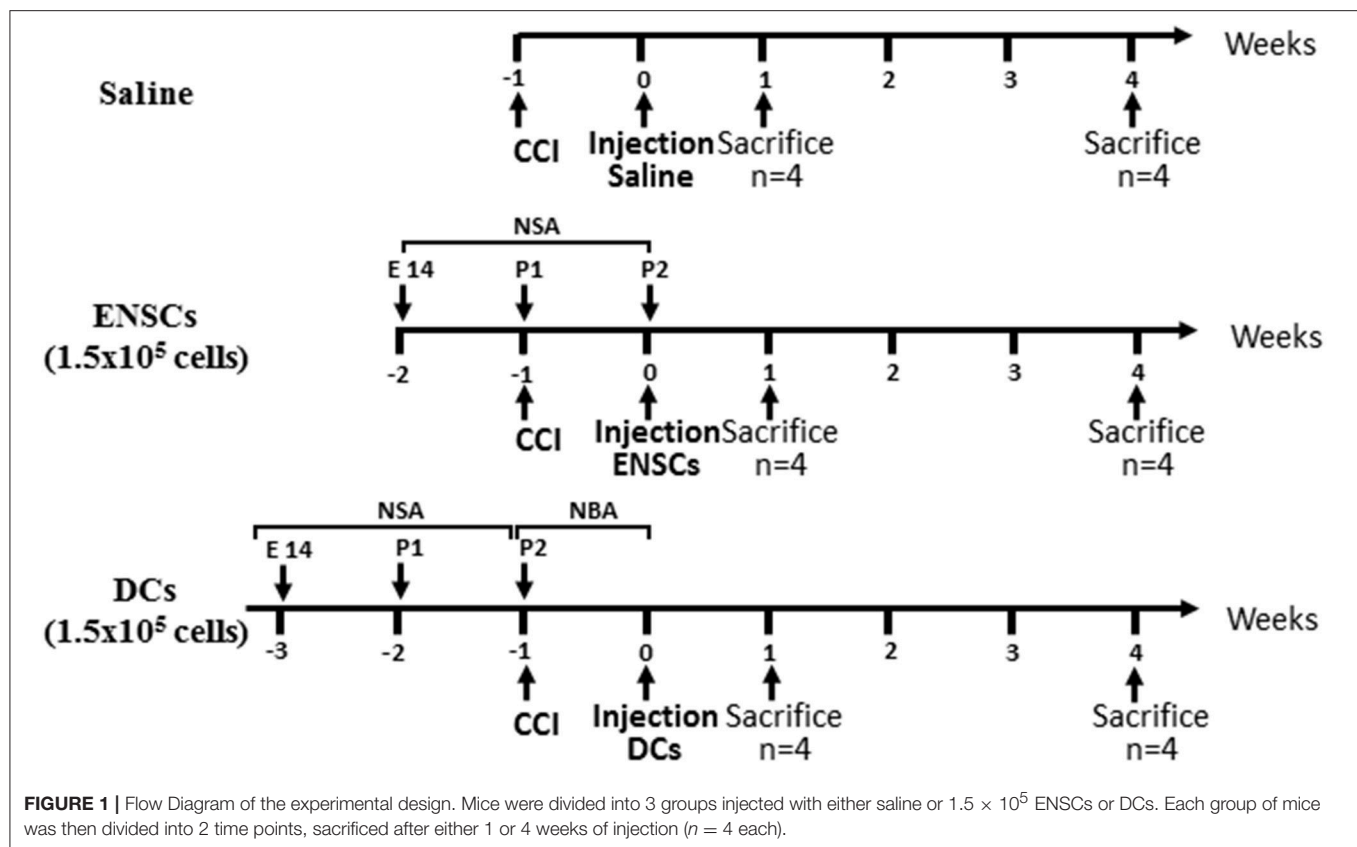
using the Hamilton syringe. The latter is then moved to “zero” in the instruments mediolateral (ML) and anteroposterior (AP) coordinates; at this point, it is right above the target site. Using the dorsoventral (DV) drive, the syringe is lowered until it reaches the lesion site (coordinates; ML: 1.88 mm, AP: -0.57 mm, DV: -1.58 mm). This is followed by lowering the DV axis by 1 mm to perform the injection at the lesion core. Following the establishment of CCI lesion, mice were maintained for 1 week and then divided into 3 groups ($n = 8$ each) which received a single injection of either saline, ENSCs or DCs (at 1.5×10^5 cells). Each group of mice was then divided into 2 time points ($n = 4$ each), sacrificed after either 1 or 4 weeks of injection (Figure 1).

Perfusion and Brain Tissue Preparation

Control mice without transplantation (CCI), and mice at different time points after transplantation of either ENSCs (CCI+ENSC) or DCs (CCI+DC) (day 7 and day 28) were anesthetized and their brains perfused, removed and sectioned into brain slices. Briefly, mice were injected intra-peritoneally with a mixture of xylazine (90 mg/kg, Panpharma) and ketamine (10 mg/kg, Interchemie), and perfused transcardially with 20 ml PBS (1X) followed by 30 ml of ice-cold paraformaldehyde (PFA, 4%). Brains were then removed and fixed in 4% PFA overnight. They were cut into 40 μ m coronal sections with a microtome (Leica Microsystems, USA) and stored at 4°C in 0.01% sodium azide (97.5 mg of NaN₃ in 100 mL PBS 1X). Brain sections were used to perform histological observations such as immunofluorescence (IF), Cresyl violet (Nissl stain) and Haematoxylin and Eosin staining (H&E).

Immunofluorescence

This was performed as previously described (44). Briefly, brain slices were first washed twice with PBST (0.1% Triton in PBS, 1X) for 5 min each, at room temperature (RT) and on a shaker. A blocking solution (5% FBS in PBST) was then added (1 mL/well) for 1 h with continuous shaking before adding the primary antibody (500 μ L/well), which was left overnight at 4°C. Polyclonal antibodies used were: rabbit anti-mouse GFAP (1:1,000, Abcam), goat anti-rabbit doublecortin (DCX, 1:500, Santa Cruz), a microtubule-associated protein localized in somata and processes of migrating and differentiating neurons, rabbit anti-mouse ionized Ca binding adaptor molecule (Iba-1, 1/1,000, Abcam) and rabbit anti-mouse NeuN (1/1,000, Abcam). After incubation, sections were washed for 3 times with PBST (15 min each, RT, on a shaker), and then incubated for 1 h at RT in the appropriate secondary antibody (500 μ L), previously diluted in blocking solution. The secondary antibodies used were either goat anti-rabbit PAb (Alexa-Fluor 488, 1/1,000, Abcam) or rabbit anti-goat PAb (Alexa Fluor 488, 1/500, Abcam) specific for primary antibodies. Hoechst stain was then added (1 ml) for 5 min and sections were washed twice for 5 min each at RT and on a shaker. Slices were then mounted on microscope slides (star frost) using a mounting solution (2–3 drops) and a cover slip. Slides were then evaluated and photographed using a fluorescent microscope (Axio Observer Inverted Microscope, Carl Zeiss, Germany). Appropriate negative controls were performed in each assay.



Hematoxylin and Eosin (H&E) Stain

Brain tissues were transferred into coated slides (star frost) and organized from anterior to posterior. Slices were stuck on the slide using soaked filter paper in PBS (1X) or the slide is left on the bench for 1 day until it dries. The prepared slides were then placed in distilled water (dH₂O) for 3 min for hydration then transferred to hematoxylin for up to 1 min. The slides were then placed in running tap water to provide the necessary alkalinity for “bluing” process. The slides were then immersed in eosin for up to 1 min. Slides were then placed in 95% ethanol for 2 times, 3 min each and then immersed in 100% ethanol for 5 min to prevent cell lysis. Finally, slides were placed in Xylol for 1 min, which acts as a clearing solvent to remove alcohol from tissues. Slides were then mounted and visualized on the microscope.

Nissl Stain

Brain tissue slides were first prepared, placed in distilled water (dH₂O) for 3 min and then transferred to Cresyl violet (0.5%) for up to 2 min. The slides were then placed in running tap water and then immersed in distilled water. Slides were then placed in 75% ethanol for 1min, 95% ethanol for 2min, and then immersed in 100% ethanol for 3 min. Finally, slides were placed in xylol for 2 min and were mounted and visualized on the microscope.

Data Acquisition and Quantification

For each mouse, images were acquired with a LSM710 confocal microscope, Carl Zeiss, Germany. At least four

sections corresponding to the areas of interest were used for quantifications using Image J program (NIH). For Iba-1 and GFAP expression, the total area covered by these markers at the lesioned site was quantified with respect to the total injury area. For DCX marker, the area covered by the DCX+ cells throughout the lateral SVZ was quantified with respect to the total length of the SVZ. For NeuN quantification, the total NeuN+ area in the hippocampus was quantified on both contralateral and ipsilateral sides among different groups.

Statistical Analysis

All results are expressed as mean $\pm$ SEM. For data concerning IF, within each experimental group, statistical significance was evaluated using two-way analysis of variance (ANOVA) having the group (control, transplantation) as a parameter. The p -value was determined and values for $p < 0.05$, $p < 0.001$, $p < 0.0001$ (*, **, *** respectively within the same group and #, ##, ### respectively among groups) were considered significant.

RESULTS

Establishment and Validation of Mild CCI Model, Neurosphere, and Neuroblast Assays

CCI lesion was established as described in the materials and methods section. CCI was then validated using H&E staining on histological sections of the mouse brain (Figure 2). Indeed, the

expected morphological changes such as damaged parenchymal tissues and reduction in the cytoplasm of the injured cortex were observed in the lesioned somatosensory region (**Figure 2**). On the other hand, no significant malformations in the hippocampal region were observed. Moreover, Nissl stain confirmed that neurons residing in the ipsilateral cortex were highly injured (**Supplementary Figure 1**).

In parallel, ENSCs were harvested from E14 embryos and cultured using the neurosphere assay. They showed microspikes morphology, characteristic of neurospheres, at 5–6 days of culture (**Figure 3A**). On the other hand, the three types of differentiated cells (DCs): flat astrocytic cells, round neuronal progenitor cells, and mature neurons were obtained using the neuroblast assay (NBA), after proliferation and differentiation stages (**Figure 3B**). The phenotype of cells was validated using immunocytochemistry where neurospheres showed nestin and GFAP staining, thus reflecting the undifferentiated state of stem cells whereas differentiated cells were positive for Tuj-1, GFAP, and NeuN markers (**Figure 3C**).

Microglia Is Activated Following CCI, but Not Enhanced After Injection of ENSCs or DCs

Iba-1 protein, a microglial specific marker, is usually used as a measure of neuroinflammation and neuroprotection during different phases of CCI. Microglial activation using Iba-1 was assessed by IF on the 6 groups of CCI mice ($n = 4$ each) injected with either saline, ENSCs or DCs and maintained for 1- or 4-weeks after injection (**Figure 4A** and **Supplementary Figure 2**). Quantification of IF data demonstrated a significant increase ($*p < 0.05$) in Iba-1 expression post-CCI in saline and DCs groups, but not in ENSC group, at 4 weeks in comparison to 1 week (**Figure 4B**). In addition, similar morphological changes were observed in different areas of the injured cortical region among the 6 groups of mice. Indeed, bushy hypertrophied Iba-1+ microglia were found to be highly expressed around the CCI region whereas resting microglia of amoeboid morphology were observed at distant places from the CCI zone (**Figure 4C**). Taken together, ipsilateral lesioned cortex expresses high levels of Iba-1+ microglia after 1 and 4 weeks of injection within the different groups (Saline, ENSCs, or DCs).

Astrogliosis Is Significantly Reduced by Treatment With ENSCs at 1 Week or DCs at 4 Weeks Post-transplantation

Previous studies have shown that acute CCI induces inflammation, glial cell reactivity and a dramatic increase in the number of activated astrocytes, with hypertrophic cellular processes, leading eventually to neuronal apoptosis and “reactive astrogliosis” (45). The latter is characterized by GFAP protein, upregulated in the activated astrocytes, and occurs mostly in the ipsilateral cortex of the injured brain, as demonstrated by immunofluorescence (**Figure 5A**). When compared to the saline group, our data demonstrated a significant reduction in the number of GFAP expressing astrocytes in the ENSCs group, at 1 and 4 weeks post-injection ($**p < 0.01$, **Figure 5B**). This

reduction was also highly significant in the ENSCs group at 1 week, in comparison to DCs ($***p < 0.001$). In addition, the DCs group also showed a highly significant reduction in GFAP expression at 4 weeks, in comparison to controls ($***p < 0.001$, **Figure 5B**). Taken together, treatment with ENSCs or DCs (glial and neuronal cells) following mild CCI demonstrated a reduction in astrogliosis, especially 4 weeks post-injection, as shown by a decrease in GFAP expression. This decrease in GFAP levels started very early in the ENSCs group (1 week), in comparison to DCs group, which was comparable in both groups at 4 weeks.

Neurogenesis Is Significantly Enhanced at the Progenitor Level in ENSCs Before DCs Groups

Previous studies reported an activation of neurogenesis and cellular proliferation in the subventricular zone (SVZ) and dentate gyrus (DG), following CCI (46, 47). Protein expression of DCX, a marker of late neuronal progenitors, was used to demonstrate the effect of injecting ENSCs or DCs, following CCI, on neurogenesis in the SVZ region (**Figure 6A** and **Supplementary Figure 3**). Our data demonstrated a significant increase in the number of DCX expressing progenitor cells at 1 week in the ENSCs group, in comparison to control or DCs groups ($***p < 0.001$) (**Figure 6B**). In addition, a significant increase in DCX was also obtained in DCs and ENSCs groups at 4 weeks post-injection, in comparison to controls ($***p < 0.001$ and $*p < 0.05$; respectively) (**Figure 6B**). In summary, treatment with ENSCs or DCs after CCI increased the expression of DCX, therefore enhancing cellular proliferation, progenitor cell survival and neurogenesis at 1 and 4 weeks post-injection. Unlike DCs, ENSC showed an enhancement in neurogenesis 1 week after transplantation as well as reduced astrogliosis. In contrast, DCs treatment is less effective at this time point.

Expression of Mature Neurons

Protein expression of NeuN, a marker of mature neurons, was used to demonstrate the effect of injecting ENSCs or DCs, following CCI, on neuronal maturation in the hippocampus region (**Figure 7A**). Results showed a significant increase in the number of neuronal cells in DCs group at 4 weeks post-injection, in comparison to controls ($**p < 0.01$, **Figure 7B**). On the other hand, our data demonstrated a significant decrease in NeuN expressing cells in control mice at 4 weeks post-saline injection, in comparison to 1 week, but also in ENSC group at 1 week, in comparison to controls ($*p < 0.05$, **Figure 7B**). Finally, no significant increase in NeuN levels was observed in the other groups and time points (**Supplementary Figure 4**). Taken together, injection of ENSCs or DCs post-CCI suggests that it may increase neurogenesis via inducing neural progenitor's proliferation and expression rather than neuronal maturation.

DISCUSSION

Traumatic brain injury, an intracranial injury, has been recognized among the major causes of death worldwide with

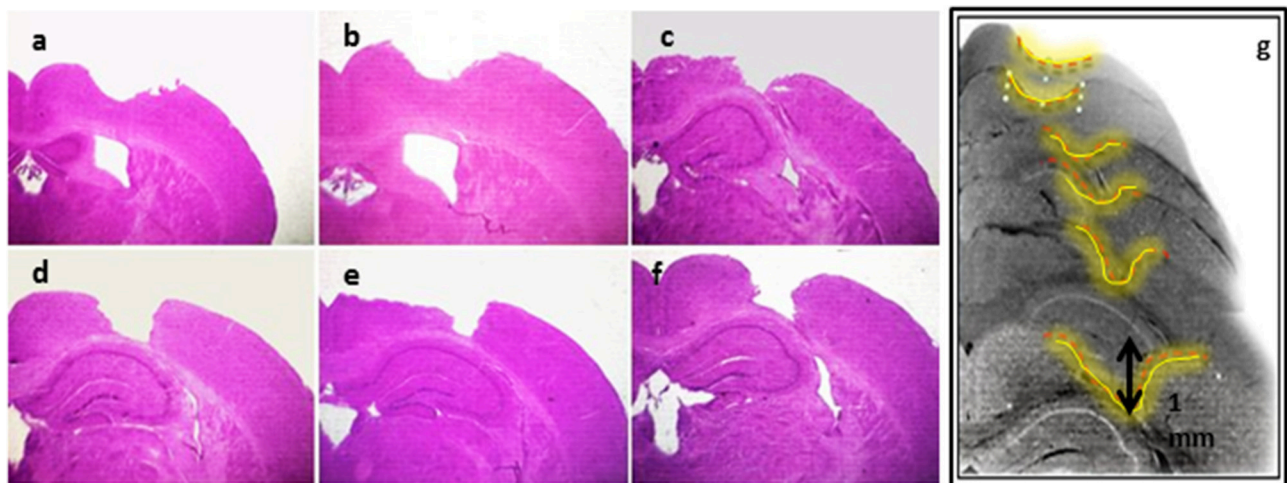


FIGURE 2 | Establishment of CCI model. H&E staining showing serial sections (a–g) of the lesioned area of the brain tissues subjected to CCI, and detecting the morphological changes in the mouse brain coronal sections.

broad devastating symptoms and disabilities. TBI causes primary injury due to mechanical forces at the time of the incident followed by a secondary injury. The latter is characterized by cytotoxicity, cerebral edema, oxidative stress, necrosis, apoptosis, mitochondrial dysfunction, and inflammation (9–11). These neuropathological sequelae happen progressively, aggravating the neural injury and inducing neuropsychological and motor deficits. Thus, several options have been proposed as a prospective therapy for TBI including stem cell transplantation using mesenchymal stem cells (MSCs) (26, 48), neural stem cells (NSCs) (27–30), induced pluripotent stem cells (iPSCs) (31) and embryonic neural stem cells (ENSCs) (32). Thus, the aim of our study was to investigate the effect of grafting different cell types (ENSCs or DCs) on neuroinflammation and neurogenesis after mild CCI. This study describes the environment surrounding the injury after transplanting ENSCs or DCs in the same conditions.

Neuroinflammation is considered as a potential therapeutic target due to its correlation with neurological symptoms and pathology (49). In fact, inflammatory response post-TBI is signaled by glial cell activation, peripheral leukocyte recruitment via disturbed BBB and rapid rise in the levels of inflammatory mediators such as cytokines and chemokines (50, 51). Mediators and cellular events activate the immune cells and induce their migration toward the injured area (52). For instance, neutrophils are the first cells infiltrating the injured brain followed by migration of microglia and activation of astrocytes (52). Neuroinflammation can be either harmful by inducing oxidative damage and neuronal death or beneficial by promoting the clearance of debris and tissue remodeling (53).

Our data showed an up-regulation of microglial activation which is in accordance with a recent study in moderate TBI, using the CCI model, demonstrating that microglial activation occurred 1-week post-CCI, which significantly increased 5 weeks after injury and persisted for 1 year (54). Since there is an interaction between astrocytes and microglia post-CCI,

DCs which contain populations of astrocytes, but not ENSCs, affect the microglial expression more significantly at 1 week. It was previously demonstrated within the cortex of a moderate diffuse TBI mouse model that the expression of tumor necrosis factor alpha (TNF α) and IL-6 peaked at 3–9 h post-TBI (55). Similarly, increased microglial activation was shown at 6 h post-TBI in pigs, indicating an association between the induction of an inflammatory response and brain pathology (56). Microglial activation is well established as one of the secondary biochemical changes following TBI, providing an evidence of neuroinflammation post-TBI (54). Indeed, microglial activation is characterized by morphological as well as functional modifications where the resting ramified microglia transforms into hypertrophic bushy cells involved in secretion of pro- and anti-inflammatory molecules (54). In fact, M1-like microglial activation is implicated in the deterioration of injury due to the release of cytotoxic mediators (57, 58) whereas M2-like microglial activation contributes to injury recovery and repair due to the release of neurotrophic and immunomodulatory factors (59, 60). Several TBI studies reported a peak in M2 microglia during the first 7 days after injury; however, this phenotype shifts to M1 microglia thereafter (61, 62). In another model of stroke, the M2 marker CD206 was highly expressed 3 days after ischemia whereas the M1 marker MHCII was found highly expressed at day 7 post-ischemia (63). Our observation of microglial up-regulation following mild CCI suggests that induced neuroinflammation may last for a long time after the initial brain insult, increasing the risk of persisting neurodegeneration and therefore the need of targeting inflammatory events using new therapies. Chronic microglial activation is associated with up-regulation of pro-inflammatory cytokines such as IL-1 β and TNF α (64) contributing to long-term neurodegeneration following TBI.

On the other hand, our data demonstrated a significant reduction in the number of GFAP expressing astrocytes in

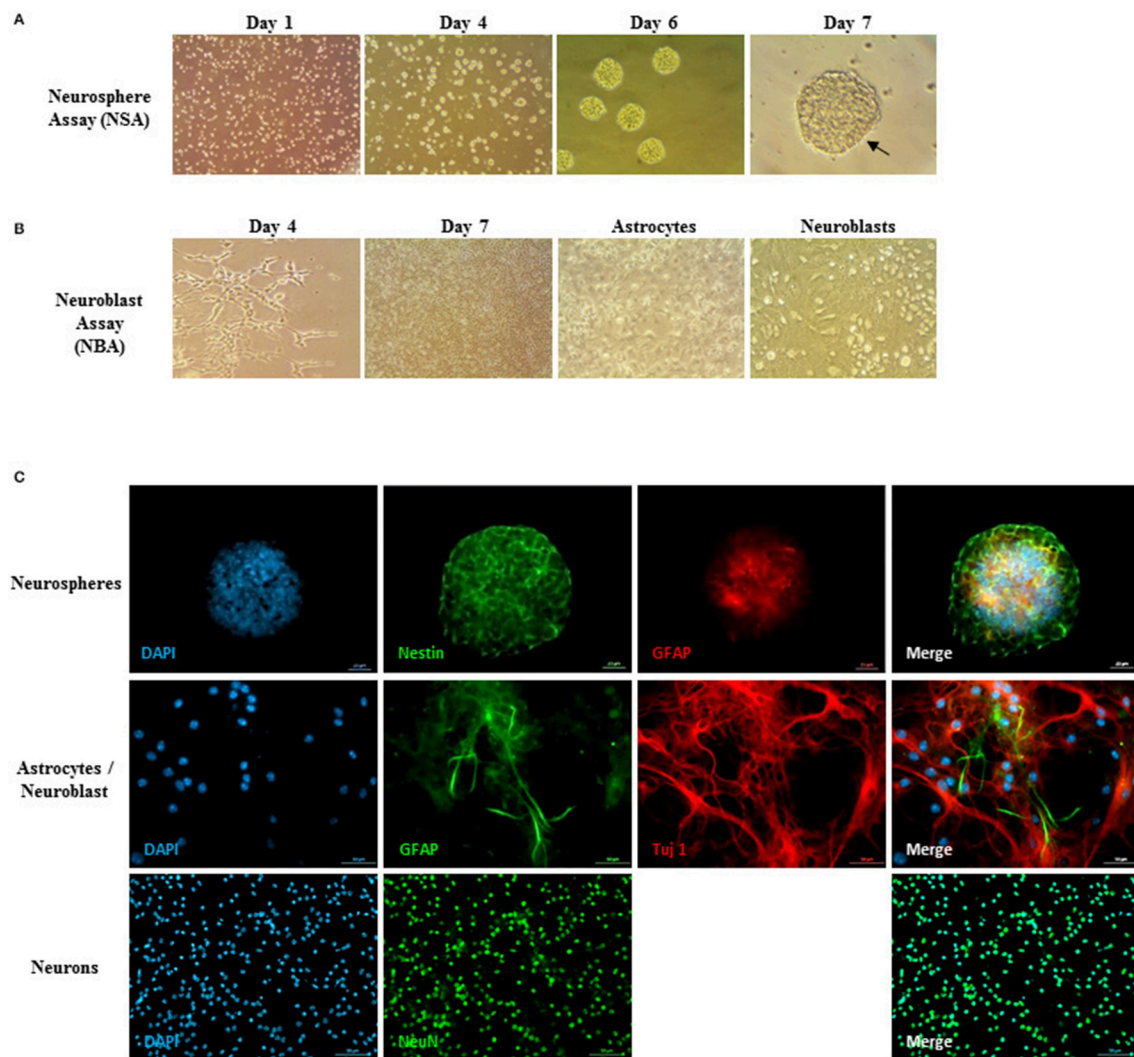
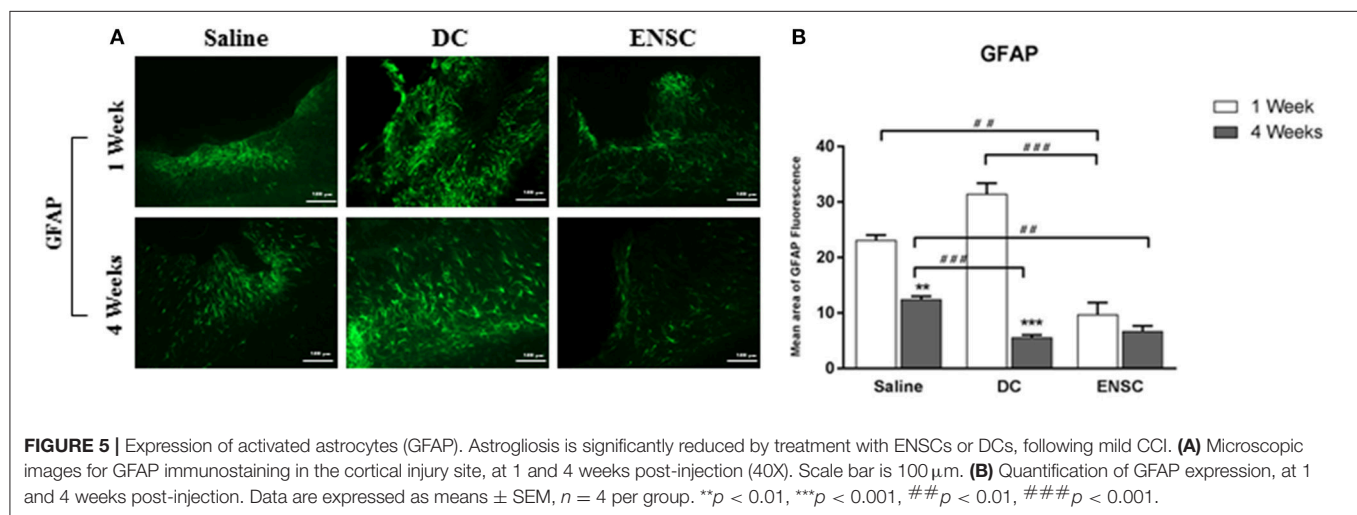
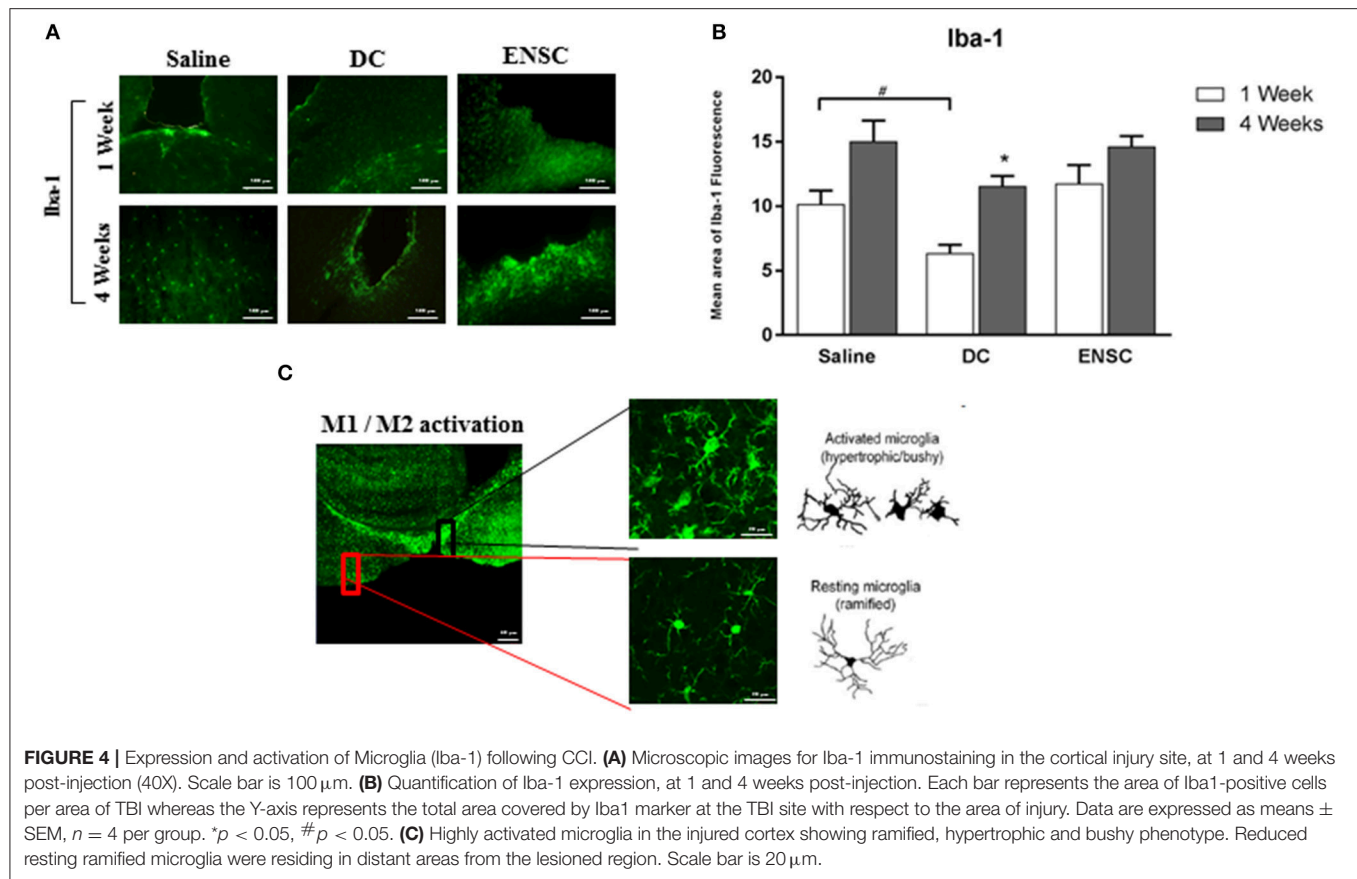


FIGURE 3 | Neurosphere assay, neuroblast assay, and neurosphere immunostaining. **(A)** Young healthy neurospheres showing microspikes at 5–6 days post-culturing. **(B)** Neuroblast assay (NBA) with proliferation and differentiation stages. Flat astrocytic cells are shown by black arrows underneath, and round neuronal progenitor cells shown by red arrows on top. **(C)** Neurosphere immunostaining assay to validate the neural embryonic origin of the spheres. Upper panel: Neurospheres showed a Nestin, GFAP positive phenotype, thus reflecting the undifferentiated state of the ENSCs and showing that not all GFAP⁺-cells are differentiated astrocytes, but have a large majority of undifferentiated NSCs. Middle and lower panels: validation of the differentiated cells (DCs) phenotype residing in the NBA cultured population. The cells show a Tuj-1, GFAP positive phenotype (middle panel) as well as NeuN, GFAP positive immunostaining (lower panel).

control and DC groups at 4 weeks post-injection, in comparison to 1 week. In addition, GFAP decreased in the ENSC group at 1 week, in comparison to control or DC groups, which was maintained at 4 weeks. Previous studies have shown that TBI causes a dramatic increase in the number of activated astrocytes with hypertrophic cellular processes resulting in “reactive astrogliosis” (45). In fact, astrocytes affect many essential neural functions in normal CNS by maintaining the extracellular balance of ions and neurotransmitters, regulating the blood flow and influencing synaptic activity and plasticity (65). Therefore, dysfunction in the mechanisms underlying scar formation and reactive astrogliosis cause detrimental effects on the CNS (65). During the acute phase of TBI,

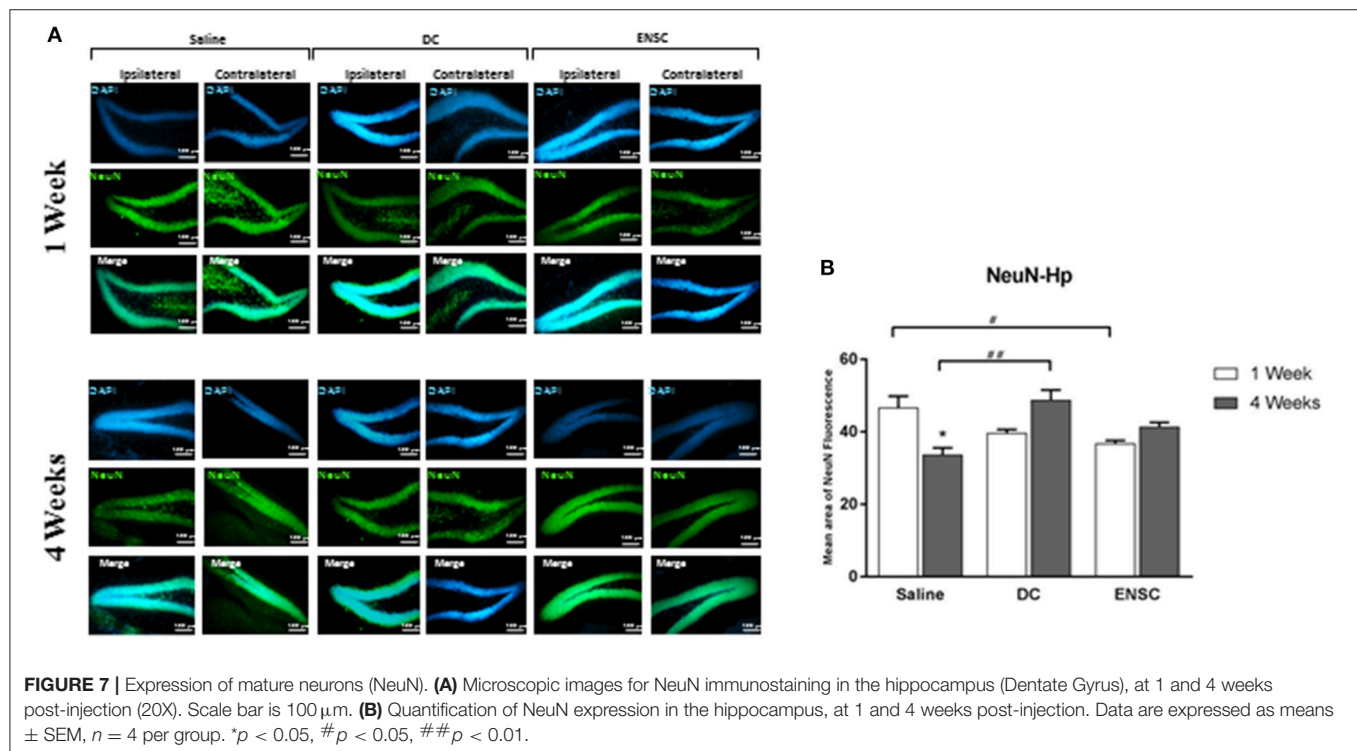
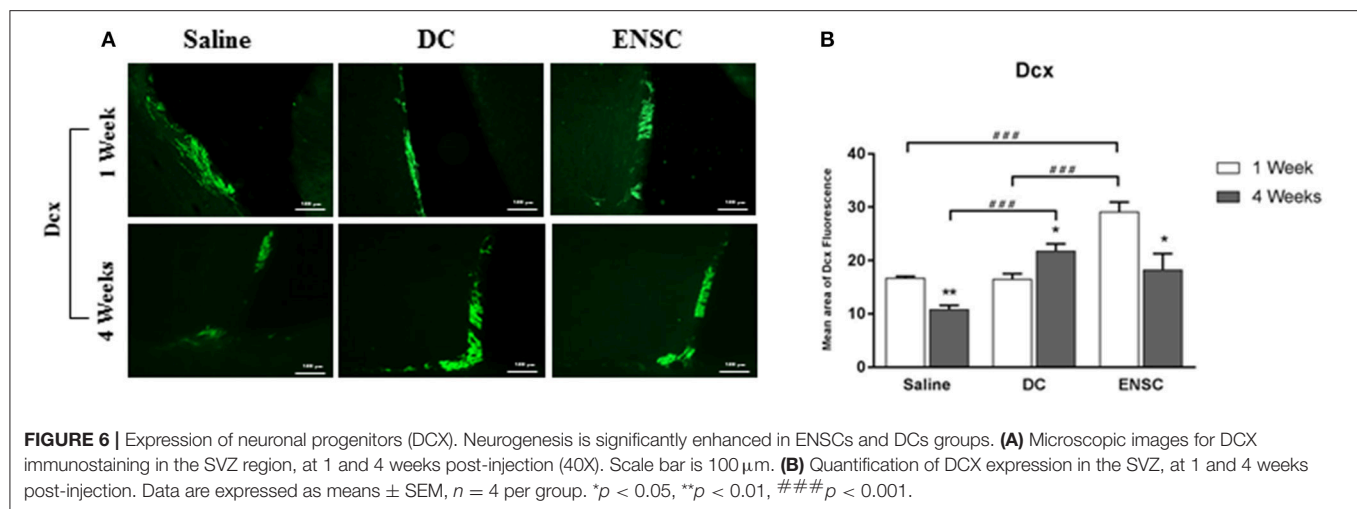
inflammation and glial cell reactivity are induced due to the alteration in the BBB leading eventually to neuronal apoptosis (45). Our results demonstrated that following mild CCI, treatment with ENSCs or DCs (glial and neuronal cells) caused a reduction in astrogliosis, suggesting that our cell therapy at 1 and 4 weeks’ time intervals provide a neuroprotective (anti-inflammatory) role which helps in recovery post-CCI.

One of the major results of this study was that Doublecortin (DCX) expression increased significantly in the CCI groups after transplantation. In fact, our data showed a significant up-regulation in the number of DCX expressing progenitor cells in DCs and ENSCs groups at 4 weeks post-injection, in



comparison to controls, in addition to a significant increase at 1 week in the ENSCs group, in comparison to control and DC groups. Indeed, it is well known that the subgranular zone (SGZ) of the DG in the hippocampus, in addition to the SVZ region, is a site of neurogenesis in adults where there is a continuous regeneration of neuronal cells (66). Recent evidence showed that neurogenesis occurs near the damaged brain regions after TBI in humans, where the newly produced

cells differentiate into mature neurons after migrating into the boundary zone of the lesioned cerebral cortex (67). In addition, spontaneous behavioral recovery is initiated after activation of endogenous neurogenesis following TBI (68). Despite this initial cellular proliferation and neurogenesis after trauma, survival and maturation of cells are not maintained since this early endogenous recovery is not sufficient to sustain the progenitor cell population (69). It was shown that only 20% of the newly



born striatal neurons survived for 2 weeks following middle cerebral artery occlusion in rats (70). Similarly, after intracerebral hemorrhage, most newly generated cells, observed between 72 h and 1 week, did not survive more than 3 weeks (71). It's important to note that it takes 4 weeks for the new progenitors to become functionally mature neurons (72). Moreover, the generated immature neurons are sensitive to environmental factors such as the release of inflammatory mediators by activated microglia and astrocytes which affect the newly produced progenitor cells, suppressing long-term neurogenesis after TBI (69). Interestingly, targeting the inflammatory events after brain injury may augment neurogenesis and maintain its activation.

Several studies have demonstrated the complex role of microglia in induced neurogenesis following TBI (20).

Using NeuN as a marker of mature neurons, we validated the effect of cell transplantation on neurogenesis. Although it takes 4 weeks to obtain mature and functional neurons, the control group showed a decrease in NeuN expression after 4 weeks of injury. On the other hand, the groups receiving DCs demonstrated a significant increase in NeuN expression.

One limitation in our study, however, was the incapacity to distinguish engrafted neurons and their axons from host tissue. In fact, it was not possible to characterize the transplanted neurons, to study their survival and migration and to assess

possible developed projections of the engrafted cells. Alternatives to overcome this limitation would be to use GFP mice (73) or fluorescently labeled cells for transplantation (74). As for the survival of the engrafted cells, it is affected by the reduced perfusion, i.e., low vascularization and oxygen levels, and the persisting neuroinflammation in the lesion core. The inflammatory response seems to impact neuronal survival depending on a balance between pro- and anti-inflammatory mechanisms induced by different factors. Chronic neuroinflammation induced after injury increases the risk of persisting neurodegeneration and therefore reduces the survival of engrafted cells. Meanwhile, in another model of lesion, it was shown that a 1-week delay between the cortical lesion and embryonic neurons transplantation can significantly enhance graft vascularization, cell proliferation, survival and density of projections developed by grafted neurons, leading to a beneficial impact on functional repair and recovery (75).

Taken altogether, 4 weeks after injection of ENSC or DCs, our data revealed no preference for the transplantation of one cell type over the other. In fact, microglial activation was enhanced in both groups whereas astrogliosis was reduced, which also suggests that ENSCs and DCs may play a critical role in targeting the inflammatory events and in enhancing early progenitor cell survival. Moreover, transplantation of both cell types, ENSCs or DCs, enhanced neurogenesis. Hence, to repair the damaged brain, concomitant use of both ENSC and DCs, targeting post-injury neuroinflammation and enhancing the survival of newly generated cells by supporting an optimal environment for the cells, may provide a new perspective for cell therapy.

AUTHOR CONTRIBUTIONS

MN, SM, FB, FN, NR, KZ, and FK carried out the experiments. MN, NB, KZ, and JS analyzed data. KZ and FK designed and supervised the study. FK, MN, and KZ wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fneur.2018.00895/full#supplementary-material>

Supplementary Figure 1 | Nissl staining. Brain tissue sections of CCI mice injected with saline, DCs or ENSC, respectively, 1 week post-CCI. Cortex at CCI region (ipsilateral) with 10× magnification showing the injured area depth of 1mm. Higher magnification confirmed damaged parenchymal tissues in the cortex post-CCI (data not shown).

Supplementary Figure 2 | Expression of Microglia (Iba-1) in the cortex following transplantation. (A,B) Microscopic images for Iba-1 immunostaining, at 1 and 4 weeks post-injection. Scale bar is 100 μm. (C) High magnification images from regions of interest showing immunolabeled microglia. Scale bar is 20 μm.

Supplementary Figure 3 | Expression of neuronal progenitors (DCX) following injection. Microscopic images for DCX immunostaining, in (A) saline, (B) DCs, and (C) ENSCs groups, at 1-week post-injection. Scale bar is 100 μm.

Supplementary Figure 4 | Expression of mature neurons. (A) Microscopic images for NeuN immunostaining, at 1 and 4 weeks post-injection. Scale bar is 100 μm. (B) Quantification of NeuN expression in the Cortex, at 1 and 4 weeks post-injection. Data are expressed as means ± SEM, $n = 4$ per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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Neuroprotection in Traumatic Brain Injury: Mesenchymal Stromal Cells can Potentially Overcome Some Limitations of Previous Clinical Trials

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Traumatic brain injury (TBI) is a leading cause of death and disability worldwide. In the last 30 years several neuroprotective agents, attenuating the downstream molecular and cellular damaging events triggered by TBI, have been extensively studied. Even though many drugs have shown promising results in the pre-clinical stage, all have failed in large clinical trials. Mesenchymal stromal cells (MSCs) may offer a promising new therapeutic intervention, with preclinical data showing protection of the injured brain. We selected three of the critical aspects identified as possible causes of clinical failure: the window of opportunity for drug administration, the double-edged contribution of mechanisms to damage and recovery, and the oft-neglected role of reparative mechanisms. For each aspect, we briefly summarized the limitations of previous trials and the potential advantages of a newer approach using MSCs.

Keywords: traumatic brain injury, mesenchymal stromal cells, brain protection, brain repair, vulnerability

INTRODUCTION

Traumatic brain injury (TBI) is the leading cause of mortality and morbidity across all ages in all countries. In Europe, it is estimated that 2.5 million people suffer a TBI each year, 1 million are admitted to hospital, and 57,000 die (1). TBI survivors have to deal with chronic post-injury motor, cognitive, and neuropsychological symptoms/dysfunctions. Even in the milder cases, TBI substantially increases the risk of epilepsy, stroke, and late-life neurodegenerative diseases (1). TBI thus implies a huge burden for patients, their families, and society.

Trauma causes primary damage to the brain by multiple mechanisms, including tearing, shearing, and stretching forces. Consequently, a cascade of metabolic, biochemical, and inflammatory changes is initiated, leading to secondary damage. Then second insults, both intracranial and systemic such as hypoxia, hypotension and intracranial hypertension, may worsen the progression of the injury.

Treatment of TBI patients has not changed much in the last 20 years, consisting only in supportive therapy directed at prevention, early detection and treatment of second insults, since all pharmacological trials testing neuroprotective agents have failed (2–5). This translational defeat may have several explanations, analyzed in numerous papers (6–8). In these critical reappraisals, many factors were identified at preclinical and clinical levels as area of improvement.

They included, but were not limited to, pharmacokinetics and pharmacodynamics, inadequate sample sizes, heterogeneity of TBI populations, the lack of relevant mechanistic early endpoints and insensitivity of global outcome measures (9).

Mesenchymal stromal cells (MSCs) may offer a promising strategy, with preclinical data showing that MSCs of human origin protect the injured brain by acting on multiple mechanisms of protection and repair (10–13), with potential advantages in terms of therapeutic window.

After initial expectations about the possibility of MSC trans-differentiation through neuronal lineage for brain reconstruction, decades of experimental data mainly show that MSCs do not protect the TBI brain through cell replacement, but by stimulating neuroprotective and endogenous neuroreparative mechanisms that this narrative review will discuss. We shall focus on three flaws of past trials that MSC-based therapy has the potential to overcome: the “window of opportunity” for drug administration, the double-edged contribution of mechanisms to damage and recovery, and the important, but often neglected, role of reparative mechanisms.

WINDOW OF OPPORTUNITY FOR PHARMACOLOGICAL NEUROPROTECTION IN TBI

The biochemical mechanisms of progressive brain damage are set in motion immediately after TBI as a consequence of the external force applied to the head. Using microdialysis in a rodent model of concussion, Katayama demonstrated a surge of extracellular potassium in the first minutes after injury, parallel with massive release of glutamate—up to 10–100 times the normal concentration (14). The time-resolution of the method, however, was limited (1 min of dead space for dialysate collection); when electrodes were used, almost immediate K⁺ release was demonstrated after trauma (15).

Early mechanisms of cellular injury act in minutes-to-hours after injury. The massive release of excitatory neurotransmitters, spreads energy failure and overload of free radicals from the contused tissue to surrounding brain regions. Energy crisis alters cell permeability, causing calcium inflow, which triggers mitochondrial dysfunction, with consequent energy failure, and apoptotic/necrotic death. Primary axotomy is uncommon, even in the case of traumatic axonal injury; the alteration of membrane permeability induces edema and impairs axonal transport, making axons more vulnerable to secondary axotomy and demyelination. These cascades clearly indicate how mechanical forces applied to the brain may evolve and propagate to healthy, potentially salvable tissue (16, 17).

The progress of secondary injury, in its sequence of deleterious events over time, is the theoretical basis for neuroprotective strategies. When neuroprotectant drugs were tested under experimental conditions, it became evident that their maximum potential was exploited by early administration or—when possible—by pre-treating the brain before insults (18). In general, however, later exposure to a protective compound gave less or no benefit (19–21).

These findings shaped the design of clinical trials, where drugs had to be administered in the first hours after injury, when there was felt to be a “window of opportunity.” A recent review (5) of 16 robust trials testing neuroprotective agents in TBI indicated a window of opportunity of 4 h in three, 6 h in two, 8 h in seven, and 12 h in one trial, while only three trials tested treatment up to 24 h after injury (**Table 1**). This narrow window of opportunity makes clinical trials more challenging, reducing enrolment rates and increasing complexity. Patients need to be rescued, stabilized, centralized to the study center, evaluated clinically and by imaging; relatives must be contacted for consent procedures so, finally, randomization and drug preparation and administration can start within the few hours permitted by the protocol. It is no surprise that a number of cases failed to meet the time limit: in several trials ~20% of potential candidates could not be enrolled because of the narrow time window (36). In the same trials the duration of the pharmacological intervention was limited to the first days after ICU admission (**Table 1**).

However, mounting evidences indicate that pathophysiological processes caused by the initial injury do not exhaust themselves in the first days but persist for months or years, and that TBI survivors are at risk of late neurodegenerative diseases (including Alzheimer’s and Parkinson’s disease, chronic traumatic encephalopathy) (37). For example, Johnson et al. reported immunohistochemical evidence of microglia activation and white matter degradation in subjects who died many years

TABLE 1 | Major randomized clinical trials (RCT) evaluating pharmacological treatments for acute moderate/severe TBI.

Study	Drug	Treatment start	Treatment duration	Sample size	Results
Skolnick (22)	Progesterone	<8 h	5 days	1,195	ND
Wright (4)	Progesterone	<4 h	4 days	882	ND
Shakur (23)	Anatibant	<8 h	4 days	228	ND
Marmarou (24)	Bradycor	<12 h	5 days	139	ND
Eurogroup (25)	Nimodipine	<24 h	7 days	852	ND
Teasdale (26)	Nimodipine	<24 h	7 days	352	ND
Perel (27)	Tranexamic acid	<8 h	1 day	170	ND
Robertson (28)	Erythropoietin	<6 h	14 days	200	ND
Maas (29)	Dexanabol	<6 h	1 day	861	ND
Yurkewicz (30)	Traxopodil	<8 h	3 days	404	ND
Morris (31)	Selfotel	<8 h	4 days	693	ND
Marshall (32)	Tirilazad	<4 h	5 days	1,120	ND
Young (33)	Pegorgotein	<8 h	1 day	463	ND
Asehnoune (34)	Steroids	<24 h	10 days	336	ND
Edwards (2)	Steroids	<8 h	2 days	10,008	ND
Grumme (35)	Steroids	<4 h	8 days	396	ND

Study selection was derived from the systematic review by Bragge et al. (5). An RCT was defined as robust if it was multicenter, included more than 100 patients, and had low risk of bias. For each study, it is reported first author and year of publication, the investigated drug, treatment start and length, number of patients included, and the effect on outcome, as mortality for Shakur, Perel and Asehnoune and Glasgow Outcome Scale for the others. ND, no statistical difference between intervention and control regarding selected outcome.

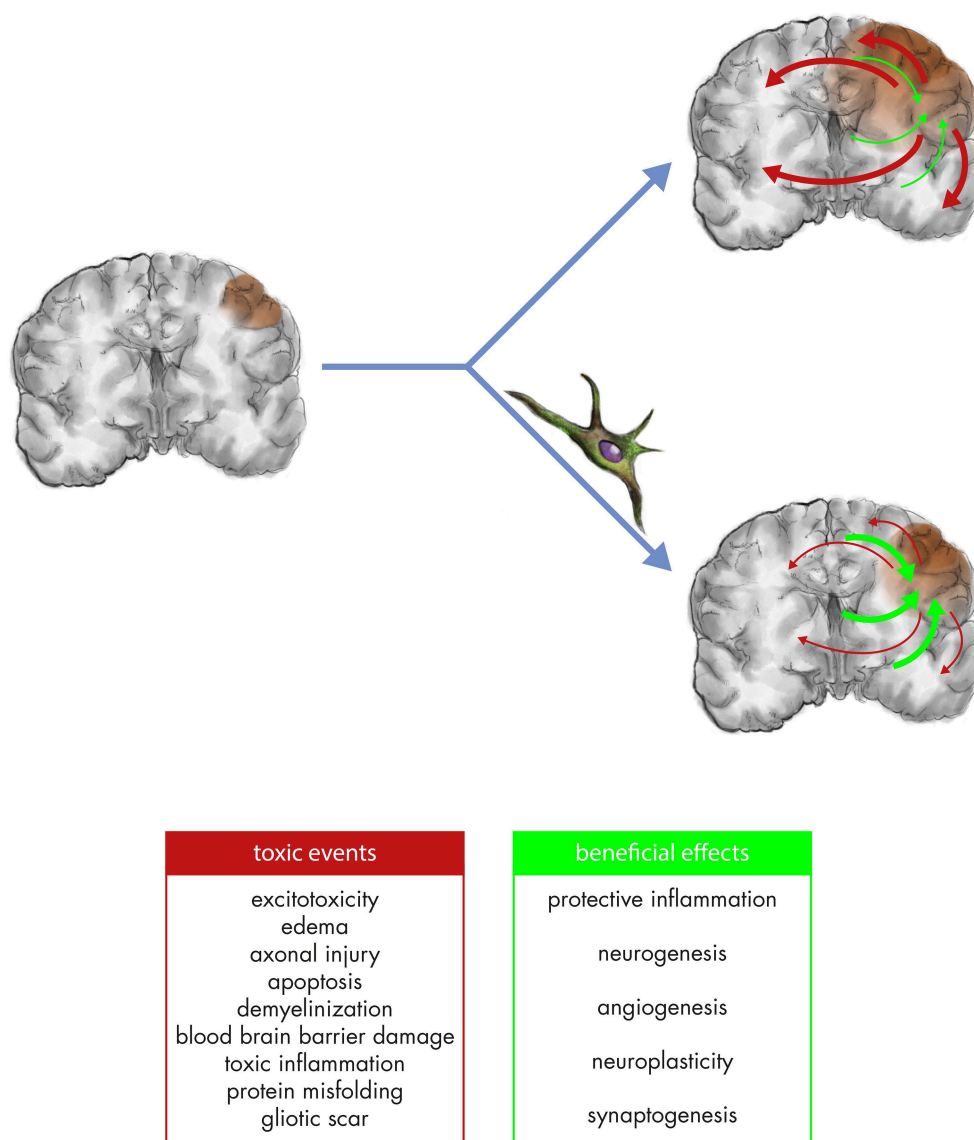


FIGURE 1 | After the biomechanical impact (left panel), toxic secondary cascades including excitotoxicity, axonal injury, apoptosis, demyelination, blood/brain barrier damage, toxic inflammation, protein misfolding, and gliotic scar (red arrows) contribute to the amplification of brain damage. Endogenous responses in TBI also comprise potentially beneficial mechanisms of protective inflammation, neurogenesis, angiogenesis, neuroplasticity, synaptogenesis (green arrows) but are too weak and short-lived to counteract the toxic cascades (right upper panel). MSC can mitigate toxic cascades and foster the regenerative ones, contributing to both neuroprotection and neurorestoration (right lower panel).

after TBI (38); and in patients there is a relation between chronic inflammation detected by positron emission tomography, up to 17 years post-TBI, and worse cognitive outcomes (39).

Inflammation is an important beneficial mechanism for clearing pathological debris and effecting repair (40–44); however, if dysregulated, it may also contribute to neuronal damage. The relative positive or negative effects of inflammation in relation to time from injury are still far from certain, and a threat of neurodegeneration associated with late microglia inhibition has recently been reported in TBI subjects chronically treated with minocycline, an

antibiotic that can inhibit microglia activation (45). With this in mind, immunomodulatory rather than inhibitory strategies contributing to the resolution of inflammatory changes may prove effective, with a wide therapeutic window.

MSCs have high immunomodulatory potential both *in vitro* and *in vivo*. It has been suggested that in response to injury these cells can sense the injured environment, leading to the promotion of injury resolution and regenerative processes through the secretion of immunomodulatory bioactive factors and trophic molecules including growth factors, cytokines, and antioxidants

(46–48), that may vary in relation to the needs of the tissue and the time from injury.

Preclinical studies in rodents address the effects of MSCs in a wide range of TBI-to-therapy intervals, with consistent data from several laboratories showing their efficacy when infused 24 h post-TBI. Both central (49–60) and systemic (60–75) administration of MSCs 24 h post-TBI have resulted in early and persistent improvements of functional and structural outcomes. It was recently shown that a double systemic infusion of MSC (at 4 and 24 h) post-TBI was more effective than a single dose at 24 h (76). The authors showed 4 and 24 h post-TBI peaks of IL1 β , TNF α , and IFN γ , suggesting that a shorter lag time between TBI and treatment may be important to counteract early pro-inflammatory changes. Whether this gain in protection was due to multiple doses or the earlier treatment still needs to be fully investigated.

MSC infusion has also given protective effects when delivered in the sub-acute phase (between 2 and 7 days after injury) either systemically (77, 78) or centrally (79–81). Kota et al. administered bone-marrow MSCs (BM-MSCs) 3 days after TBI, showing IFN- γ and TNF- α reductions of ~50% (82), with significant inhibition of brain permeability, edema, microglial activation and systemic levels of norepinephrine, while promoting neurogenesis (83).

Effect size relative to the time from experimental TBI to MSC administration has been evaluated in a recent meta-analysis (10). The analysis confirms MSC efficacy when infused from 2 h up to 7 days, with no significant differences in effect sizes relative to the time from TBI to intervention.

So far there are only few reports of MSC given in the chronic phase of TBI. At 2 months post-TBI, MSC transplant into the lesion core improved sensorimotor deficits and promoted neuro-restorative processes (84, 85). However, at this stage iv injection was not effective (86), suggesting that MSC may act through complementary mechanisms when infused locally into the brain or systemically, the latter no longer being sufficient at later stages.

Compelling data on MSC rationale, efficacy, immune tolerance, and feasibility are fostering the design of clinical studies. Pragmatically, to optimize the reduction of toxic cascades and the promotion of endogenous reparative mechanisms, an administration of MSC within 48 h from TBI would seem to be desirable. However, only data from clinical trials will provide a definitive answer in term of the best timing for intervention.

DOUBLE-EDGED CONTRIBUTION OF MECHANISMS TO DAMAGE AND RECOVERY

Counteracting specific damage pathways should reduce the extent of tissue injury, and ultimately contribute to a better outcome. This is the logic behind several lines of investigation in TBI, from studies on calcium blockers to N-methyl-D-aspartate (NMDA) antagonists.

Under physiological conditions, glutamate plays an important role as a neurotransmitter; it is also involved in coupling glucose utilization and neuronal activity (87). However, high concentrations of glutamate (100–500 micromoles) are lethal for

neurons *in vitro*, and have been measured in the extracellular space of experimental TBI in rodents. In humans too there is evidence of high extracellular concentrations of glutamate after TBI, particularly in the elderly (88). This supported the hypothesis that blocking glutamate receptors (like the NMDA subtype) might attenuate the deleterious effects of the glutamate surge induced by TBI. Several compounds inhibiting the NMDA receptors, with competitive or non-competitive mechanisms, have therefore been tested in experimental and clinical settings. While in the laboratory evidence of neuroprotection was found (89, 90), clinical trials all failed to show benefit (91). Among the possible explanations for these repeated failures, there is the hypothesis that NMDA receptor activity is essential for neuronal function and integrity, so that NMDA blockage at critical time points (92), especially in vulnerable phases after TBI, could be deleterious rather than protective.

Another failed neuroprotective treatment is corticosteroids, which were tested in the first mega-trial in TBI at the end of the last century, “Corticosteroid randomization after significant head injury” (CRASH) (2). The hypothesis leading to this trial was that inflammation is a key component of the brain response to TBI and that blocking the inflammatory cascade could therefore be protective. Soluble mediators and cellular components of inflammation were investigated and related to the extent of brain damage. Unfortunately, however, the CRASH results showed no improvement in favorable outcomes and there was in fact a higher risk of mortality in the treatment group, not fully explained by systemic complications (such as infection and gastric bleeding); this may call into play the complex double-edged function of inflammation involved not only in toxic but also in regenerative processes, as discussed above. In this context MSCs affect the biology of the injured cells and tissue through the secretion of cytokines, morphogens, small molecules, and cargo-bearing exosomes (93, 94), which skew the activation of immune cells from a toxic to a more permissive phenotype, thus contributing to injury resolution and tissue repair (55, 58).

THE NEGLECTED ROLE OF REPARATIVE MECHANISMS

Besides toxic cascades TBI also induces neuro-restorative processes including neurogenesis, gliogenesis, angiogenesis, synaptic plasticity, and axonal sprouting (95–97). These events are induced by biochemical factors such as growth factors, steroids, and neurotransmitters, released in response to injury, with the potential for counteracting progression of the injury and contributing to functional recovery. However, all these spontaneous processes are short-lived and the efficacy of the self-repair responses is limited. Providing the injured tissue with a facilitatory milieu that increases endogenous reparative mechanisms may open up new therapeutic opportunities.

In the adult brain the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus (DG) are populated by neural stem cells, which can differentiate into functional neurons (95, 98). Proliferation in the DG is age-dependent, with higher potential in the juvenile brains. The new cells can

differentiate into functional mature neurons, involved in higher functions.

The neurogenic response after TBI comprises three phases: proliferation of precursors/progenitors cells, migration to injured tissue, and differentiation into proper cell types (99). An increased proliferative response in the hippocampus 2 days after TBI, with a peak in the first week after injury, has been described in different TBI models (100). These proliferating cells may differentiate into astrocytes, oligodendrocytes, and neurons, and extend projections alongside the hippocampal mossy fibers participating in recovery of function.

TBI induces a proliferative response in the neurogenic niche in the SVZ and hippocampus (101), under stimulation by growth factors. Preclinical studies have shown that intracerebral administration of single growth factors including fibroblast growth factor 2 (FGF-2) and epidermal growth factor (EGF) can promote endogenous neurogenesis after TBI (102, 103) and improve cognitive outcome. Similarly, infusion of VEGF into the lateral brain ventricle in TBI mice promotes cell proliferation in the SVZ and the peri-lesional cortex after TBI (104); VEGF in fact mediates the survival of newly generated neurons rather than proliferation of neuroblasts (105).

On account of their neurogenic and neuroprotective effects, growth factors are an interesting tool to stimulate reparative processes after TBI. However, their administration after injury is linked to temporal issues related to their rapid kinetics and limited effects. Studies in TBI rodents have shown increased amounts of growth factors after MSC treatment (52, 55, 64, 106–108), leading to the promotion of endogenous restorative processes and suggesting that MSCs may act as a local bioreactor able to produce and release a multitude of growth factors, depending on the specific requirements of the injured tissue.

It has been shown that MSCs stimulate endogenous neurogenesis with an higher proliferation rate in the SVZ and SGZ (64) and an increased number of developing neurons in the SVZ (detected as doublecortin marker) (57, 60); they also stimulate axonal regeneration, as documented by increased GAP-43 expression (58, 107) in MSC-treated TBI animals. Likewise, their ability to promote plasticity in TBI has been documented by infusing a fluorescent dye into the contralateral cortex 5 weeks after injury and measuring its transport from the injection site to the injured hemisphere through the corpus callosum 1 week later (79). Functional outcome and axonal fiber length were increased in MSC-treated animals, suggesting an MSC mediated effect on neuronal connectivity by directing axonal projections, neurite outgrowth and elongation in the injured cortex.

Another aspect linked to neuroplastic processes is represented by glial activation and extracellular matrix composition, both aspects possibly being modulated by MSCs. Acute glial activation is needed to clear excessive glutamate release and remove cellular debris (109, 110). However, at chronic stages, excessive gliotic scar may hamper remodeling processes (111). MSCs reduce

the gliotic scar surrounding the contusion 1 month after TBI and this effect is associated with a smaller lesion and better functional recovery (55, 57). MSCs can also alter the extracellular matrix composition, allowing restorative plasticity by circuit reorganization (112).

MSCs act also on vascular cerebral compartment, increasing vessel density in the pericontusional tissue after acute (24 h post-TBI) (57, 60, 113, 114), sub-acute (7 days) (115), and chronic (84) administrations. This suggests that rescue effects on injured vessels as well as regenerative action on brain vasculature involve mechanisms stimulated by cell therapy. In fact, gene expression microarray analysis showed MSC expression of genes involved in angiogenic processes possibly sustaining both neurovascular repair in the acute phase after injury and neovascularization later on (81).

CONCLUSIONS

Despite its high prevalence and heavy social burden, TBI remains a neglected syndrome. Acute care for TBI patients relies on maintenance of cerebral and body homeostasis, blunting or avoiding further insults. After more than 30 years looking for treatments, broadly defined as neuroprotective strategies, to reverse or mitigate injury progression in TBI, we still lack any effective therapy. The reasons for this failure have been extensively analyzed, and new therapeutic approaches for dealing with them could have higher translational potential. Experimental studies support the hypothesis that MSCs may overcome three of the major limitations. First, MSCs in animal models show efficacy when administered within the acute, sub-acute and delayed phases post-TBI, not being limited by a narrow window of opportunity. Second, preclinical data support the notion that MSCs influence a complex pathway such as inflammation, favoring restorative over deleterious aspects. Finally, the recognition that MSCs act on the injured environment fostering reparative processes, relies on a new paradigm, exploitation of the endogenous ability of self-repair.

In conclusion, MSCs have the potential to be the next candidate for neuroprotective trials in TBI patients.

AUTHORS CONTRIBUTIONS

MC, TZ, ERZ, and NS designed the review, assembled a preliminary draft, and incorporated further contributions from each author into subsequent versions. All the authors revised it critically for important intellectual content and approved the final version.

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Early Microglial Activation Following Closed-Head Concussive Injury Is Dominated by Pro-Inflammatory M-1 Type

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Microglial activation is a pathological hallmark of traumatic brain injury (TBI). Following brain injury, activated microglia/macrophages adopt different phenotypes, generally categorized as M-1, or classically activated, and M-2, or alternatively activated. While the M-1, or pro-inflammatory phenotype is detrimental to recovery, M-2, or the anti-inflammatory phenotype, aids in brain repair. Recent findings also suggest the existence of mixed phenotype following brain injury, where activated microglia simultaneously express both M-1 and M-2 markers. The present study sought to determine microglial activation states at early time points (6–72 h) following single or repeated concussive injury in rats. Closed-head concussive injury was modeled in rats using projectile concussive impact injury, with either single or repeated impacts (4 impacts, 1 h apart). Brain samples were examined using immunohistochemical staining, inflammatory gene profiling and real-time polymerase chain reaction analyses to detect concussive injury induced changes in microglial activation and phenotype in cortex and hippocampal regions. Our findings demonstrate robust microglial activation following concussive brain injury. Moreover, we show that multiple concussions induced a unique rod-shaped microglial morphology that was also observed in other diffuse brain injury models. Histological studies revealed a predominance of MHC-II positive M-1 phenotype in the post-concussive microglial milieu following multiple impacts. Although there was simultaneous expression of M-1 and M-2 markers, gene expression results indicate a clear dominance in M-1 pro-inflammatory markers following both single and repeated concussions. While the increase in M-1 markers quickly resolved after a single concussion, they persisted following repeated concussions, indicating a pro-inflammatory environment induced by multiple concussions that may delay recovery and contribute to long-lasting consequences of concussion.

Keywords: microglia, inflammation, polarization, concussion, traumatic brain injury

INTRODUCTION

The prevalence of closed-head concussive injury is high in both military and civilian population. In fact, closed-head mild traumatic brain injury (mTBI) is the most common form, comprising 70–90% of all TBI incidence (1–3). While patients typically recover quickly from a single concussive injury, cumulative effect from multiple concussions can cause enduring damage that may evolve into neurodegenerative diseases (4, 5). Notably, athletes with a history of repeated concussions show late-life memory problems, psychiatric illness and increased risk of progressive neurodegenerative diseases such as Alzheimer's disease, and chronic traumatic encephalopathy (CTE) (4–7). Although, the association between repetitive TBI and the increased risk for CTE is well-recognized, the pathological mechanisms leading to neurodegeneration are poorly understood. Repetitive TBI in athletes is associated with chronic activation of microglia and appears to mediate pTau pathology and dementia in CTE (8). We have developed a rat model for closed-head mTBI called projectile concussive impact (PCI) injury that mimics several aspects of human concussive injury (9–11). Using this injury model, we have demonstrated acute increases in inflammatory cytokines, persistent gliosis, chronic functional neurological impairments, and white matter thinning following single or repetitive hits (9).

Neuroinflammation is considered as an important pathological mechanism leading to brain damage. Following physical trauma, inflammatory responses occur. These responses, such as the activation of microglia and macrophages, as well as the local release of inflammatory cytokines, have both positive and negative effects on the brain (12–14). Beneficial effects of inflammatory responses include wound healing and repair, but if left uncontrolled, inflammation can lead to neuronal damage and impede recovery. Persistent neuroinflammation has been observed years after a single TBI (15). Additionally, a recent positron emission tomography (PET)-study using translocator protein 18 kDa (TSPO), a marker of activated glial cell response, reported higher glial reactivity in retired football players, suggesting ongoing neuroinflammation that may contribute to later onset of neuropsychiatric problems (16). Microglia is rich in damage-associated molecular patterns (DAMPs) sensors and therefore responds rapidly to injury after detecting DAMPs (17). Once activated, microglia release cytokines, and chemokines that attract peripheral immune cells to infiltrate the brain parenchyma (18). Additionally, microglia responds to the inflammatory environment by changing their morphology and by assuming specific activation phenotypes (17). Similar to peripheral macrophages, microglia modify their activation state depending on stimuli. Two unique microglial polarization states have been discovered, the M-1 type (classical phenotype) and the M-2 type (alternative phenotype) (19). While M-1 are pro-inflammatory in nature and detrimental to recovery, M-2 acts as anti-inflammatory and supports tissue repair (19). M-1 microglia secretes high levels of IFN- γ , TNF- α , IL-1 β , chemokines, and reactive oxygen species (20). The M-2 phenotype secretes neurotrophic factors and anti-inflammatory cytokines (IL-6, IL-10) and is sub-divided into M2a, M2b, and M2c depending

on their specific phenotype markers, and are induced by different conditions or triggering factors (21). However, recent research findings question the microglial polarization concept. Histological and single-cell RNA-sequencing experiments have shown that microglia co-expressed the markers for both “polarized” states following TBI (22, 23).

Microglial activation, acute increase in cytokine production, and white matter abnormalities are the major pathological features found across various animal models of concussive injury (9, 24–27). Although inflammatory responses such as glial reactivity and increased cytokine production are studied following concussive injury, the role of different microglial activation states in the development of mTBI pathology and their association with injury severity are largely unknown. Here we examined whether single or repeated concussions can alter M-1 and M-2 activation states in different brain regions. Furthermore, the present study characterized acute changes in microglial morphology following concussion(s) using the PCI model. We used gene expression profiling in both the cortex and hippocampus to understand M-1 and M-2 marker expression following either single or repetitive injury. Immunohistochemical staining and quantification were used to determine the location and ratio of M-1 to M-2 microglia. We found that both single and repetitive concussions induced microglial activation and M-1 phenotype dominated over M-2 state. While a single concussion induced M-1 marker expression resolved quickly, multiple hits prolonged the pro-inflammatory cytokine gene expression. In addition, we observed unique morphological changes in cortical microglia following repeat hits. Overall, our findings support the hypothesis that concussive injury altered expression of M-1 and M-2 markers and provide a novel target for early therapeutic interventions.

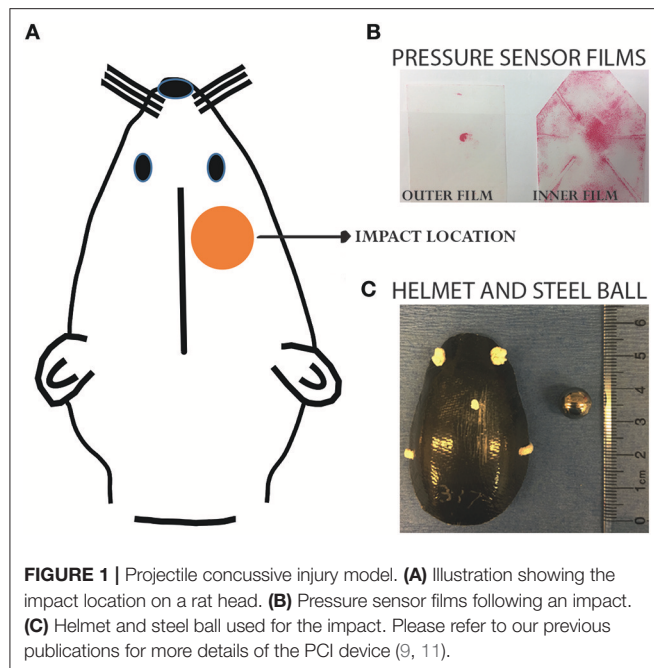
MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (300–330 g, 6–8 weeks, Charles River Labs, Raleigh, VA) were used in all experiments. During the 1 week quarantine period, animals were housed in pairs and then housed individually. Animals were housed in a normal 12 h light/dark cycle with access to food and water *ad libitum*. The animal housing facility was accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animal procedures were approved by the Institutional Animal Care and Use Committee of Walter Reed Army Institute of Research (WRAIR). Animal research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals, and adhered to the principles specified in the Guide for the Care and Use of Laboratory Animals, National Research Council (NRC) Publication, 2011 edition.

Projectile Concussive Impact (PCI) Model

Animals were randomly assigned to four groups: single sham (SS), single concussion (SC), repeated sham (RS), and repeated concussion (RC). The SC group received one projectile impact,



the RC group received 4 impacts spaced 1 h apart, and sham groups received the same procedures except projectile impact. Our previous studies have extensively characterized this closed-head concussive injury paradigm (9, 11). Briefly, rats were anesthetized with 4% isoflurane for 4 min in an induction chamber. A custom-designed carbon-fiber helmet (U.S. Army Research Laboratory, Aberdeen Proving Ground, MD) was placed on the rat's head. Pressure sensor films (Fujifilm pre-scale pressure sensitive film) were adhered to the inner and outer surface of the helmet to record the distribution and magnitude of pressure from the impact (**Figure 1**). The rat was then placed on an elevated platform in a supine position with its head positioned above an oval opening in the platform. The projectile was made of a stainless steel ball (3.5 g weight and 10.05 mm diameter) placed in a tightly fit silicone tube directly beneath the oval opening of platform at a distance of 5 cm. A computer-controlled program was used to trigger the pressurized, rapid-release of projectile aiming at the right, dorsal–frontal quadrant of the brain (**Figure 1**). Following the impact, the helmet and sensor films were removed and the animal was returned to its home cage. Animals were euthanized at 6 h and 72 h after the final impact.

Immunohistochemistry and Cell Counts

For immunohistochemistry processing, animals ($n = 5$ –6 per time point/condition) were deeply anesthetized with intramuscular injection of ketamine/xylazine mixture (70 and 6 mg/kg, respectively,) and transcardially perfused with saline followed by 4% paraformaldehyde (FD Neurotechnologies, Columbia MD). Whole brains were collected, post-fixed in 4% paraformaldehyde and cryoprotected using 30% sucrose solution. Cryoprotected brains were shipped to FD Neurotechnologies for further processing and staining. Coronal free-floating sections

(40 μ m) were processed for immunohistochemical staining using specific antibodies for Iba-1 (1:1,000, Rb polyclonal, Wako Chemicals, Richmond, VA), CD163 (1:100, Ms monoclonal, Hycult Biotech Plymouth Meeting, PA) and MHC-II (1:100, Ms monoclonal, Abcam, Cambridge, MA). Secondary antibodies were conjugated with either Alexa-488 or Alexa-594. Images were captured using a BX51 microscope equipped with multichannel filters (Olympus, Waltham, MA). Co-localized cells (Iba-1/CD163 and Iba-1/MHC-II) from 6 sections/brain (400 μ m apart) were counted from the cortex directly at 40X magnification using FITC/TRITC double filter.

Inflammatory Gene Array and Single Tube Real-Time PCR (qRT-PCR)

For measuring inflammatory gene expression, a separate cohort of animals ($n = 10$ per group) which received sham procedure, single concussion or repeated concussions were used. At 6 h and 72 h post-injury, animals were deeply anesthetized with intramuscular injection of ketamine/xylazine mixture (70 and 6 mg/kg, respectively) and euthanized using a guillotine. Ipsilateral cortex and hippocampus were quickly dissected out and snap frozen using liquid nitrogen. Total RNA was isolated from snap-frozen ipsilateral cortex and hippocampal tissue using the mirVana total RNA isolation kit (Ambion, AM1560) in accordance to the manufacturer's instructions. RNA concentration and quality were determined using NanoDrop Lite (Thermo Scientific, Pittsburgh, PA). Extracted RNA was immediately used to synthesize cDNA. One microgram (1 μ g) RNA was reverse transcribed using TaqMan Reverse Transcription reagents (Applied Biosystems, Carlsbad, CA). For custom designed TaqMan inflammatory profiling arrays, equal volumes of cDNA from each sample were pooled and run on a single 96 well PCR-array plate per cohort (e.g., SC 6 h, RC 6 h). M-1 and M-2 specific genes included in the profiling arrays were chosen based on literature evidence (22, 28–30). Selected genes from the profiling arrays were later validated using individual sample qRT-PCR. Amplifications of these gene transcripts were carried out in triplicate using TaqMan universal PCR Master Mix on an ABI Step One Plus real time PCR machine (Applied Biosystems). Gene expression was normalized to the endogenous control, succinate dehydrogenase complex flavoprotein subunit A (Sdha) and the fold change per condition was calculated using the $2^{-\Delta\Delta C_t}$ (C_t is the threshold cycle) method, compared to the sham group.

Data Analysis

Target genes of interest were determined by examining PCR-array. Genes were classified as either upregulated or downregulated when the change in expression relative to sham was greater or equal to +1.5 fold or less than or equal to -1 fold, respectively. To generate heat maps, changes in individual gene expression levels were expressed as fold change over respective sham at corresponding time points and injury condition. qRT-PCR data and immunostained cell counts were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test using GraphPad Prism version 7 software (La Jolla, CA). All data

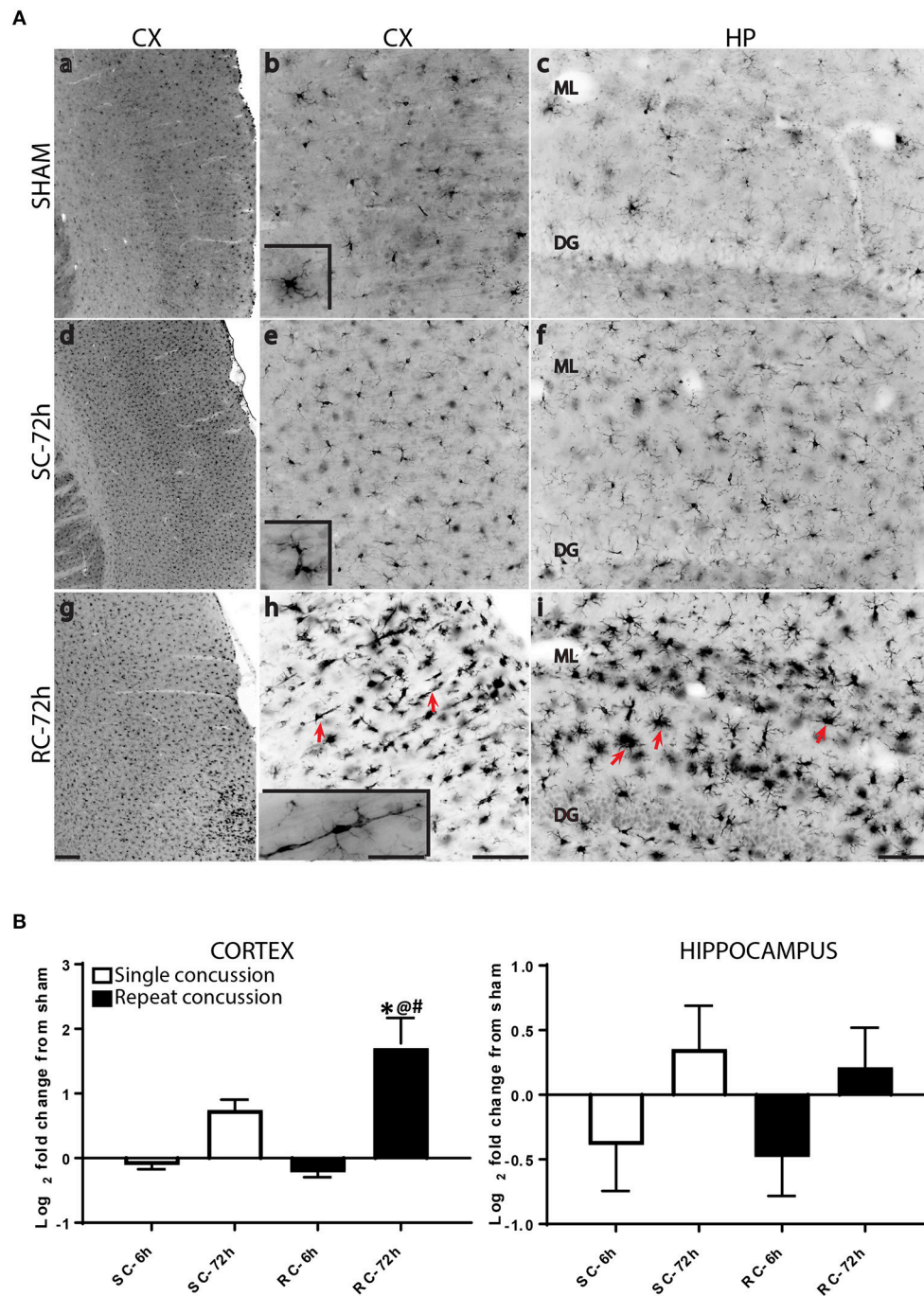


FIGURE 2 | Changes in microglial morphology and Iba-1 expression following concussive injury. **(A)** Iba-1 immunohistochemical staining in the ipsilateral cortex and hippocampus at 72 h following either single or repeated concussion. Resting, ramified microglia was observed in the sham cortex and hippocampus (top panel, **a–c**). Following single hit, microglia showed activated morphology with retracted processes (middle panel, **d–f**). Repeated hits showed elongated microglia (**g,h**, arrows) in the cortex and bushy, hypertrophied microglia (**i**, arrows) in the hippocampus. **(B)** Quantification of Iba-1 gene expression in the cortex and hippocampus. One-way ANOVA with Tukey's *post-hoc* test. **p* < 0.05 compared to single hit 6 h. @*p* < 0.05 compared to repeated hit 6 h. #*p* < 0.05 compared to Single hit 72 h. SC: single concussion, RC: repeated concussion, CX: cortex, HP: hippocampus, ML: molecular layer, DG: dentate gyrus. Scale bar in g (for left panel) = 200 μ m, in h (middle panel) and i (left panel) = 100 μ m and inset (for insets in middle panel) in h = 50 μ m.

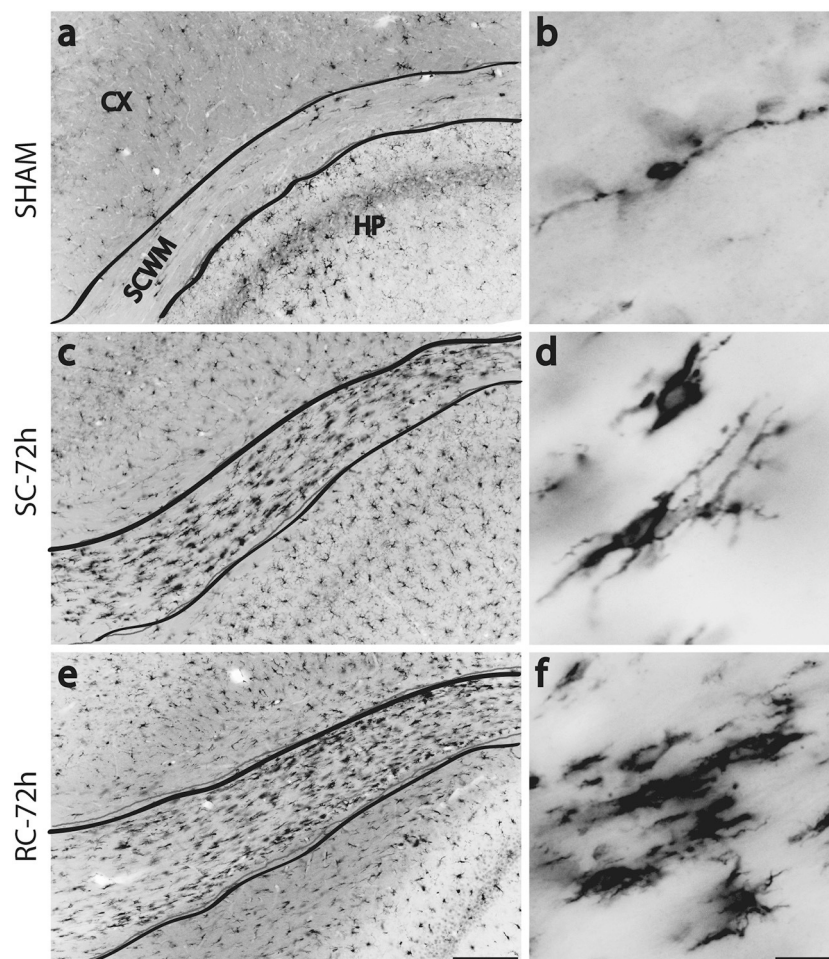


FIGURE 3 | Changes in Iba-1 immunoreactivity in sub cortical white matter (SCWM) at 72h post injury. Few scattered microglia with migrating morphology (long processes) were found in sham rat's SCWM (**a,b**). Numerous, hypertrophied microglia were found in the SCWM following either single (**c,d**) or repeated concussions (**e,f**). SC: single concussion, RC: repeated concussion, CX: cortex, HP: hippocampus, SCWM: subcortical white matter. Scale bar in e (for left panel) = 200 μ m and in f (for right panel) = 50 μ m.

are presented as mean + SEM and for all comparisons a p -value <0.05 was considered significant.

RESULTS

Concussive Injury Altered Microglial Morphology and Iba-1 Gene Expression

While resting microglia has a ramified morphology, activation is characterized by a hypertrophied, bushy phenotype. We observed ramified microglia following sham injury (**Figure 2A** top panel) and activated phenotypes following either single (**Figure 2A** middle panel) or repeated concussion (**Figure 2A**, bottom panel). Although mild microglial reactivity was observed at 6h post-injury, microglial morphological change was clearly noticeable at 72h after repeated hits in entorhinal cortex, hippocampus and sub-cortical white matter (SCWM). While cortical microglia retracted processes following a single concussion (**Figures 2A,d,e**), multiple concussions induced

both hypertrophied and elongated, radial microglia in the entorhinal cortex (**Figures 2A,g,h**). Some of these elongated radial microglia were seen coupled together to form train-like elongated structures (**Figures 2A,h-inset**). Hypertrophied bushy microglia were seen in both hippocampus (**Figures 2A,i**) and SCWM (**Figure 3**) following concussive injury.

We also quantified Iba-1 mRNA levels in both the cortex and hippocampus, using qRT-PCR. While cortical Iba-1 gene expression mirrored the histological observations, hippocampal Iba-1 gene expression showed a modest increase at 72 h following concussive injury (**Figure 2B**).

Concussive Injury Altered Inflammatory Gene Expression Profile in the Cortex and Hippocampus

Inflammatory gene profiling was performed separately in cortex and hippocampus tissue using a custom designed PCR-array plate. Based on literature searches, we included 86 total M-1 and

M-2 targets and 2 housekeeping genes (**Table 1**) in the PCR-array. Comparative analysis revealed 36 M-1 markers and 16 M-2 markers that were altered relative to their respective sham groups in the ipsilateral cortex (**Figure 4**). Single hit induced a brief upregulation of 25 M-1 related targets evident at 6 h post-injury, most of which returned to sham levels by 72 h post-injury, except for tissue inhibitor of metalloproteinases-1 (Timp1) (**Figure 4**). The only M-1 target that showed a downregulation at 72 h after a single hit was Chemokine (C-C motif) ligand 19 (Ccl19). Following repeated hits, 29 M-1 genes in the ipsilateral cortex were altered at 6 h post-injury. In contrast to the profile after a single hit, most of these genes remained altered at 72 h post-injury, including the downregulation of Ccl19, indicating a persistent response following repeated injuries. Although fewer number of altered M-2 markers were detected compared to M-1 markers, M-2 targets in the cortex also exhibited a similar pattern with prolonged expression shifts following repeated hits than a single concussion (**Figure 4**). While several of M-1 markers showed expression levels higher than 6 fold, only Ccl22 among M-2 markers was upregulated 6 fold, indicating a clear dominance in M-1 marker upregulation following concussion (**Figure 4**).

Both single and repeated concussions also induced a transient change in M-1 marker expression in the hippocampus (**Figure 5**). Comparative analysis revealed that 25 M-1 markers and 9 M-2 markers were altered relative to sham expression levels in the ipsilateral hippocampus (**Figure 5**). Most of the hippocampal M-1 targets were upregulated except for downregulation of Tlr3, Timp3, and Cxcl6 after concussion. The expression patterns of several M-1 markers, including Nos2, Ccl2, and Il1b, were similar between cortex and hippocampus. The hippocampal M-2 markers were mostly downregulated (example: VEGF, Gata3, IL25) except for Ccl22, which was upregulated as observed in the cortex following concussion (**Figure 5**). Interestingly, the M-1 target, Timp1, is the only common gene that was upregulated in all four conditions (single and repeated concussions at 6 and 72 h) in both the cortex and hippocampus.

We validated the expression levels of selected targets using individual qRT-PCR in both the cortex and hippocampus (**Figures 6, 7**). Target TaqMan array IDs are given in **supplementary Table 1**. We have also included RT-1HA, a component of MHC-II complex (**Figures 6A, 7A**) and Arg-1, a well-characterized M-2 marker previously shown altered expression following TBI (**Figures 6B, 7B**). Our individual RT-PCR runs echoed the inflammatory array pattern demonstrating a predominance for M-1 related gene upregulation following concussive injury.

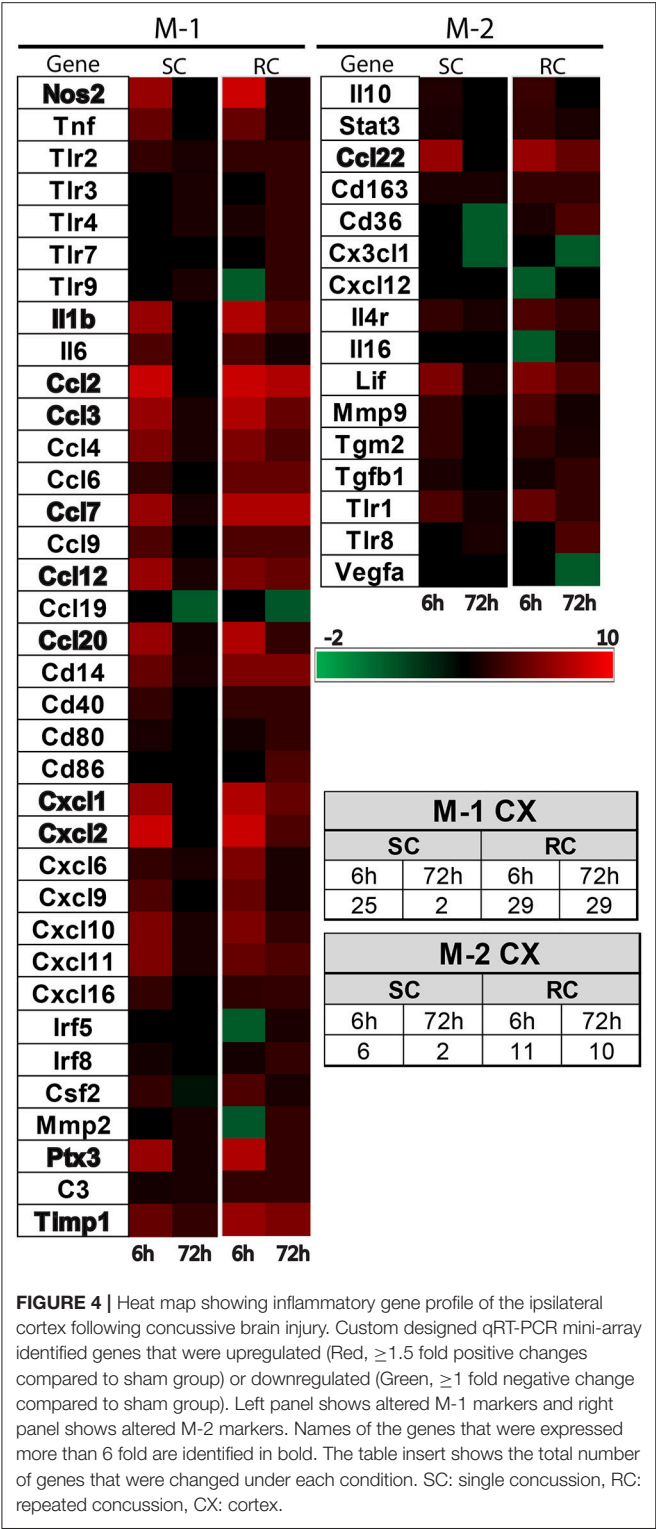
Pro-Inflammatory M-1 Type Cells Predominates Microglial Milieu Following Concussive Injury

We used MHC-II/Iba-1 for M-1 and CD163/Iba-1 for M-2 microglial labeling. Although we did see gene expression changes following qRT-PCR in the hippocampus, only very few, scattered M-1 or M-2 positive cells were seen in the hippocampus

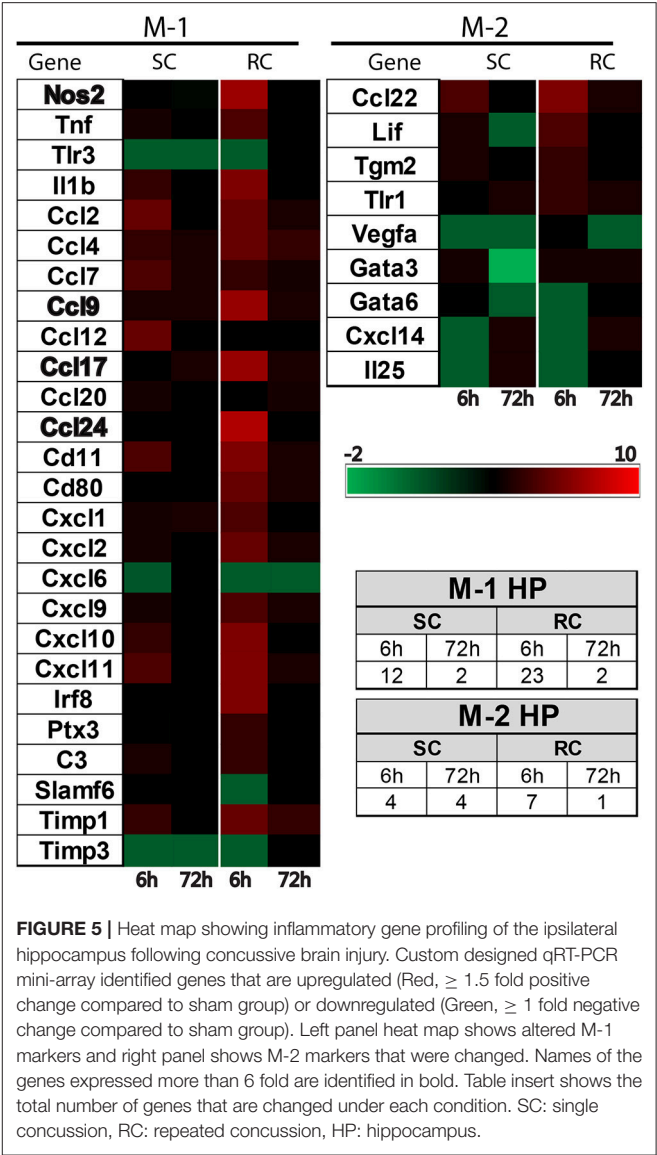
TABLE 1 | Gene table for inflammation profile array genes and their TaqMan IDs.

Rn00691090_m1	Rn01464736_g1	Rn00584362_m1	Rn01483988_g1	Rn01762214_m1	Rn00567818_m1	Rn00671924_m1	Rn00709368_m1	Rn01625859_g1
Arg1	Ccl3	Cd40lg	Il10	Irf8	Slamf6	Ccl4	Cd80	Tnf
Rn00587615_m1	Rn00676341_m1	Rn00583505_m1	Rn01752376_m1	Rn00571654_m1	Rn00680715_m1	Rn00567011_m1	Rn01505881_m1	Rn00569995_m1
Il13	Jak2	Stat1	Ccl1	Cd86	Il15	Stat5a	Stat6	Ccl11
Rn01467286_m1	Rn01471276_m1	Rn01464638_m1	Rn01536936_g1	Rn01400117_g1	Rn01536591_m1	Rn01481451_m1	Rn01403352_m1	Rn00572656_g1
Ccl7	Ccl9	Ccl12	Ccl17	Ccl20	Ccl2	Ccl24	Ccl25	Ccl14
Rn00580728_m1	Rn01423584_g1	Rn00593186_m1	Rn00578225_m1	Rn00788261_g1	Rn00573260_m1	Rn01496393_m1	Rn00595504_m1	Rn00569848_m1
Cd36	Cd40	Cx3d1	Cxcl1	Cxcl11	Cxcl12	Cxcl14	Cxcl9	Tlr4
Rn04224332_u1	Rn01640054_m1	Rn00678341_g1	Rn01441684_s1	Rn00569873_m1	Rn00594078_m1	Rn01477715_m1	Rn00580432_m1	Rn01759835_m1
Tlr8	Tlr9	Dusp1	Fpr1	Gata6	Irfng	Il1b	Il25	Il33
Rn01410330_m1	Rn01435145_m1	Rn01500522_m1	Rn00573491_g1	Rn00821234_g1	Rn01538170_m1	Rn00561646_m1	Rn00440945_m1	Rn01769850_m1
Il6	Irf4	Irf5	Lif	Marco	Mmp2	Nos2	Pparg	Ptx3
Rn00571440_m1	Rn01430873_g1	Rn00441826_m1	Rn04181452_s1	Rn01488472_g1	Rn01507024_m1	Rn00573587_g1	Rn01767369_m1	Rn01456850_m1
Tgm2	Timp1	Timp3	Tlr1	Tlr2	Il4r	Cxcl6	Cd209a	Cst2
Rn01511602_m1	Rn01490246_m1	Rn00566486_m1	Rn01414231_m1	Rn01492519_m1	Rn01456866_m1	Rn00572010_m1	Rn00590475_m1	Rn99999916_s1
Vegfa	Chit1	C3	Cxcl3	Ccl6	Il4	Tgfb1	Sdha	Gapdh
				Cd163	Tlr7			

Bold items indicate gene name, followed by their respective ID.



following immunohistochemistry and therefore was not included in the counting. Co-labeled cells were counted directly from the ipsilateral cortex (6 sections/brain) using a double filter. While most of the MHC-II positive cells co-localized with Iba-1 microglia, there were cells that did not co-localize (Figure 8A).



While few occasional MHC-II positive cells were observed following sham procedure or single concussion, repeated concussion induced robust expression of MHC-II (Figure 8A), mainly at 72 h post-injury. MHC-II positive glia (M-1) were mainly located in the entorhinal part of the cortex (Figure 8A). In contrast to MHC-II cells, CD163 positive M-2 cells were found throughout the cortex in both sham and injured rats. Although there were more CD163/Iba-1 positive cells (M-2) than MHC-II cells at 6 h post-injury, this anti-inflammatory M-2 expression appeared to be transient (Figure 8B).

The M-1/M-2 ratio can be used to evaluate pro- (above 1:1 ratio) or anti- (below 1:1 ratio) inflammatory environment. The M-1/M-2 ratio showed a significant increase at 72 h following multiple impacts (Figure 9) compared to sham and single concussion, indicating that a majority of the activated microglia following repeated impacts were pro-inflammatory. Low M-1/M-2 ratio indicating an anti-inflammatory environment was

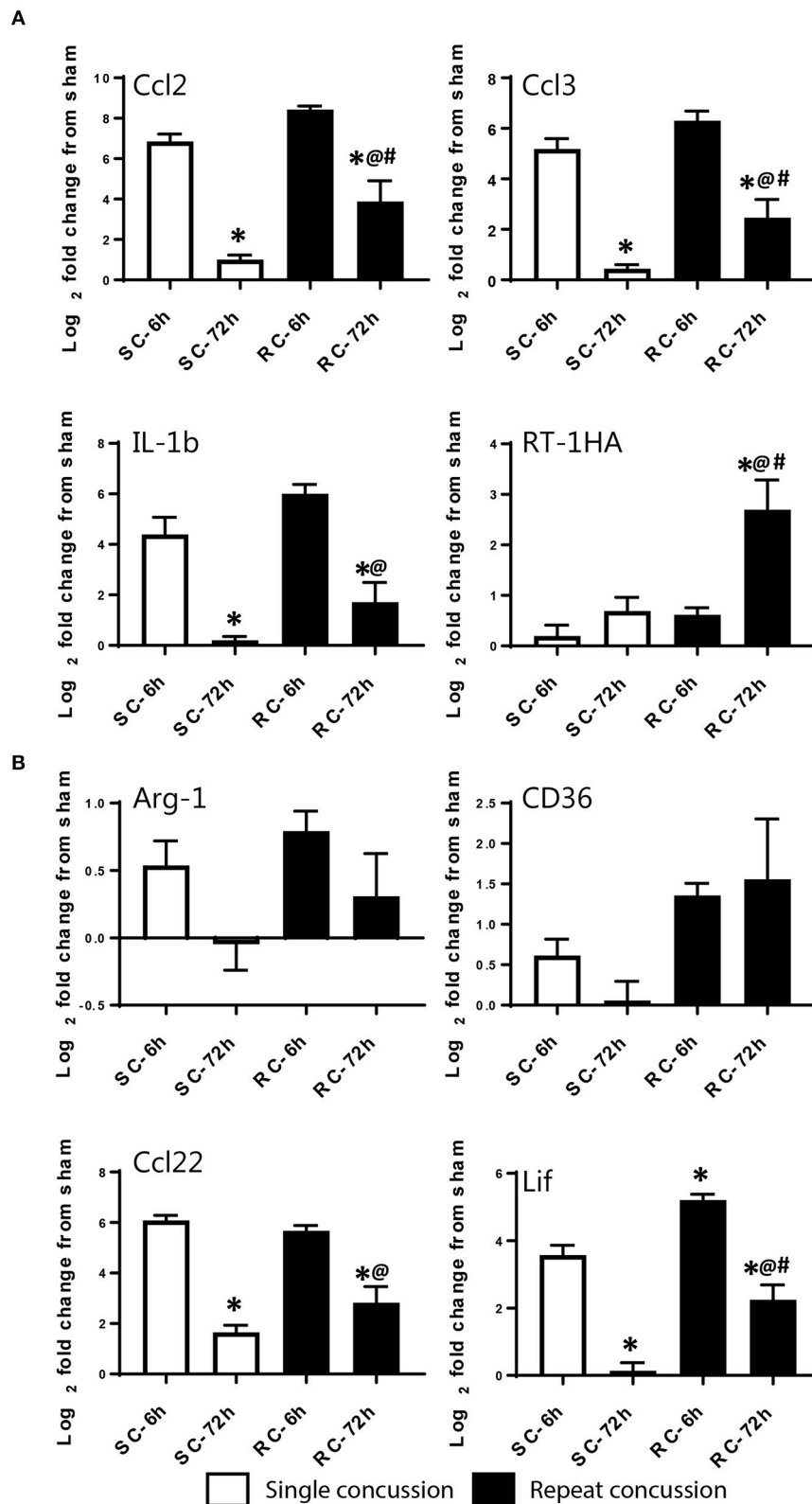


FIGURE 6 | Single gene qRT-PCR of selected targets in the ipsilateral cortex. Selected gene targets including both (A) M-1 and (B) M-2 markers are run separately using individual samples ($n = 10/\text{condition}$). RT-1HA was included as this is part of MHC-II complex. One-way ANOVA with Tukey's *post-hoc* test. * $p < 0.05$ compared to single hit 6 h. @ $p < 0.05$ compared to repeated hit 6 h. # $p < 0.05$ compared to Single hit 72 h. SC: single concussion, RC: repeated concussion, CX: cortex.

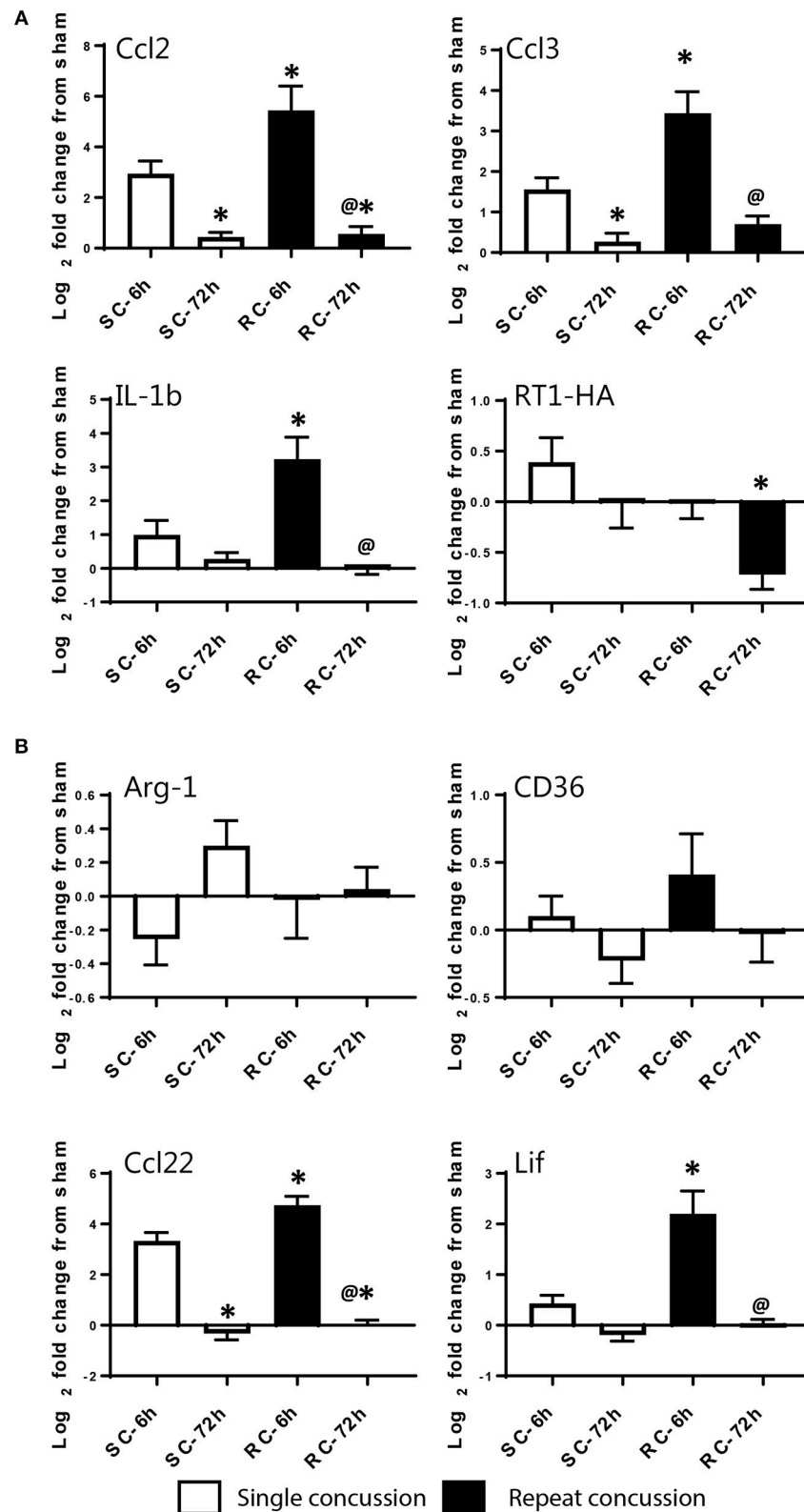


FIGURE 7 | Single gene qRT-PCR of selected targets in the ipsilateral hippocampus. Selected gene targets including both (A) M-1 and (B) M-2 markers are run separately using individual samples ($n = 10/\text{condition}$). RT-1HA was included as this is part of MHC-II complex. One-way ANOVA with Tukey's *post-hoc* test. * $p < 0.05$ compared to single hit 6 h. @ $p < 0.05$ compared to repeated hit 6 h. SC: single concussion, RC: repeated concussion, HP: hippocampus.

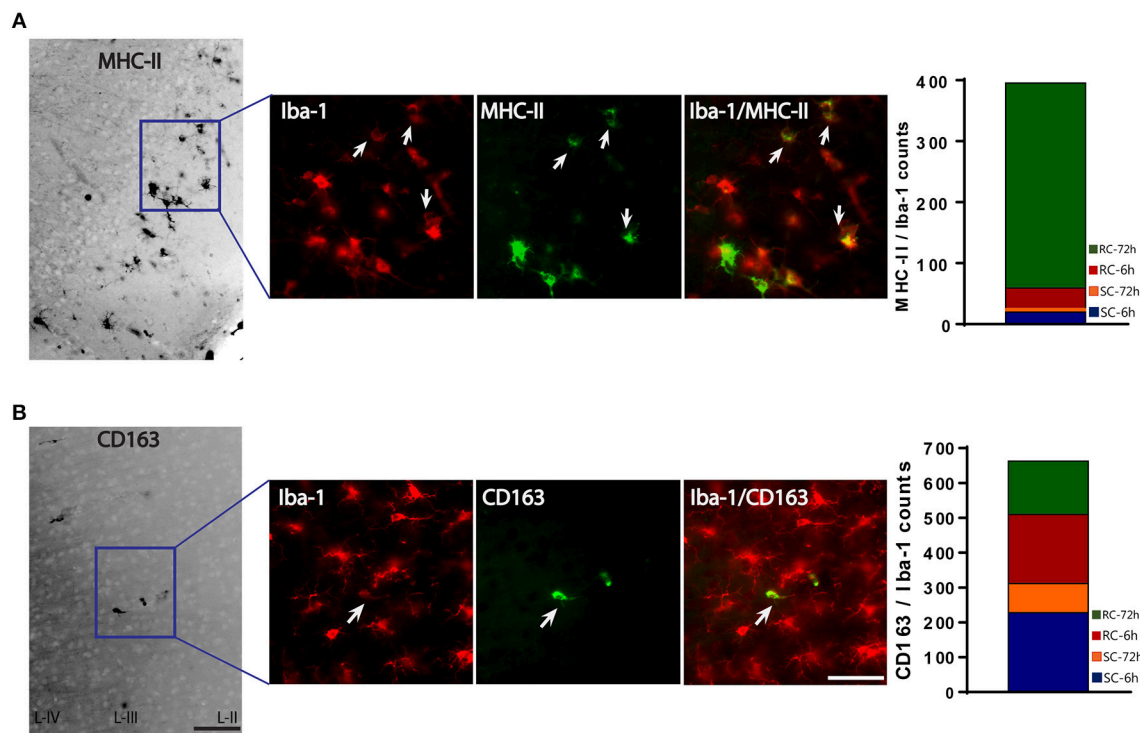


FIGURE 8 | M-1 and M-2 marker expression by activated microglia in the ipsilateral cortex. **(A)** MHC-II (M-1 marker) stained microglia co-labeled with Iba-1 were found mainly in the entorhinal cortex. Co-localized cells were counted from entire cortex. **(B)** CD163 (M-2 marker) stained microglia co-labeled with Iba-1. Co-localized cells were counted from entire cortex (bar graphs). Arrows indicate co-localized cells. Scale bar = 50 μ m.

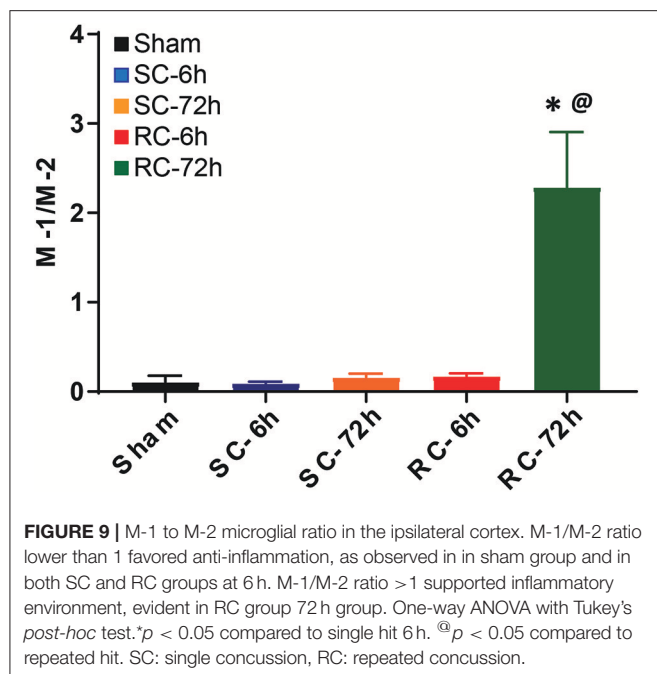
observed in sham rats, because of the presence of CD163 positive cells (**Figure 9**). Although single concussion and 6 h repeated concussion groups had low M-1/M-2 ratio, this was not significantly different from sham group (**Figure 9**).

DISCUSSION

The goal of the current study was to analyze early changes in microglial activation following single or repetitive closed-head concussive injury. Although the link between concussive injury and chronic neurodegeneration is well-established, early pathological changes following concussion is less explored. Increased glial reactivity (both microglia and astrocytes) was visualized by PET imaging in young, active NFL players indicating the onset of early neuroinflammation in the absence of gross morphological or functional changes (16). This highlights the importance of studying early changes following concussion, which may help to design therapeutics that could be administered before the onset of neurodegenerative pathology. We have previously reported increased levels of inflammatory cytokines in the cerebro-spinal fluid (CSF) and serum following repeated concussive injuries in rats (9). Cytokine-induced neutrophil chemoattractant 1 (CINC-1), TIMP-1 and L-selectin were increased as early as 1 h following multiple concussions (9). These cytokine changes in biofluids may reflect acute brain inflammation, as well as cytokine synthesis and secretion from reactive microglia/macrophages. To better understand the

microglial changes in concussed brain, we performed a detailed phenotypic analysis of M-1 or M-2 like marker expression at 6 and 72 h following single and repeated concussions by using custom designed qRT-PCR array. We observed an increase in the expression of several M-1 targets and a few M-2 markers following both single and repeated hit injuries. mRNA levels of several cytokines including Ccl2, Ccl3, IL-1b, Ccl22, and Lif were found to be upregulated. While the increase was transient following single hit, it appeared to continue for days following repeat hits. Further, we found unique morphological changes in the microglia following repeated injuries. Collectively, these data suggest early microglial changes including both morphological and phenotypic alterations following concussive brain injury.

Microglia respond to the injury milieu in part through their morphology. Brain injury alters the activation state of microglia that is clearly characterized by distinct morphological features (28). Although morphology does not reliably reflect the functions or RNA expression profile phenotypes, it indicates that the cell is responding to altered homeostasis and researchers consider morphological change as an indication of neuroinflammation. Compared to healthy ramified microglia, reactive microglia comes in various shapes including amoeboid, hypertrophied and bushy (31). In our study, microglial morphological changes were more prominent following multiple impacts. By 72 h, many hypertrophied, bushy microglia were found in the cortex, hippocampus, and SCWM. This was not surprising, as active hypertrophied microglia has been observed in both single and



repeated closed head injury (CHI) (24, 32, 33). However, we did not observe amoeboid microglia as opposed to focal TBI where amoeboid glial cells were found throughout the contused tissue (34). Besides the classical activation morphology, we also observed radial, train-like microglial formations in the entorhinal cortex following repeated hits. This distinct, rod shaped microglial morphology was previously reported following fluid percussion injury (FPI) in rats (35, 36). The exact function of these train-like glial formations are not yet known. In our histological studies, they did not co-localize with either M-1 marker MHC-II or M-2 marker CD163. However, this does not necessarily mean that they are not M-1 or M-2. It is possible that they may express other M-1 or M-2 targets. It appears that this interesting microglial shape is unique to diffuse type brain injury (35–37), suggesting that concussive injury produced by the PCI model shares pathological similarities with other diffused injury models.

Although shifts in microglial phenotypes are well-studied in moderate TBI using controlled cortical impact (CCI) or FPI models (22, 38–41), dynamics of microglial polarization is largely unknown following CHI. To our knowledge, only one study reported increased M-1 marker OX-6 or MHC-II staining in microglia like cells following closed head blast injury (42) that however did not examine multiple M-1 and M-2 markers to determine their polarization bias. In the present study, we designed a custom inflammation gene array plate which contained probes for known M-1 like and M-2 like markers (21, 22) in order to specifically profile inflammatory cytokines and chemokines in the brain following single and multiple concussive injuries. While several M-1 markers (Nos2, IL-1b, Ccl2, Ccl3, Ccl7, Ccl12, Ccl20, Cxcl1, Cxcl2, Ptx3, Timp1) showed significant upregulation, only a few M-2 markers (Ccl22, Lif) showed an increase, indicating the predominance of pro-inflammatory

M-1 phenotype following concussion. These findings are akin to other TBI studies, where similar M-1 targets showed an increase following TBI (22, 40, 43). While focal TBI studies show changes in inflammation-related cytokine/chemokine expression lasting for weeks post TBI, in our model of single concussion, majority of the M-1 markers that upregulated at 6 h was resolved by 72 h. However, we observed persistent inflammatory response following multiple hits, indicating that the cumulative pathological response following repetitive injury took longer to resolve. Increased neuroinflammation as early as 24 h that lasted for weeks to years has been reported in preclinical studies of repetitive CHI (32, 44–46). Our previous study using PCI model also showed more robust pathology following multiple impacts. Compared to a single impact, acute increase in cytokine levels and GFAP expression were observed following repeated concussions (9–11). It is possible that this acute neuroinflammation may lead to long-lasting behavioral deficits. We have observed chronic functional impairment following both single and repeated impacts in the PCI model (9). Limiting acute neuroinflammatory responses may delay or halt chronic behavioral impairments that develop following concussion. Notably, acute administration of MW151, an inhibitor of brain proinflammatory cytokine upregulation have been shown to prevent chronic cognitive impairment following CHI in mice (37).

M-2 marker expression following concussion(s) in the PCI model differs from that of focal-TBI models. Our immunoprofiling did not show a change in M-2 marker Arginase-1 as it was found to be upregulated following focal TBI (22, 43, 47). In another study, Arginase positive M-2 microglia was detected following CCI injury and its number was increased by IL-2/anti-IL-2 complex treatment (26). It is possible that in our studies, PCI induced a more diffused pathology that is different from the focal-CCI injury model. Supporting this notion, Arginase-1 expression was not found to be altered after FPI in rats (35), a diffuse form of TBI. Arginase-1 is the arginine degrading enzyme that plays a role in anti-inflammation, cell survival and regeneration (48). Arginase-1 expression in microglia and macrophages are thought to drive them toward a M2 phenotype. However, flow cytometric analysis of isolated macrophage/microglia from injured brain demonstrated that 42% of Arginase positive cells co-expressed iNOS, a M-1 marker, indicating concurrent expression pattern of both M-1 and M-2 markers following TBI (40). Co-existence of M-1 and M-2 markers are reported in other TBI studies as well. Using a reporter mouse model for Arginase-1 expression, brain infiltrating macrophages following TBI identified as Arg1+ or Arg1-, revealed simultaneous gene expression of pro- and anti-inflammatory chemokines (49). Following severe CCI injury, a spectrum of macrophagic and microglial phenotypes were observed rather than showing a bias toward M-1 or M-2 (41). Mixed microglial phenotype with simultaneous expression of M-1 and M-2 markers were also reported following CCI in mice (22). In our concussion model, although we observed a mixture of M-1 and M-2 phenotypes, M-1 markers clearly dominated the acute injury environment. Both cortex and hippocampal regions showed similar M-1 response.

One of the highly expressed (>8) M-1 target was *Nos2*, the gene that codes for the enzyme inducible Nitric oxide synthase (iNOS) that is responsible for producing pathological form of nitric oxide (NO). Once activated, *Nos2* can produce NO for hours to days contributing to oxidative damage to neighboring neurons (50). Acute increase in iNOS expression was observed in isolated microglia/macrophages following TBI (40) and inhibition of iNOS is shown to be neuroprotective following TBI (51). Another interesting neuroinflammatory marker upregulated across all conditions was TIMP-1. TIMP-1 is the inducible form of TIMPs that is known to be upregulated by inflammatory stimuli such as IL-1b and TNF- α (52, 53). Elevated TIMP-1 levels were observed in the CSF of TBI patients (54). Our previous study also demonstrated increased TIMP-1 levels in both serum and CSF following concussion (9). It is possible that the increase in TIMP-1 expression in the brain may have contributed to its elevation in biofluids. The elevated expression of M-1 markers such as *Nos2* and TIMP-1 points to the presence of an early inflammatory environment following CHI. Interventions to curb M-1 phenotype expression may provide therapeutic benefits. Recent research on microglial phenotype emphasizes the therapeutic importance of shifting M-1 type to M-2 in reducing neuroinflammation. Treatments with Omega-3, HMGB-1 inhibitor glycyrrhizin, stem cell exosomes, atorvastatin and therapeutic hypothermia (55–58) were all shown to enhance M-2 polarization while reducing neuroinflammation following TBI.

We did not perform qRT-PCR studies using isolated microglia, and therefore, multiple cell types may have contributed to the cytokine/chemokine gene expression. To confirm the localization of M-1 and M-2 markers to microglia, we performed co-localization studies using microglial marker Iba-1. Quantification of MHC-II/Iba-1 (M-1 type) and CD163/Iba-1 (M-2 type) from cortical regions confirmed the RT-PCR results that showed a dominance in M-1 type following multiple hits at 72 h. However, at 6 h following single or repeated injuries, more CD163 cells were observed compared to MHC-II positive cells indicating more M-2 type cells than M-1 type at 6 h following concussion. Therefore, it appears that immediate post-injury environment is anti-inflammatory. However, the ratio of M-1/M-2 was not significantly different from sham following single concussion or 6 h repeated concussion. However, following focal-injury, transient upregulation of M-2 like phenotype later replaced by M-1 type was observed (40). At 6 h post-injury, the mRNA levels did not directly translate into the histological results. It is probable that the cell's defensive mechanisms to control neuroinflammation such as microRNAs that block inflammatory mRNAs are active immediately following a concussion. Interestingly, we previously observed increased mir-145 levels in circulation following concussive injury (9). In polarized microglia, mir-145 increase is strongly associated with M-2 phenotype (29), supporting our notion that microRNA-mediated regulatory mechanisms may be involved in favoring M-2 phenotype immediately after injury.

There are several limitations in the current study, particularly in examining microglial phenotypes following concussive injury. Although qPCR results show a clear dominance in

pro-inflammatory markers, our study did not examine the source of these mRNAs. Repeated concussive injury is reported to have macrophage infiltration into the injured parenchyma (59) and it is likely that in our study macrophages contributed partly to the post-injury inflammation. Although we used Iba-1 immunohistochemistry to detect microglia, macrophages also express Iba-1 and may result in false positive staining. Recent research identified neurotoxic A1 astrocytes and inflammatory neurons that secrete cytokines and chemokines triggering secondary damage cascade (60, 61). To some extent they may be also responsible for the M-1/M-2 marker upregulation. Our study did not address the possibility of co-expression of M-1 and M-2 markers in the same microglia. On the other hand, we observed upregulation of both M-1 and M-2 markers indicating that inflammatory milieu following concussive injury was not exclusively M-1 or M-2 type microglia. Another limitation for the current study is the lack of functional correlates. Although the current study did not include any functional outcome metric, our previous study has extensively characterized the behavioral changes following concussion(s) at both acute and chronic time points (9). Using the same concussion paradigm in rats, Mountney et al observed that repeated concussions impaired motor function and produced gait abnormalities. It is possible that the early neuroinflammatory changes detected in the present study may have contributed to the development of behavioral dysfunction following repeated concussions in the PCI model.

Our study describes early changes in microglial phenotype and morphology following closed-head concussive injury in rats. Although pro-inflammatory M-1 phenotype dominates the early post-injury environment, a few M-2 markers were also elevated demonstrating that both types co-exist following injury. While a single concussion induced transient upregulation in inflammatory markers, multiple impacts exacerbated and sustained the response providing caution against sustaining multiple concussions while the brain is susceptible. Targeting microglial sub-types as opposed to reducing global microglial activation may provide a novel therapeutic approach for treating CHI.

DISCLOSURE

This material has been reviewed by the Walter Reed Army Institute of Research (WRAIR). There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army, Department of Defense, the Uniformed Services University of the Health Sciences or any other agency of the U.S. Government.

AUTHOR CONTRIBUTIONS

SM and BW designed the study. SM, BW, SU, WY, and LL performed data collection and data analysis. SM wrote the article.

BW, SU, LL, JG, and DS contributed to data interpretation and critical evaluation of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fneur.2018.00964/full#supplementary-material>

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Enduring Neuroprotective Effect of Subacute Neural Stem Cell Transplantation After Penetrating TBI

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Traumatic brain injury (TBI) is the largest cause of death and disability of persons under 45 years old, worldwide. Independent of the distribution, outcomes such as disability are associated with huge societal costs. The heterogeneity of TBI and its complicated biological response have helped clarify the limitations of current pharmacological approaches to TBI management. Five decades of effort have made some strides in reducing TBI mortality but little progress has been made to mitigate TBI-induced disability. Lessons learned from the failure of numerous randomized clinical trials and the inability to scale up results from single center clinical trials with neuroprotective agents led to the formation of organizations such as the Neurological Emergencies Treatment Trials (NETT) Network, and international collaborative comparative effectiveness research (CER) to re-orient TBI clinical research. With initiatives such as TRACK-TBI, generating rich and comprehensive human datasets with demographic, clinical, genomic, proteomic, imaging, and detailed outcome data across multiple time points has become the focus of the field in the United States (US). In addition, government institutions such as the US Department of Defense are investing in groups such as Operation Brain Trauma Therapy (OBTT), a multicenter, pre-clinical drug-screening consortium to address the barriers in translation. The consensus from such efforts including “The Lancet Neurology Commission” and current literature is that unmitigated cell death processes, incomplete debris clearance, aberrant neurotoxic immune, and glia cell response induce progressive tissue loss and spatiotemporal magnification of primary TBI. Our analysis suggests that the focus of neuroprotection research needs to shift from protecting dying and injured neurons at acute time points to modulating the aberrant glial response in sub-acute and chronic time points. One unexpected agent with neuroprotective properties that shows promise is transplantation of neural stem cells. In this review we present

(i) a short survey of TBI epidemiology and summary of current care, (ii) findings of past neuroprotective clinical trials and possible reasons for failure based upon insights from human and preclinical TBI pathophysiology studies, including our group's inflammation-centered approach, (iii) the unmet need of TBI and unproven treatments and lastly, (iv) present evidence to support the rationale for sub-acute neural stem cell therapy to mediate enduring neuroprotection.

Keywords: traumatic brain injury, inflammasome, pyroptosis, neural stem cell, cell transplantation

INTRODUCTION

TBI is a critical public health problem and one of the leading causes of death and disability around the globe (1–5). The World Health Organization (WHO) and the World Bank estimate that 69 million (95% CI 64–74 million) individuals suffer from TBI every year, with Southeast Asian and Western Pacific regions experiencing the greatest overall burden (6). A recent estimate of the Global Incidence of TBI puts it at ~939 cases per 100,000 people each year with 79% being mild TBI. The calculated incidence of TBI in the Americas (including United States (US) /Canada) is 1,299 cases per 100,000 people each year. The calculated incidence for Latin America is about 909 per 100,000 people each year (7). Worldwide about 90% of all TBI-related deaths occur in developing countries (8). In 2016, road traffic injuries were among the three leading causes of death from injuries independent of gender. The economic status (a surrogate for investment in health care, trauma centers, and road safety) of the country rather than its global location appear to influence trauma outcomes. For example, in the poorest country in the Western Hemisphere, road traffic accidents accounted for >40% of TBI incidence (9). Similarly, African and Eastern Mediterranean regions are above the global average while the rest are on par or below (6, 10). Within the US, road traffic accidents have been on the decline and apart from age related vulnerability to falls, firearm injury has become an increasingly serious problem (11, 12). Overall, TBI affects 1.7 million people in the US with ~50,000 fatalities annually. Timely and aggressive management of acute trauma patients has lowered the fatality rate but does not eliminate the socioeconomic consequences of TBI (13–17). The annual cost of TBI in the US is estimated to be between \$168 billion in medical spending and \$223 billion in work losses (18). Globally it is estimated at \$400 billion (19). Despite the outpouring of resources for TBI management and research, 5.3 million TBI patients in the US continue to live with disabilities, a consequence that is independent of injury severity (20). Improved clinical care has led to increased post injury survival, while return-to-work has remained static for the past five decades (21–24). The current clinical management of severe TBI exploits the limits of physiological interventions and addresses issues mainly at the systemic level and sometimes at the cellular and biochemical levels but rarely at the subcellular organelle dysfunction level. As an example, TBI induced mitochondrial dysfunction has remained intractable (25). Consequently, TBI survivors experience the full wrath of secondary mechanisms

(26–30). This “secondary mechanism fueled” histopathology seen in human TBI is recapitulated with preclinical TBI models (31–36) and offers an opportunity to test interventions.

CURRENT TBI TREATMENT

The Brain Trauma Foundation (BTF), a non-profit group of TBI expert clinicians, has dictated the management of severe TBI since its establishment in 1996 (37, 38). Since that time, there has not been much change in the treatment of TBI despite a better understanding of the destructive events inherent to the disease process. Though adherence to these guidelines decreased overall healthcare costs and improved patient survival (39, 40), the latest fourth edition offers no class I and few class II recommendations in regards to severe TBI management. The major focus of current neurointensive care is (i) metabolic stabilization of the patient, (ii) prevention of further deterioration, and (iii) facilitation of “spontaneous” brain recovery. Along with prompt neurosurgical interventions when warranted, optimizing hemostasis, oxygenation, ventilation, temperature, blood pressure, blood glucose, and acute seizure prophylaxis increased positive outcomes after severe TBI (38, 41, 42). Contrary to previous guidelines, a Glasgow Coma Scale (GCS) of lower than 5 is no longer a contraindication to surgery because of advances in modern surgery and the neurointensive care unit which have improved survival of these patients (43, 44). Early management and proper monitoring of parameters such as intracranial pressure and sodium levels have limited certain types of secondary brain injury (42, 45). Compliance with BTF guidelines is proportional to the strength of evidence (46). For implementation of an efficient trauma system in under-privileged areas, the organization of low cost resources such as trauma registries are required (47–50). For example, Latin American neurosurgeons have advocated for improving clinical research methodologies and topics in the region (51), to better understand implications and relationships between intervention and outcomes. Aggressive surgical therapy seems to be an option for improving survival even in penetrating TBI (PTBI) (48) in developed countries. Intensive critical care management and less aggressive surgical therapy based on the military experience acquired during the 1970's war in Lebanon also produces favorable outcomes especially in pediatric and adult severe TBI (52, 53). Severe TBI patients are treated with a combined medical-surgical approach, managed initially in the intensive care unit (ICU) with neuromonitoring (54, 55), in conjunction with BTF “living” guidelines (updated to

incorporate the findings of randomized clinical trials (RCT), the gold standard for proving the efficacy of new treatments) (37, 38, 56). The next section revisits a few trials and identifies TBI pathophysiological processes that may have led to their failure.

CAN INSIGHTS INTO TBI PATHOMECHANISM EXPLAIN FAILURE OF PAST NEUROPROTECTION TRIALS?

Primary injury, which occurs at the time of impact, includes tissue laceration, cerebral contusion, axonal damage and hemorrhage. Following hospital care, TBI patients can also remain disabled, rendering them worse off which led to the conclusion that secondary injury. It was deduced that secondary insults also significantly influenced outcomes (20, 56, 57). Investigations into the secondary injury process revealed several concurrent processes with distinct spatiotemporal peaks occurring within seconds after injury and lasting for years (58, 59). The result is a complex cascade of molecular and cellular damage, which magnifies the primary injury causing delayed and remote secondary injury (60–64). Initial descriptions of secondary mechanisms included clinical parameters necessary for decision-making, which led to the invention and adoption of the Glasgow coma scale (GCS) (57, 65). The list now includes parameters known to influence TBI outcome such as cerebral blood flow (66), hypoxia-ischemia (67), mitochondrial dysfunction (25), cerebral metabolism (68), cell death (69), glutamate excitotoxicity (70, 71), calcium dysregulation, edema (72) culminating in inflammation, the most enduring of the secondary damage mechanisms (73–75). Inflammation has also been linked to depression like symptoms causing depression like symptoms *via* failure of neurogenesis (76, 77) in multiple CNS conditions including TBI. All these processes have been recapitulated in animals model (**Figures 1**) (78). In the early post-traumatic period (seconds to days), injured neurons in contusions appear swollen, but over time (days or weeks), they become shrunken and eosinophilic, with pyknosis of the nuclei (79). Neuronal and glial “apoptosis” was observed after TBI in human tissue prior to description of the process (69) and later confirmed (80).

Over the three decades, the improved survival of TBI patients upon management with Glasgow coma score (21, 65) and the adoption of cerebral cardiopulmonary resuscitation (CCPR) protocols based upon quantitation of physiological measures (81) led to RCTs that attempted to block/reverse the TBI pathological processes. Such RCTs mostly failed to yield any class I evidence necessary to improve TBI outcomes. These trials included surgical interventions, which unlike decompressive craniectomy (DC) in stroke (82), did not find benefit and had to be stopped due to adverse effects and low recruitment. For e.g., both Decompressive Craniectomy in Patients with Severe Traumatic Brain Injury (DECRA) and Randomised Evaluation of Surgery with Craniectomy for Uncontrollable Elevation of Intracranial Pressure (RESCUEicp) showed poor outcome (55). DECRA was criticized for excluding second tier treatments often used in “real life,” not representing the “real world population,” and because the duration of high ICP was too short (83, 84).

Further negating the DECRA findings, a retrospective analysis revealed benefit of DC and/or barbiturate combination for refractory intracranial pressure management after severe TBI (85). More recently another DC trial (with 80% of patients similar to DECRA and 38% to RESCUE-ICP) showed that the addition of a barbiturate step following DC was more effective than DC alone, barbiturate alone or barbiturate before DC (86). RESCUEicp reported that at 6 months post-decompressive craniectomy, mortality was lowered but at the cost of higher rates of vegetative state, and severe disability. The trial evaluating Early Surgery vs. Initial Conservative Treatment in Patients with Traumatic Intracerebral Hemorrhage was halted after enrolling <20% of the planned number (87, 88). The limited success of surgical intervention is unsurprising as numerous secondary processes (discussed below) are initiated after primary insult and cannot be surgically targeted. More perplexing is the failure of neuroprotective pharmacological RCTs (11, 89–92) including the ProTECT trial (93) which were based on robust preclinical data. Therefore, in the next section we explore the possible reasons that single TBI pathological mechanism targeting RCTs failed.

Mitochondrial Dysfunction/Calcium Dysregulation

TBI induced mitochondrial dysfunction is the rate-limiting step in metabolic restoration of a patient with clinical management (25, 94, 95). Persistently elevated intracellular calcium levels play a central role in activating cellular death mechanisms. Dysfunction of mitochondria (96), production of pro-inflammatory cytokines, as well as axonopathy (97) are all related to calcium dysregulation (98). Upon binding of calcium, the calmodulin-calcineurin complex upregulates the expression of IL-2 by activating the transcription factor NFAT. IL-2 stimulates the proliferation of T lymphocytes, which then recruit more immune cells and amplify the process (99). This pathway is exploited in the treatment of cancer, transplant rejection, and autoimmune diseases. Insights into what constitutes mitochondrial dysfunction came from studies in cardiomyocytes. In these cells low ATP, high calcium caused mitochondrial dysfunction due to the opening of high conductance pores in the inner mitochondrial membrane, uncoupling mitochondrial oxidative phosphorylation and promoting ATP hydrolysis. Cyclosporine A (CsA) was found to prevent such pore opening in isolated mitochondria (100) and cells (101). However, prevention of neurological deterioration was incorrectly attributed to CsA (102), albeit unknown at that time (103). CsA was found to be beneficial in transient forebrain ischemia rodent models upon intracerebral injection provided it could cross the blood brain barrier (BBB) (104). It was therefore given before and after the injury to enable entry to the brain during the opening of BBB and stabilized isolated mitochondria in rodent TBI brains (105). However, despite safety in humans (106, 107), the drug failed to meet the OBTT criteria for advancing to translation (108). This could be in part due to inadequate dosing or possible adverse effects associated with the vehicle Cremaphor. The drug also has a short therapeutic window and needs continuous infusion over the first 3 days post injury to stabilize the mitochondria (109–112). Recent work with a new

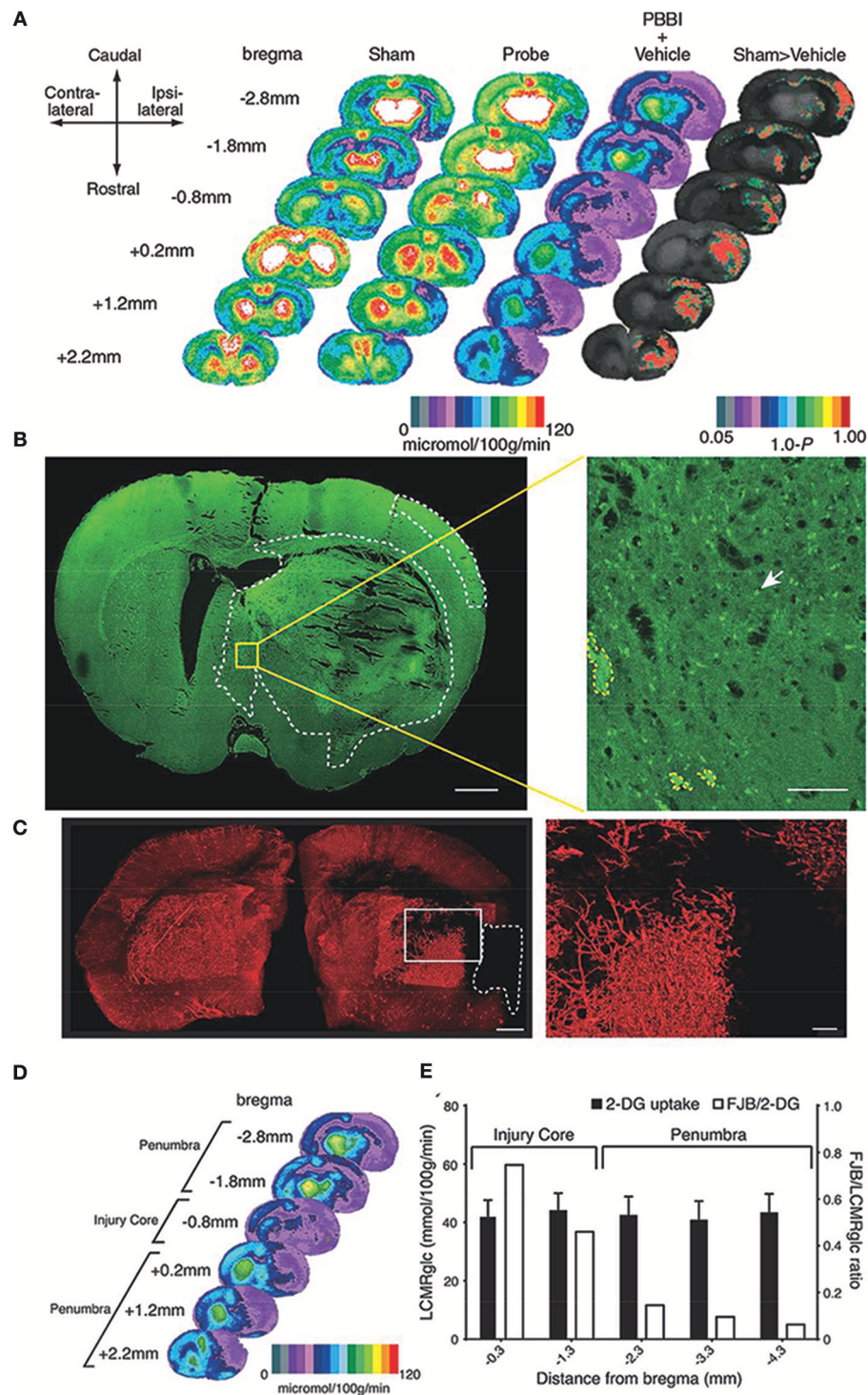


FIGURE 1 | Local cerebral glucose metabolism after penetrating ballistic-like brain injury (PBBi) **(A)** is shown as color-coded maps of average local cerebral metabolic rate for glucose (LCMRgic) at 2.5 h after injury. Each coronal section is a representation of multiple animals within a group at that particular level. Rat brain atlas levels are given on the left column as millimeters from bregma. Compared with controls (columns 1 and 2) in PBBi (column 3), LCMRgic decreased radially from injury core into perilesional areas and globally across the entire brain. P-maps of average local cerebral glucose utilization were produced by comparing the values of pixels corresponding to the same anatomic position across groups. **(B)** Confocal image of a Fluoro-Jade B (FJB)-stained coronal section at 0.8 mm distance from bregma shows regions with FJB+ cells (circumscribed by white-dotted line). Greater neurodegeneration was observed in the injury core and peri-injury zone in the ipsilateral than those in the contralateral cerebral cortex. **(C)** Composite light sheet microscopy image shows ipsi and contralateral hemispheres perfused with fluorescent (Continued)

FIGURE 1 | tomato-lectin at 2.5 h post PBBI. Region with injury induced hypoperfusion is circumscribed by white-dashed line. Surface reconstruction renders the labeled vasculature in 3D. **(D)** Hypoperfused region overlaps with the 2-deoxy glucose (2-DG) uptake impairment heat map. **(E)** The incidence of neurodegeneration was proportional to 2-DG uptake impairment at the injury core but not in regions caudal to the injury core. Fluorojade B (FJB)/LCMRglc ratio decreased from injury core toward more caudal regions, decreasing maximally at -2.3 mm from bregma and plateaued (penumbra). Further details are present in the original article (78).

carrier in gyrencephalic animals is reportedly neuroprotective (113). A related drug, minocycline, although not tested in OBTT and initially used for a different purpose i.e., reducing neuroinflammation via ablation of activated microglia, was tested in human TBI and found to have no benefits (114) though the marker of inflammation was reduced. Minocycline, a well-known antibiotic in the tetracycline family with anti-inflammatory qualities that include inhibition of NF κ B prevents the transcription of pro-inflammatory cytokines and activation of M1 microglia (99, 115). Consistent with equivocal data in rodent TBI (116), a clinical trial using minocycline in chronic TBI patients (6 to 142 months post TBI) showed reduced microglial activation but increased neurodegeneration over time (114). Microglial activation, measured with ^{11}C -PBR28 PET was reduced after 12 weeks of treatment with minocycline but plasma axonal neurofilament light levels, a marker of neurodegeneration, increased and white matter atrophy was more prominent on MRI (114). This suggests that mitochondrial function in even activated microglia are necessary to prevent neurodegeneration, and that there is a need to separate the anti-inflammatory regenerative properties e.g., phagocytosis and proinflammatory degenerative properties e.g., pyroptosis that reside within the same activated microglia, rather than merely ablating all microglia. It is possible that attempts to reduce proinflammatory activated microglia via cell ablation does not mitigate their pyroptosis, an inflammatory process that causes damage to intact tissue. Instead there may be a need to ramp up the anti-inflammatory microglia activity (117). Collectively, preclinical data and the minocycline TBI trial suggest that non-specifically targeting activated glia through these corresponding classes of drugs [Consistent with such data mitochondrial uncoupling agents are also capable of tissue sparing (118)] is not sufficient for neuroprotection in neither an acute nor chronic time point when used with certain dosing regimen.

Calcium is also required for the activation of calpain proteases that cause the breakdown of the cytoskeleton and the cessation of axonal transport (97). Neurofilaments and microtubules accumulate and swelling ensues, forming axonal bulbs that eventually separate the axon. Years after injury, extensive axonopathy is associated with atrophy of the brain, expansion of the ventricles, and premature dementia (119). Of the several clinical trials that evaluated the effect of the calcium channel blocker nimodipine in acute severe TBI, only one improved neurological outcome at 6 months (120). However, subgroup analyses from failed studies revealed that the patients with evidence of subarachnoid hemorrhages on CT benefited from the drug (121). Nimodipine is now widely used in patients with SAH from traumatic and non-traumatic etiology as vasospasm prophylaxis.

Glutamate Excitotoxicity

Another contributory mechanism to TBI is a surge of excitotoxic amino acids, primarily glutamate, that occurs which causes irreparable disturbances in ion fluxes (71). Binding of glutamate to the N-methyl-D-aspartate (NMDA) receptor results in depolarization of neurons, followed by a massive influx of calcium, and efflux of (71, 122). Loss of GABAergic inhibition (possibly due to higher numbers of GluR3 subunit containing but GluR2 subunit depleted AMPA receptors which allow influx of calcium mediating greater vulnerability to excitotoxicity), and downregulation of astrocytic glutamate transporters which allow excitatory neurotransmitter accumulation in the synapse culminating in neuronal and glial (oligodendrocyte) cell death (123, 124). The integrity of GABAergic inhibition needed to contend with excitotoxicity may be compromised by reduced glutathione activity. Glutathione activity was reduced in a rodent model of TBI (125), and increased super oxide production decreased parvalbumin expression in parvalbumin (PV+) GABAergic cells (126). PV+ cells have dendritic arborizations receiving fast converging excitatory inputs (127–130) as reviewed earlier (131) and are endowed with a fast spiking phenotype (132). Higher cognitive functions such as perception (133), and the deterrence of epileptiform activity (134, 135) is contingent upon reliable PV+ cell mediated inhibition. As such, pharmacological disruption of glutamatergic signaling onto fast spiking parvalbumin GABAergic cells disinhibits the circuitry mediating gamma oscillations which facilitate information storage and transfer (136). Promising preclinical results with magnesium blockade of the neuronal NMDA receptor (70), did not translate into neuroprotection in clinical trials, in part due to narrow therapeutic windows, adverse side effects, and interference with normal electrical activity of the brain (11, 19, 90, 137). Glutamate NMDA receptor antagonists (competitive receptor antagonists, ion channel blockers, and glycine antagonists)—such as selfotel, aptiganel, eliprodil, licostinel, and gavestinel—failed to show efficacy in clinical trials of stroke or traumatic brain injury. Deficient properties of the molecules used in human trials or inappropriate design of clinical studies may have contributed to failure. It is possible that excitotoxicity kills inhibitory GABAergic cells (such as hippocampal parvalbumin neurons) due to their higher amounts of GluR3 containing and GluR2 lacking AMPA receptor subunits than glutamatergic neurons, as mentioned above. The clinical NMDA antagonists dose then targets only the glutamatergic neurons, oligodendrocytes, and astrocytes impairing brain metabolic capacity. An alternative hypothesis suggests that glutamate may be involved in the acute neuro-destructive phase immediately after traumatic or ischaemic injury (excitotoxicity), but later, is required for normal physiological functions except during spreading depolarizations (138). Thus

blockade of synaptic transmission mediated by NMDA receptors may hinder neuronal survival (139). Ikonomidou speculated that these drugs could be useful if used just prior to a TBI event, akin to an unexpected decrease in TBI related mortality in alcohol and methamphetamine abusers (140, 141), despite neurotoxicity of the abused substance (142). It remains to be seen what role such drugs can play in TBI management.

Hyperglycolysis

Human brain normally uses glucose as the sole substrate and due to lack of fuel stores; the brain requires a continuous supply. Seymour Kety and Carl Schmidt introduced the first quantitative measurements of human, whole-brain blood flow and metabolism (143). Kety, Sokoloff and their colleagues noted that, while the human brain is only 2% of the body weight, it accounts for 20% of the body's energy consumption. Their technique laid the foundation for studies of brain metabolism in terms of rates of glucose and oxygen consumption (144). When the technique was applied to TBI, both cerebral metabolic rate of oxygen (CMRO₂) and cerebral metabolic rate of glucose changes (CMRO₂) were evident (66, 145). During the first 6 days after moderate or severe TBI, CMRO₂ and arterial lactate levels are the strongest predictors of neurologic outcome (146). Relative to the impaired glucose uptake in the TBI brain, some regions exhibited a high level of glycolysis. This process called "hyperglycolysis" is defined as an increase in glucose utilization two standard deviations above the normal. Hyperglycolysis is thought to be mediated by the release of catecholamines in response to injury to meet increased energy demand upon cells to drive pumping mechanisms in order to restore membrane ionic balance (147). In a study of 28 patients, Hovda et al. reported that hyperglycolysis was observed in 56% of patients on fluorodeoxyglucose-positron emission tomography (FDG-PET) within the first week of injury and persisted for several weeks (148, 149). In the context of TBI, it is not clear which cells are undergoing hyperglycolysis. Lactate accumulation in the injured brain can stem from neuronal mitochondrial dysfunction (150) and/or due to massive influx of lactate from peripheral tissues (151). In turn, elevated lactate levels contribute to pan necrosis (plasma membrane damage, cerebral edema, BBB permeability and overall cell breakdown) (152). In preclinical (153) and clinical studies (149) of TBI, hyperglycolysis and related metabolic crisis (non-ischemic high lactate) increased the incidence of spreading depolarization (138), seizures (154) and were associated with poor outcomes (148). Misled by an "insufficient fuel" concept, glucose supplementation was explored in preclinical studies but surprisingly turned out to be harmful (155–157). However, as in the case of mild TBI, mere fasting was found to be neuroprotective (158). Accordingly, there is no consensus on glycemic control after TBI. Novel metabolic imaging techniques (159) or combination of metabolic studies and neuromonitoring with imaging will be key to gaining insights into the TBI metabolic crisis (94, 138, 150, 154). In three clinical trials, intensive insulin therapy—when compared to conventional insulin therapy—consistently increased the risk of hypoglycemia in moderate to severe TBI patients and failed to decrease mortality at 6 months (160–162). Among these

studies, only Yang et al. reported better neurological outcome, measured with Glasgow Outcome Scale (GOS), at 6 months. Supply-demand mismatch, generated from increased metabolism in the setting of decreased cerebral blood flow (CBF), provokes an energy crisis that promotes further damage (94).

This led to the search for alternative substrate to improve cellular metabolism. Ketogenic diet (KD) via induction of ketosis is known to increase cerebral metabolism of ketones. Age-dependent neuroprotection after TBI in part could be due to younger animals achieving significant β -hydroxybutyrate levels earlier than adults do. In both juvenile rats subjected to weight drop model and adolescent rats to cortical contusion injury (CCI), KD resulted in decreased brain edema, cytochrome c release, apoptotic and oxidative stress marker expression, mitochondrial calcium loading, improved cellular energetics, increased expression of brain-derived neurotrophic factor, smaller contusion volumes and better motor, and cognitive performances. Ketosis mediated by fasting or calorie restriction was also neuroprotective in adult rats with TBI. One of the prominent mechanisms of KD includes inhibition of glycolysis (and subsequently dependent proinflammatory cytokine synthesis), thus lowering inflammation and upregulating bioenergetics via mitochondrial biogenesis (163). At the organelle level a ketogenic diet was found to reduce onset of seizures by preventing the opening of mitochondrial membrane permeability transition pores (164) effectively acting as a neuroprotective uncoupling agent (165).

In contrast to the beneficial effects of ketone metabolism, poor nutritional support can exacerbate TBI (166). Currently, clinical studies are underway to determine the optimal method to induce cerebral ketone metabolism in the post-injury brain, and to validate the neuroprotective benefits of ketogenic therapy in humans (167). Improvements in the understanding of human brain metabolism (168) led to the documentation of metabolism perturbations in injured brains (94, 169–171) and the ability to test if supplementation can bypass these impairments (172).

Hypoxia

In order to maintain its high metabolic activity, the brain receives a substantial proportion of the cardiac output and is therefore highly susceptible to hypoxia (173, 174). Under normal circumstances, a decrease in arterial partial oxygen tension (pBTiO₂) is balanced by increases in cerebral blood flow (CBF) to prevent cerebral ischemia, sometimes at the expense of rising ICP (175). In TBI, loss of autoregulation, as evidenced by concurrent reduction in CBF and pBTiO₂, is one of the mechanisms that exacerbate injury. Hypoxia accelerates uncoupling of the electron transport chain and mitochondrial permeabilization, which induces the release of pro-apoptotic signals, such as reactive oxygen species (ROS) and cytochrome c, inside the cytosol (96). Leakage of cytochrome c in conjunction with elevated cytoplasmic calcium activates the caspase cascade, leading to cell apoptosis. HIF-1 α is a constitutively expressed protein whose activity depends on oxygen availability (176). In normoxia, HIF-1 α is hydroxylated by prolyl hydroxylase (Lee et al.) then tagged with ubiquitin for degradation in proteasomes (177). In contrast, hypoxia decreases the activity of PHD and

HIF-1 α is translocated to the nucleus where it binds HIF-1 β and pyruvate kinase M2. This complex induces the transcription of molecules that stimulate the expression of genes involved in glycolysis, angiogenesis, neurogenesis and the synthesis of proinflammatory cytokines (177–179).

Methods to alleviate TBI-induced brain tissue hypoxia by using blood substitutes, increasing hemoglobin, oxygen saturation, or oxygen tension are currently part of the TBI critical care armament. Attempts to improve cerebral oxygenation with blood substitutes such as perfluorocarbons (PFCs) alleviated hypoxia in animal models of PBBI (78, 180, 181). However, clinical translation of this artificial oxygen carrier was deemed unsafe due to the development of thrombocytopenia, an abnormality that could be detrimental in TBI patients, which led to the cessation of the Safety and Tolerability of Oxybyte in Patients With Traumatic Brain Injury (STOP-TBI) trial (182). Insights into PFC cellular distribution facilitated its repurposing to instead identify injury penumbra: perfluorocarbon enhanced Glasgow Oxygen Level Dependent (GOLD) magnetic resonance metabolic imaging (183).

Because of the prevalence of anemia in TBI patients and associated worse outcomes, administration of erythropoietin (EPO) was evaluated after head injury. The discovery of EPO, a hormone principally produced by the peritubular interstitial cells of the kidney, revolutionized the treatment of anemia in chronic kidney disease patients. Identification of EPO of neuronal and astrocytic origins and the hormone's non-hematopoietic functions (184) have been investigated. Binding to the EPO receptor prevents apoptosis of mature neurons and enhances the proliferation of neural progenitor cells (185). Its anti-apoptotic properties are mediated by the inhibition of pro-apoptotic molecules -including apoptosis regulators bcl-2-like protein 4 (BAX) and cytochrome c, and the activation of the NF κ B pathway, which results in the stimulation of the adaptive immune system cells. EPO also promotes proliferation of the endothelium and the production of nitric oxide (NO) (185). In the multicenter international EPO-TBI trial, EPO did not display neuroprotective effects in patients with moderate to severe TBI (186, 187). Perhaps EPO-induced inflammatory NO may have abrogated any beneficial effect as it increased lesion volume after PBBI (188).

Although, it could seem counterintuitive to use hyperoxia in TBI (189) (i.e., to avoid reperfusion injury), researchers found that markedly increasing oxygen (O₂) delivery to the traumatized brain, with hyperbaric oxygen therapy (HBOT) or normobaric hyperoxia (NBH), could reverse the lack of O₂ for e.g., vascular stenosis (**Figure 1**) that precipitates cellular energy failure and subsequent neuronal death. A recently published review identified eight phase I and phase II clinical trials evaluating the role of acute and subacute HBOT and/or NBH in severe TBI patients. Overall, HBOT alone or in combination with NBH improved physiologic markers of metabolic function (microdialysate LPR, glycerol, ICP) and decreased long-term morbidity and mortality to a greater extent than NBH alone or standard of care (67, 190–192). The “Hyperbaric Oxygen Brain Injury Treatment” (HOBIT) trial: (193) is a proposed adaptive clinical trial designed to answer questions about dosage and

safety parameters of HBO₂ and to provide important data to plan a definitive phase III efficacy trial.

Edema

After TBI, edema develops because of cellular dysfunction (cytotoxic edema) and blood brain barrier (BBB) disruption (vasogenic edema). Increased permeability of the cell membrane to Na⁺ and K⁺ followed by failure of the Na⁺/K⁺ ATPase pump traps osmotically active molecules inside the cell. Mechanical destruction of endothelial cells causes the capillaries to leak a protein-rich exudate into the brain parenchyma. CBF reduction, glutamate excitotoxicity, osmolar gradients additionally participate in extending the edematous state and can contribute to elevations in ICP (194). Different osmotic therapies (mannitol, hypertonic saline, hypertonic lactate, barbiturate) have been examined but none have improved long-term neurological outcome or survival (38, 186). The “BRAIN” trial tried to exploit the role of the kallikrein-kinin system in TBI but it was terminated because Anatibant, an antagonist of the bradykinin B2 receptor, caused more deaths than control 15 days post-injury (195, 196). The kinin family also is known to have a neuroprotective role via the attenuation of microglial proinflammatory secretion through actions of prostaglandin E and microsomal prostaglandin E synthase (197, 198). Bradykinin receptor B1 but not B2 deficiency protects from focal closed head injury in mice by reducing axonal damage and astroglia activation (198, 199). Anatibant may have selectively inhibited the neuroprotective effects while allowing proinflammatory signaling to persist leading to poor outcomes. Nevertheless, the historical failure of acute neuroprotective interventions (11, 114, 137, 200–202) has exposed the limitations of preclinical TBI models in guiding clinical trials in TBI. Similarly, limitations inherent in pre-clinical testing such as insufficient rigor in pre-clinical studies may also have contributed to RCT failure (203). To offset this two groups have come up a similar suggestion regarding data reporting in preclinical studies that would help compare preclinical studies. Use of “delta Score” i.e., summing the change in outcome that may occur in patients in the placebo vs. drug-treated groups over time or effect size used to run meta-analyses are helpful (204, 205). In aggregate, the RCTs failed as they allow for persistence of dual edged inflammation. To provide insights into how unmitigated inflammation underlies progressive tissue loss, our laboratory research uses a rodent model of penetrating TBI (PTBI). PTBI and TBI secondary damage mechanism are similar and may differ only in magnitude. Acute and delayed consequences of human PTBI (64, 206–208) are replicated in Penetrating ballistic-like brain injury (PBBI), a survivable rat PTBI model (34, 35, 78, 209, 210). We detail the results from using this model below.

WHY IS THE PENUMBRA VULNERABLE?

Our recent study with rat PTBI showed that the ipsilateral cortical region at 48 h post injury is replete with activated microglia (boxed regions in **Figures 2A–C**) (210). Only the “penumbra” (yellow highlight in **Figure 2D**) disappears by 10 weeks post injury (35) while regions more dorsal persist

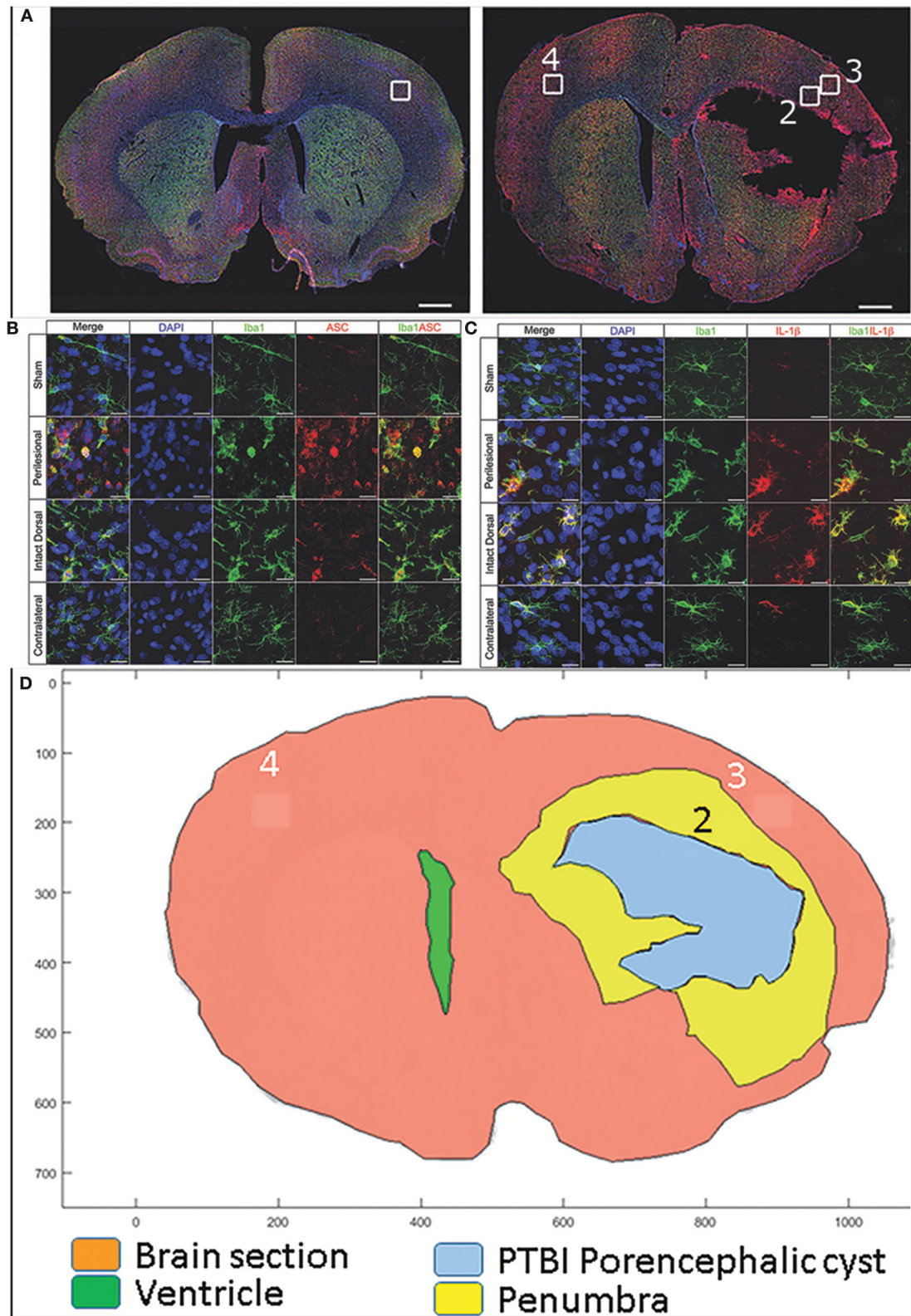


FIGURE 2 | Confocal images of free-floating rat brain sections stained with 2-(4-amidophenyl)-1H-indole-6-carboximide (DAPI; blue), ionized calcium-binding adapter molecule 1 (Iba-1; green), and apoptosis speck-like protein containing caspase-activation and recruitment domain (ASC) or interleukin (IL)-1 β (red). Top panels (A) show whole-brain sections from a representative sham (left) and 10% penetrating ballistic-like brain injury (PBTBI) animal 48 h after injury (right). Sections show (Continued)

FIGURE 2 | Iba-1+ microglia widely dispersed throughout the brain. White boxes (1–4) in the whole-brain images are shown at 100x magnification in panels below. ASC immunoreactive cells are absent in sham cortex (box 1), numerous ASC positive cells are present in PBBI perilesional area (box 2), but to a lesser extent in PBBI intact ipsilateral dorsal cortex (box 3), and absent in contralateral cortex (box 4) **(B)** Iba-1 and ASC double positive cells are present in the ipsilateral hemisphere. In **(C)** Iba-1 and IL-1b co-labeled cells are predominantly present in the ipsilateral cortex. Double positive cells are morphologically large with round/hypertrophied cell bodies and short processes. Additional details are presented in the original publication (210). By subtracting the traces of the brain sections at 48 h post PBBI from those at 10 weeks post PBBI, the PBBI penumbra (box 2 within yellow highlight) is identified **(D)**. The penumbra (box2) was occupied by highly activated microglia at 48 h post injury is lost by 10 weeks post PBBI. In contrast, in box 3 microglial were activated to a lesser extent and at 10 weeks post injury such tissue survives.

despite the presence of proinflammatory activated glia (210). It is possible that a gradient of DAMPs or NAMPs exists with the high concentration at the injury core gradually diminishing radially in a tissue architecture dependent manner. Consistent with this concept, activated microglia in penumbra surrounding the core region may undergo pyroptosis unlike microglia in distal regions. Consequently by 10 weeks post injury the entire penumbra with “critical” levels of DAMPs/NAMPs” disappears while the regions with sub-critical levels survive. This data is consistent with human TBI study where molecular characterization revealed greater numbers of activated microglia in pericontusion (penumbra) than contusion (211). Hence, if reversal of penumbra vulnerability changes TBI outcomes, rescuing the tissue may become a priority.

VULNERABLE TBI EVENTS THAT CAN AND NEED TO BE TARGETED BY CLINICAL THERAPY TO SPARE PENUMBRA

Inflammation

Within minutes of injury, damaged cells release damage-associated molecular patterns (DAMPs)—high extracellular potassium, adenosine triphosphate (ATP), mitochondrial DNA, heat-shock proteins (HSPs), high mobility group binding proteins (HMGB1) molecules, and Amyloid beta. These can activate an inflammatory response in nearby cells (212, 213). Consequentially, the assembly of inflammasomes, activation of complement pathways and local immune cells, and production of pro-inflammatory chemicals (chemokines, cytokines, ROS, NO) trigger inflammatory cell death mechanisms (79, 210, 214). The production of interleukin 1 beta (IL-1 β) by activated microglia peaks 48 h post-injury and favors polarization of microglia into the pro-inflammatory type.

Broad anti-inflammatory interventions such as hypothermia (215) or neuropeptide blockade (216) appear to be promising based on biomarker profiles. The anti-apoptotic and anti-inflammatory effects of hypothermia have also been investigated in TBI (217). Use of hypothermia for refractory ICP after TBI was beneficial in some centers (217). Particularly in China where three of the four trials had positive effects (acute reduction in ICP and long-term improvement of neurological deficits and mortality at 6 months), however all other trials failed to show similar results (186) e.g., Eurotherm3235 Trial, POLAR RCT failed to reproduce the benefits and stopped due to adverse effects. In the Eurotherm trial, titration with therapeutic hypothermia successfully reduced ICP in participants with TBI + ICP of >20 mmHg, but also led to a higher mortality rate and worse functional outcome (218). *Post-hoc* subgroup analysis

of the NABIS-HII trial revealed that hypothermia improved outcomes in patients with evacuated subdural hematomas (219). The failure of the latest hypothermia trial in TBI (220) provides insights into the barriers of translating preclinical findings into human TBI and may unfortunately lead to suboptimal use of this potentially powerful therapeutic in potentially treatable severe trauma patients (221). However, anti-inflammation is not the only consequence of hypothermia, as this approach continues to remain controversial in TBI due to its risk of altering mortality or leading to poor outcomes or new pneumonia (222, 223).

Based on anti-inflammatory action in rheumatoid arthritis, anakinra, FDA approved competitive inhibitor of an interleukin 1 (IL-1) receptor, role of such signaling was evaluated in controlled cortical impact (CCI), a TBI model in rodents, injured IL-1R1 null and wild type mice did not differ in respect to brain lymphocyte numbers (224). In a less severe TBI model, ablation of IL1R signaling or exogenous IL-1Ra was sufficient to reverse TBI induced deficits (225). Both IL1-alpha (IL-1 α), IL-1 β signal through the same IL1R. Elevated IL1alpha/IL1 beta are associated with favorable outcomes after TBI (226, 227). Off label, use of anakinra for human TBI is reportedly beneficial (228) in that it shift the microglia to less inflammatory phenotype (229). Based on the preclinical data the detrimental effects of IL1R signaling seem to dominate over the beneficial effects in TBI context hence seeking total IL1R blockade in human TBI needs to be tested next. Another inflammatory cytokine of interest is tumor necrosis factor alpha (TNF-alpha), which upon interacting with TNF receptor 1 but not TNF receptor 2 was found detrimental in neurodegenerative disorders (230). This made an FDA approved TNF antagonist Etanercept, an attractive candidate for decreasing microglia activation after human TBI (231). However, further studies are needed to achieve selective blocking of the TNF receptor 1 rather than broad TNF receptor blockade.

Example of an anti-inflammatory not useful in TBI is statin. Statins downregulate the expression of vascular adhesion molecules and chemoattractant molecules, and were thought to be potential candidates in lowering the infiltration by immune cells into injured brain. However, in a clinical trial, administration of rosuvastatin 11 h after injury did not display any differences in terms of disability (amnesia and disorientation time) with the control group at 3 months but increased IL-6 levels were seen 3 days after injury (232). Consistent with these results, the OBTT study found no beneficial effects of simvastatin administration over 2 weeks post-TBI using the oral route of administration in multiple rodent models (233). Statins are known to inhibit mitochondrial complex III (234) and can produce myopathy as a side effect (235). Thus, statins possibly

exacerbate TBI mitochondrial dysfunction (95) which may be the reason that they failed to provide any benefit.

Although on the surface it appears that since inflammation after TBI and SCI are mediated by activated microglia these two pathologies could be identical, there is evidence to suggest that the extent of mitochondrial impairment (a measure of inflammation amplification) is different (236), which becomes apparent with aging. For example, a neuroinflammatory modulator, FTY720, which was unable to improve lesion size or functional outcome in both trauma models at either stage, acute vs. chronic, when given as a single dose (237), improved neurological outcome when dosed over 3 days as was seen with CsA (230), and was more effective in SCI even though the inflammation in SCI is different from that in TBI (238).

While microglia are major effectors of inflammation and mediated neuronal death, neutrophils are the first peripheral immune cells to reach the site of injury (239). Over the following hours to days after trauma, neutrophils infiltrate the CNS and migrate across the BBB in response to chemoattractants secreted by the choroid plexus (99). Recruitment of monocyte-derived macrophages and T lymphocytes then follows. Antibody blockade of cluster of differentiation (Cd) Cd11d/CD18, a type of integrin found on both neutrophils and macrophages, reduced systemic inflammatory response syndrome and improved neurological outcomes in rodent models of TBI (240, 241). In contrast to microglia, circulating immune cells such as neutrophils in TBI only produce short duration inflammation that resolves in part due to gasdermin (242). It remains to be shown if this molecule can be exploited to resolve TBI induced microglial inflammatory response.

The role of the adaptive immune system in TBI is still unclear. While infiltration of T cells in the lesion site promoted inflammation in a rat model of SCI, T cell-deficient mice were found to have poorer outcomes than controls after CNS injury (243, 244). Though T cells could have neuroprotective function in TBI, maladaptive response to self-antigens in conjunction with M1-like microglia action can extend damage and maintain a chronic state of low-level inflammation (117).

Inflammation modulation could reduce the loss of neurons, oligodendrocytes, and neural stem cells. In addition, clearance of debris could help resolve and prevent secondary tissue loss. This approach has been found to mitigate injury-induced cognitive decline at 3 weeks post TBI. Inflammation reduction by suppressing polarization into pro-inflammatory microglia (115, 116), promoting anti-inflammatory microglial activity (245) or enhancing clearance of apoptotic cells (246) may confer greater neuroprotection than focusing solely on inhibiting neuronal death mechanisms. Immunomodulatory therapies for TBI need to be developed with a goal to guide inflammation toward the reparative phenotype (99, 247). To better target such therapies, biochemical and imaging biomarkers can be considered to quantitate TBI consequences, validate preclinical research findings, and track effectiveness of therapeutic interventions in humans (201, 248–254).

PRECONDITIONING PENUMBRA AGAINST VULNERABILITY TO SECONDARY INJURY

Consequences of TBI are not limited to the immediate results of primary and secondary injury mechanisms. Years after initial injury, TBI survivors can develop non-convulsive seizures/post-traumatic epilepsy (255–257) and progressive brain atrophy due to “accelerated brain aging” (258–260) that render them susceptible to further neurodegeneration (261, 262). Recurrent post-traumatic seizures, or “post-traumatic epilepsy” (PTE), are highly prevalent in TBI patients with a history of combat and are a major cause of morbidity in veterans (263). TBI severity, dural penetration, loss of consciousness, and post-traumatic amnesia are some of the risk factors that contribute to the development of PTE (263). In multiple models of TBI, it has been found that the formation of epileptogenic foci stems from excitatory/inhibitory neurotransmitter and receptor imbalances and loss of GABAergic cells (264), and tauopathy (263). Although prevention of acute seizures with anticonvulsants can manage immediate glutamate excitotoxicity, PTE tends to be refractory to current medical treatment (263). Axonal debris generated at impact (62, 209) are interrogated by microglia as early as 6 h post-TBI (265). Failure of activated microglia to phagocytose persistent axonal fragments may lead to development of TBI-induced autoantibodies (266). The persistence of axonal fragments and chronic inflammation has been documented several years after primary injury (207, 259, 267). This suggests that poor clearance of axonal debris may provoke the chronic inflammation that underlies neurodegenerative diseases (262). This may be in part due to the presence of “do not eat me” or the absence of “find me/eat me” signals, as seen in mice without CD47 (a ubiquitously expressed surface glycoprotein that provides “do not eat me” signals) which improved outcomes after TBI compared to wild type (268). Although it is clear that modulation of neuroinflammation may improve outcomes, the pharmacological and molecular tools needed to achieve this goal remain to be determined.

Beta-amyloid is another contributor to the long-term degeneration after TBI. The release of inflammasomes from activated microglia promotes seeding and polymerization of beta-amyloid (34, 210, 269) in the synapses (270). The accumulation of insoluble plaques in the extracellular space and tau neurofibrillary tangles inside neurons is already known to precede clinical symptoms of Alzheimer’s disease. Tau protein deposits are also the hallmark of chronic traumatic encephalopathy (CTE), another degenerative brain disease associated with TBI. CTE tends to develop in people with a history of repetitive mild TBI such as military veterans and collision sports athletes. Retired players of the National Football League are three times more likely to die from a neurodegenerative disorder than matched controls (271). Similar to PTE, no TBI therapy has been able to address the neurodegenerative consequences modulated by the chronic inflammation that lingers years after injury.

ADULT NEUROGENESIS (REPARATIVE ENDOGENOUS NEUROGENESIS) AS A “NEUROREGENERATIVE THERAPY”?

Since the discovery of CNS blast cells/neural stem cells in mammalian brain in 1989, the ability of these cells to become neurons became a topic of interest (272). Several lower-order mammals, reptiles, amphibians, and birds continue to experience neurogenesis well into adulthood. However, in adult humans, neurogenesis remains a topic of controversy. In order to understand the role of endogenous neurogenesis, it is desirable to consider how development of the cell types of the brain and the spinal cord occurs. In the mammalian fetus, NSCs are the fundamental ancestor cells for the central nervous system (CNS), as defined by their ability to self-renew and produce all three major CNS cell types: neurons, astrocytes, and oligodendrocytes (272, 273). In humans, these predecessor cells are the first neurons observed at 5-weeks after conception, even before closure of the neural tube which is lined by the neural stem cells of the ventricular zone (274). These cells are found in a layer just below the pial surface of the prospective cortex and migrate tangentially into the telencephalon (275). The two principal colonies of neural stem cells so far identified are located in the subventricular zone (SVZ) and the subgranular zone (SGZ) (276). They give rise to striatal and olfactory bulb neurons (274, 276), and hippocampal neurons, respectively.

Because neuronal loss is the single most important consequence of TBI, efforts to replace neurons have become a fundamental part of TBI research. In rodents, adult neurogenesis is robust even after TBI (277–279). In humans, the evidence for effective reparative adult neurogenesis is controversial, and is probably insignificant at best (280–282). NSCs persist in adult human brains and can produce astrocytes but not neurons (283). Apart from cell death, cell proliferation has also been observed after TBI. This regeneration raises the possibility of therapeutic manipulation of multipotent precursors *in situ* to repair the injured brain. The poor outcomes after TBI characterized by marked gray and white matter loss (259) do not support the notion that proliferating endogenous cells could replace lost neurons in mammals. However, physiological markers of neurogenesis and cell proliferation, measured in tissue samples one to 16 h after TBI, may indicate that the adult injured brain has the potential to replace lost cells and needs to be correlated to patients' outcomes (284). Complicating this, inflammation also contributes to the apoptosis of neural stem cells (285) and possibly oligodendrocytes (286) **Figure 3**. Providing neurotrophic factors, such as S100B or FGF, seems to enhance endogenous neurogenesis in experimental animals and correlates with better cognitive function (287). Failure to boost endogenous proliferation of NSCs in clinical studies (288) and the inability to produce mature neurons *in vitro* from cultured adult human NSCs reiterate that humans are incapable of adult neurogenesis (289). Proneurogenic compounds that have been found to be beneficial in preclinical TBI (290) as well as other CNS dysfunction models (291–294).

Although the exact mechanism of action for these compounds is still an area of active research, the effect of proneurogenic compounds in human TBI remains to be explored. In contrast, clinical treatments with exogenous, transplanted NSCs are moving to Phase II trials, for non-TBI indications (295–300).

ANTI-INFLAMMATORY EFFECTS OF TRANSPLANT vs. CELL REPLACEMENT EFFECTS

Several preclinical studies support the hypothesis that TBI-responsive neuroinflammation is a clinically relevant therapeutic target; however, few clinical trials target traumatic inflammation (117). Cell ablation pharmacological inhibition studies (114, 116, 233, 301–305) suggest that neural stem cells (NSCs), astrocytes, and activated microglia stabilize the brain lesion and prevent further neurodegeneration. However, unlike NSCs, reactive astrocytes and activated microglia are also known to exacerbate TBI (210, 270, 306, 307). It is in this context, that exogenous NSC transplantation alone may be a means to reduce neuroinflammation. Anti-inflammatory properties of mesenchyme-derived stem cells have been extensively reviewed (308) and their utility in TBI has been described elsewhere (309). Intra carotid artery delivery of human MSC was in fact found to be safe in stroke patients (310) as well as ALS (311). See **Table 1** for MSC use in clinical trials.

Similar to other chronic inflammatory diseases, addressing the impaired debris clearance by microglia may be essential in converting degeneration into regeneration (162, 312). How does chronic inflammation negatively influence TBI outcomes? As mentioned earlier, microglia are the main phagocytic cells of the brain and are responsible in part for ECF microenvironment homeostasis (313). As shown in **Figure 3** following injury, the accumulation of myelin debris, beta-amyloid, and other DAMPs could impair microglia phagocytosis and exclusively activate their proinflammatory phenotype (210, 270, 314). Recent insights into biochemical differences in myelin between normal in comparison to injured subjects show how injury induced autoimmune demyelination may progress (315). This situation could potentially benefit from cell transplantation. Transplantation of human fetal NSCs within 24 h of TBI has been shown to reduce microglial pro-inflammatory activation (316) and can alleviate post-traumatic cognitive deficits (316–319). It is not known if such transplantation after PBBI would produce sustained beneficial effects. Recently, our lab demonstrated robust and durable engraftment of hNSCs when transplanted 7–10 days after the injury, in models of PBBI (309). Furthermore, the FDA has already approved of these cells for clinical trials in other CNS disorders but not yet in TBI (217, 305, 320).

One of the impediments for the long-term implementation of stem cell-based therapies is lack of insight into their mechanism of action (321). However, restorative neuroscience has been energized following the discovery of NSCs (272),

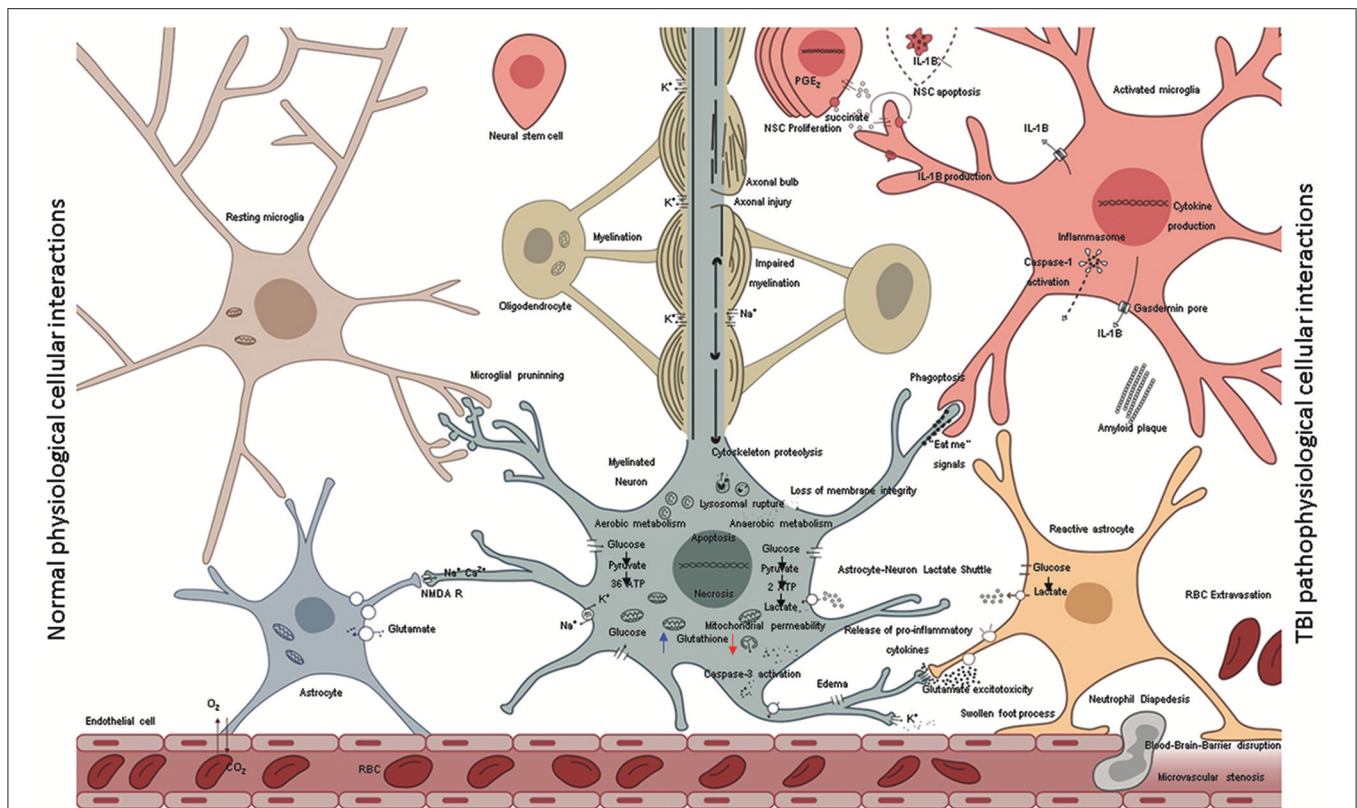


FIGURE 3 | A schematic representation shows the normal cellular interactions in the intact brain (left) in contrast to neurotoxic interactions post injury (right). The intact vasculature (left bottom) is held in place by astrocytic end-feet; astrocytic blood brain barrier keeps immune cells out of the parenchyma but allows diffusion of glucose. Glucose is taken by neural cells metabolized through glycolysis in cytoplasm and oxidative phosphorylation in mitochondria. High neuronal glutathione levels (blue arrow) mitigate oxidative damage due to inherent metabolic activity. The multipartite synapses are pruned by microglia, the axons wrapped by myelin from oligodendrocytes facilitate rapid neurotransmission. Astrocytes soaking up excess glutamate in synapses, which in turns increases glucose uptake from blood. Normal microglia phagocytose various extracellular debris (including amyloid β) produce by metabolic activity. Aberrant cellular interactions after TBI (right) as consequence of mechanical forces disrupt the blood-brain-barrier causing leakage of intravascular contents into the brain parenchyma and facilitate invasion of the CNS with non-resident cells such as RBCs and neutrophils. Initial trauma causes the release of glutamate and other excitatory amino acids and potassium efflux. Lowered neuronal glutathione levels lead to decreased capacity to inhibit excitatory neurotransmission. Ions with their water shells enter astrocytes swelling the cells such that end-feet of astrocytes fail to maintain blood barrier or clear synaptic glutamate. Excess glutamate binds NMDA receptors on neurons and oligodendrocytes. Resulting neuronal depolarization and accumulation of calcium in mitochondria abolishes normal electrochemical gradient required to generate ATP. ATP dependent ion pump activity is required to work against electrochemical gradient to hyperpolarize neurons. Due to irreversible ionic imbalance membrane integrity is lost, unraveling the myelin and death of neurons and oligodendrocytes. This process is called “glutamate excitotoxicity” produces the second wave of TBI related cell death after the primary mechanical injury. Calcium pollution renders mitochondria depolarized, builds up oxidative damage, opening of the permeability transition pore, lipid peroxidation, cytochrome c release, assembly of caspase dependent proteases, and apoptosis. In addition, with injured axons calpain-induced lysosomal rupture, cathepsin-induced cytoskeletal proteolysis set into motion the self-destructive axonal degeneration. Hypoxia stabilizes HIF-1 α facilitating expression of pro-inflammatory cytokine genes (IL-1 β , IL-18, TNF α). Succinate acts as a signal that positively feeds inflammation. NSC disrupt such feedback and mediate inflammation resolution by rendering microglia anti-inflammatory. Pro-inflammatory cytokines are released in the extracellular environment via pores (ex: IL-1 β via gasdermin D) and spread inflammation to adjacent cells including mitochondrial dysfunction and secondary death of oligodendrocytes, neural stem cells and neurons. Presence of proinflammatory microglia corrupts astrocytes turning them into agents of neurotoxicity. Transient “eat-me” signals on the surface of neurons activate glial Phagoptosis leading to further loss of tissue that was otherwise intact at the time of primary injury. Proinflammatory microglia and neurons may undergo pyroptosis or other inflammatory cell death further spreading the inflammation.

their mitogens (322), their ability to be cultured from adult rodent brain (323), and embryonic (324) human brains (289). Recapitulation of human neuronal development after transplantation of human fetal neural stem cells in rodent embryos (325, 326) suggests that transplantation of NSCs could rebuild injured brains by emulating aspects of CNS development, such as tract forming and target cell innervation.

Human Neural Stem Cells as Agents of Neuroprotection After TBI

Successful transplantation of fetal tissue in adult rat brains (327), led to the first neuroprotective fetal cortical tissue transplants in TBI rats (328). The source of NSCs was cortical tissue (329–331). Preclinical studies of TBI showed that transplantation was acutely neuroprotective but not past 2 weeks post injury. The lack of neuronal replacement was attributed to robust host immune

TABLE 1 | MSC trials.

	Trial sponsor (Location)	Disease target	Cellular characteristics	Phase (# participants)	Response to treatment	References
1	Neurogen Brain and Spine Institute (India)	Acute TBI	Autologous bone marrow mononuclear cells	Completed (43)	BMSC therapy is safe and effective on patients with severe TBI complications	NCT02028104
2	The University of Texas Health Science Center, Houston (United States)	Acute TBI	Autologous bone marrow mononuclear cells	Phase 2b-55	–	NCT02525432
		Acute TBI	Autologous bone marrow mononuclear cells	Completed-25	Treatment is safe and effective on structural preservation and the global neuroinflammatory response	NCT01575470
3	Bioquark Inc. (India)	Brain death secondary to TBI	Mesenchymal stem cells	Recruiting-20	–	NCT02742857
4	Robert W. Alexander, MD, FICS (United States)	Acute TBI (concussion)	Adipose-Derived cellular stromal vascular function	Recruiting-200	–	NCT02959294
5	SanBio, Inc. (United States)	Chronic motor deficit from TBI	SB623 cells: adult bone-marrow-derived cells that are transiently transfected with a plasmid construct encoding the intracellular domain of human Notch-1	Phase 2-52	Statistically-significant improvements in motor function, and no serious adverse events	NCT02416492
6	MD Stem Cells (United States)	Neurological Disorders	Intravenous and intranasal BMSC	Recruiting-300	–	NCT02795052

response. In order to overcome this limitation, researchers initiated a number of studies, including transplantation of human neuronal precursors, where experimental subjects receive immunosuppression (205). One of the mechanisms of action for hNSC transplants was elucidated in a multiple sclerosis model. In that study, NSCs appeared to sense the extracellular succinate that accumulates in the chronically inflamed CNS and ameliorated neuroinflammation via succinate-SUCNR1-dependent mechanisms (332). Consistent with these findings iPSC derived NSC as well as oligo precursor transplants are reported to spare tissue in rodent spinal cord injury model (333). Such tissue sparing occurs following inflammation resolution. As outlined in **Figure 4**, in the absence of timely resolution of injury induced activated microglia, the injury is magnified over time and space producing progressive increased tissue destruction in part *via* microglial pyroptosis (210) and facilitates worse outcomes such as antibody generation against cellular breakdown products (266) and neurodegeneration (**Figure 5**). Neural stem cell transplants could confer neuroprotection to alleviate such tissue loss and lead to a desired outcome via inhibiting microglial pyroptosis, disrupting the succinate based inflammation amplification, and promoting phagocytosis by surviving activated microglia (**Figure 5**). Perhaps all stem cells confer neuroprotection via efferocytosis (334).

Cell Replacement

Transplanted NSC-derived neurons can integrate and contribute electrophysiologically in both sham as well as injured rodent and primate brains (321, 335–339). However, previous studies have reported limited neuronal replacement after hNSCs transplantation in rat models of TBI (318). Recently our lab and others have shown robust and durable engraftment of hNSCs with delayed differentiation into mature neurons, for as many as 20% of transplanted cells, up to 16 weeks in a rodent PBBI model. Nevertheless, their integration into injured rat CNS and contribution to reversal of TBI induced motor and cognitive deficits has yet to be fully demonstrated (309, 340). Assessing electrophysiological properties and their contribution to amelioration of TBI-induced deficits would provide crucial mechanistic insight. It is not yet known if it is possible and/or necessary to guide transplant-derived neurons to a specific target (such as anterior horn cells, or substantia nigra) and how this can be done. The use of clinically relevant neurogenic compounds could be key to assist in targeting the migration of transplant-derived neurons. In primates with SCI, researchers have found that transplanted NSI 566 cells can be harnessed to restore lost function 9 months after grafting by differentiating into neurons and supportive glia (339). If cell replacement is indeed achieved it could positively steer outcomes (**Figure 5**).

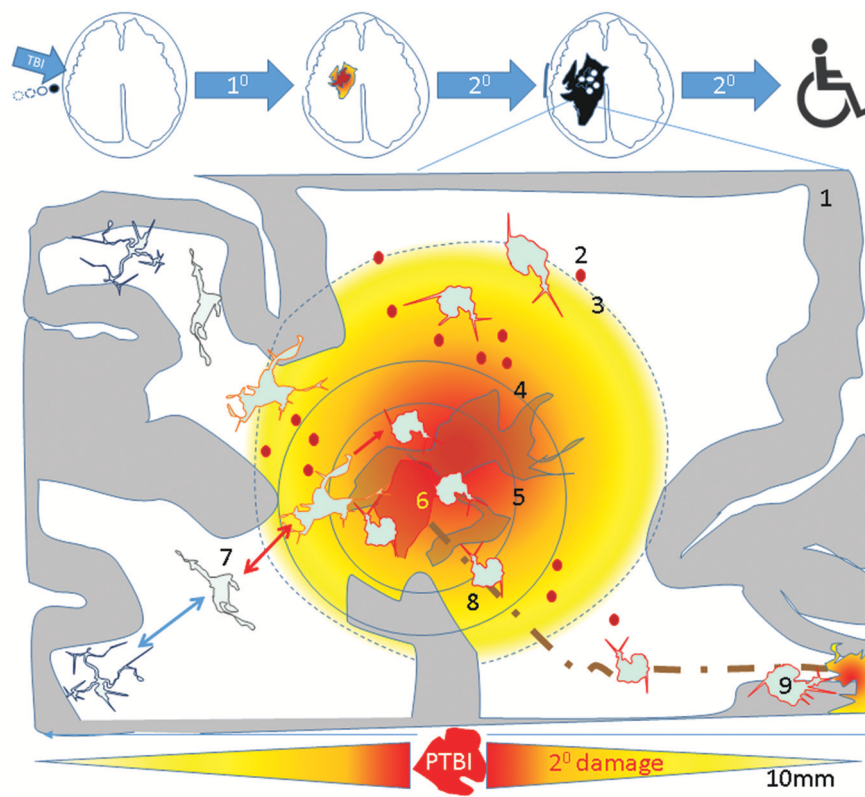


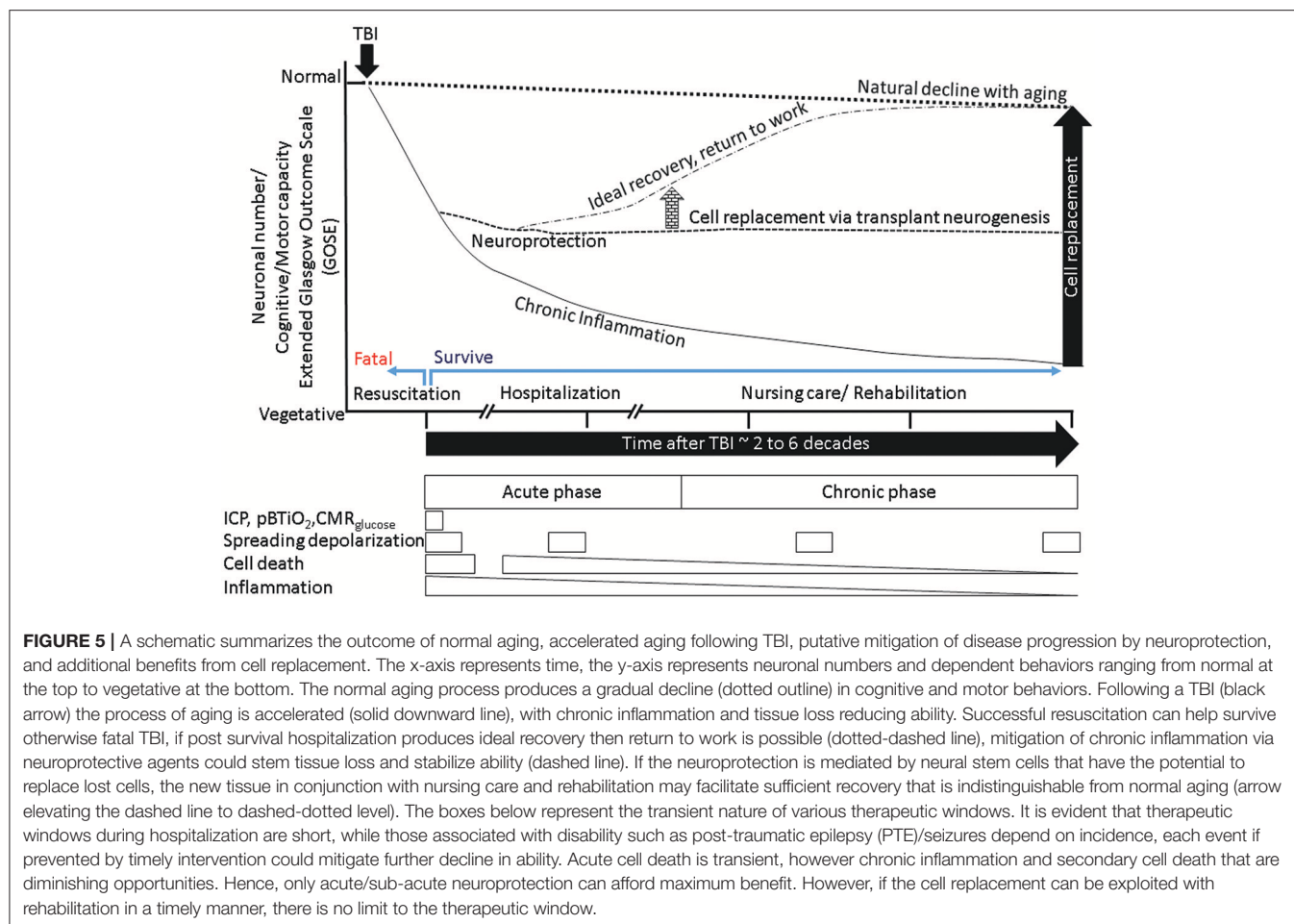
FIGURE 4 | A theoretical schematic (top) shows evolution of the primary PTBI injury into disability. A PTBI brain schematic focused on inflammatory microglia over nine regions outlines possible mechanisms underlying magnification of primary injury and spread to remote sites. 1- intact brain tissue, 2-petechiae in injured brain which perpetuate blood brain barrier compromise, hemorrhage, delayed cell death, 3- a gradient of local DAMPs/NAMPs may combine with circulating inflammatory mediators (via broken BBB) to recruit and activate microglia in penumbra rendering tissue vulnerable to secondary damage, 4- region with neuronal apoptosis/pyroptosis and neural stem cell recruitment and apoptotic death., 5- regions of astrocyte destruction and reactive gliosis which stems from 6-Focal injury. The focal injury turns into a permanent cavity in part due to oncotic cell death and axonal destruction (brown dotted line). 7- Normal surveying microglial acquire various reversible/irreversible activation states as they travel along the DAMP/NAMP gradient. 8. Irreversibly activated microglia could migrate away from injury core toward remote deeper regions. 9-Pyroptosis of activated microglia in remote regions connected to injury site (via pathways that were axotomised by injury) may mediate secondary axotomy and remote neurodegeneration (210, 270).

The current literature suggests that NSC mediated neuroprotection (332, 333) could be achieved more easily than cell replacement, especially in severe TBI. It is true that the incidence of severe TBI is small and cell therapy to treat will not be appealing to mild and moderate TBI patients. However, the costs associated with TBI requiring hospitalization necessitate use of cell therapy to treat severe TBI (18, 341). Following the discovery of the mechanistic insights, “the neuroprotective factor” could be delivered via non-cell transplantation means even for less severe TBI where cell therapy is not warranted.

RATIONALE FOR USE OF hNSC TO TREAT TBI AND GUIDELINES FOR CELL THERAPY

After examining several RCTs and gaining insights into their failure to confer neuroprotection, (11, 90, 114), identification of anti-inflammatory mechanisms as leading neuroprotective

agents, the neuroprotective properties sub-acute use of human NSCs (332, 333, 342) is worthy of exploration in TBI. NSCs could potentially mitigate secondary damage by (1) reducing inflammation; (2) promoting regeneration with appropriate pharmacological interventions (e.g., drugs promoting neurogenesis such as NSI-189) and rehabilitative measures; and (3) Slowing down TBI-induced delayed disability. Accomplishing this set of objectives in itself would be an important goal of NSC therapy. As of mid-2018, a total of 316 patients with various reported CNS disorders have received clinical grade hNSC transplants (Table 2). None of these patients had any safety issues yet. Neuralstem Inc., has sponsored phase I and phase II clinical trials evaluating hNSC transplantation as a potential therapy for ALS (354). In these, a *post hoc* analysis compared ambulatory limb-onset ALS participants who were administered open-label intraspinal hNSC and followed for up to 3 years after transplant. Due to lack of controls, participants in these phase 1 and 2 trials were matched to subjects from the Pooled Resource Open-Access ALS Clinical Trials (PRO-ACT)



and ceftriaxone datasets to provide required analyses in order to inform future clinical trial designs. The ALS Functional Rating Scale revised (ALSFRRS) and a composite statistic combining survival and functional status (ALS/SURV) were assessed to monitor changes in function. Results from These Ph1/2 studies revealed significantly improved survival and function (346) when compared to historical datasets. In another study where non-ambulatory ALS patients received either unilateral or bilateral injections, no increase of disease progression after the transplants was observed for up to 18 months after surgery. Rather, two patients showed a transitory improvement of the subscore ambulation on the ALS-FRS-R scale (from 1 to 2). A third patient showed improvement of the MRC score for tibialis anterior, which persisted for as long as 7 months. Three of the patients died due to disease progression (353). More recently a study of stereotactic, intracerebral injection of CTX0E03 neural stem cells from ReNeuron into patients with moderate to moderately severe disability as a result of an ischemic stroke has progressed from a Phase I to Phase IIb as the clinical endpoints are being met albeit slower than expected (295, 299, 300).

PATHWAY TO ADDRESS UNMET PATIENT NEED, CLINICAL TRIALS, TO ARRIVE AT PROVEN TREATMENTS

The previous sections suggest that the unit of intervention for TBI should be at the cellular level i.e., at the unit of life. However, it is important to be wary of moving too hastily. The compelling unmet TBI medical need and desperation on the part of patients, in the absence of multicenter clinical trials, can lead to unproven therapies being administered to patients. Three such cases of unproven stem cell therapies (mix of multiple fetal NSCs or MSCs and NSCs) have been documented (355–357). Fortunately, all of the issues could be resolved by corrective measures i.e., removal of the transplanted cells. The aforementioned events have their roots in premature and unapproved use of treatments that were initiated by investigator/patient. “Stem cell tourism” that exploits the therapeutic hope of patients and families with incurable neurological diseases can jeopardize the legitimacy of stem cell research. Julian et al posit that an improvement in education, regulation, legislation, and involvement of authorities in global health in neurology and

TABLE 2 | Human fetal neural stem cells in Clinical Trials.

	Trial sponsor (Location)	Disease target	Cellular characteristics	Phase (#. treated)	Response to treatment	References
1	City of Hope (CA, USA)	recurrent high grade gliomas	carboxylesterase-expressing neural stem cells	phase I (15)	Initial safety and proof of concept regarding the ability of NSCs to target brain tumors	NCT01172964 (343–345)
2	Neuralstem Inc. (MD, USA) in collaboration with Emory University Atlanta, Georgia, United States, Massachusetts General Hospital Boston, Massachusetts, United States, University of Michigan, Ann Arbor, Michigan, United States	ALS	Eight wk fetal-derived neural stem cells	phase I (18)	Safe	NCT01348451 (346)
				phase II (18)	Improved survival compared to standard treatment	NCT01730716
		chronic spinal cord injury		phase I (8)	On going	NCT01772810 and NSI website (347)
		stroke		phase I/II (9)	Safe with behavior modification	NSI website
3	ReNeuron Ltd. (UK) Division of Clinical Neurosciences, Glasgow Southern General Hospital, Glasgow, UK, G51 4TF, ReNeuron, Queen Elizabeth Hospital, Birmingham, UK, NHS Southern General Hospital, Glasgow, UK, G51 4TF, Kings College Hospital London, UK, University College London Hospital London, UK, Royal Victoria Infirmary Newcastle, UK Nottingham City Hospital Nottingham, UK Salford Royal NHS Foundation Trust Salford, UK Royal Hallamshire Hospital Sheffield, UK Southampton Hospital Southampton, UK	stroke	12 wk fetal cortex derived, genetically modified CTX0E03 neural stem cells	phase I (12)	Safe	NCT01151124 (299)
				phase II (41)	Safe with behavior modification slightly delayed than expected.	NCT02117635
4	ReNeuron, Division of Clinical Neurosciences, Glasgow Southern General Hospital, Glasgow, UK, G51 4TF	lower limb ischemia		phase I (9)		NCT03333980
5	Stem Cells Inc. (CA, USA)	neuronal ceroid lipofuscinosis	16 wk fetal-derived neural stem cells	phase I (6)		NCT00337636 (348)
		cervical spinal cord injury		phase II (50)		
		macular degeneration		phase I/II (15)		
		thoracic spinal cord injury		phase I/II (12)		NCT01321333 (349, 350)
		Pelizaeus-Merzbacher disease		phase I (4)		NCT01005004
6	TRANSEURO (UK) STEM-PD	Parkinson's disease	fetal-derived dopaminergic cells	phase I (40)		NCT01898390 (351, 352)
7	Azienda Ospedaliera Santa Maria, Eastern Piedmont University, Novara and Terni Hospital, Terni, Italy.	ALS	8 wk fetal-derived neural stem cells	phase I (3)		EudraCT:2009-014484-39 (353)

TABLE 3 | Unproven application of cell therapy.

1	Moscow Hospital	Ataxia telangiectasia (AT)	8–12 wk aborted fetal periventricular tissue isolated from fresh-autopsy cultured for ~16 days, 1–2 fetus/procedure, 3 procedures total	1	Tumor formation	(355)
2	Commercial stem-cell clinics in China, Argentina, and Mexico.	Residual deficits from an ischemic stroke	the infusions were described as consisting of mesenchymal, embryonic, and fetal neural stem cells	1	Debilitating glioproliferation	(356)
3	"stem cells" at a clinic in Georgia, USA Private clinic,	Exudative macular degeneration	autologous adipose tissue-derived "stem cells"	1	Bilateral Retinal Detachments	(357)
4	Thailand-Canada	Lupus nephritis	autologous CD34+ hematopoietic stem cell transplantation, mobilized with GCF and collected from peripheral blood	1	angiomyeloproliferative lesions	(359)
5	Multiple US based Stem cells clinics in FL, CT and MD	Patient for each of the following conditions: Two for non neovascular AMD, and one for Quiescent neovascular AMD	autologous adipose tissue-derived "stem cells"	3	Loss of vision	NCT01736059: NCT02320812, (360), NCT01920867, NCT0024269
6	Stem Cell Ophthalmology Treatment Study (SCOTS) FL,CT,MD	Stargardt's macular dystrophy	autologous bone marrow-derived stem cells in the right eye	1	recurrent retinal detachment with proliferative vitreoretinopathy.	(361)
7	Stem Cell Ophthalmology Treatment Study (SCOTS) FL,CT,MD	optic neuropathy	autologous bone marrow-derived stem cells	1	Improved vision	NCT 01920867 (362)

Unproven stem cells use with beneficial vs. debilitating consequences

1 success vs. 8 failures

neurosurgery is required to prevent such exploitation (358) (Table 3).

CONCLUSION

For over 30 years of TBI research, neuroprotection via RCTs has been elusive. Progressive tissue loss in severe TBI is an unmet need that turns TBI into a disease process with no hope for recovery. Analysis of the trial failures has led to insights into the mechanisms that need to be targeted, specifically neuroinflammation. Preclinical animal model studies that recapitulate human severe TBI have led to the identification of mechanisms underlying the vulnerability of the penumbra and evaluating the extent of penumbra sparing will likely give insights into the neuroprotective ability of an intervention. Additionally, the continued exploration of neural stem cells transplantation, which was bolstered by initial efforts with fetal cortical tissue transplants that were neuroprotective, resulted in the discovery that cell transplants can resolve inflammation via disruption of proinflammatory pyroptotic signaling and without interfering with activated glial functions such as phagocytosis. Multiple independent studies in a variety of CNS conditions suggest

use of clinical trial grade human neural stem cells which have been found to be safe and meeting the clinical end points. Thus, the rationale for using human neural stem cell based transplantation for TBI is well supported as both enduring neuroprotection and cell replacement can be achieved with single agent.

AUTHOR CONTRIBUTIONS

AK, AC, DC, SL, MS, KR, HP, LD, and JW reviewed the preclinical articles and prepared the draft, AM wrote the section on trial cost. AA, SY, ZH, SL, AR, RB, and RT worked clinical trials articles and prepared draft. SN, HP, and MS generated the data for figures. LL, AA, SY, SG, and RB conceived and revised the draft to produce final manuscript. AK produced original artwork to encapsulate the literature on cellular interactions with RB and SG.

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Feasibility of Human Neural Stem Cell Transplantation for the Treatment of Acute Subdural Hematoma in a Rat Model: A Pilot Study

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Human neural stem cells (hNSCs) transplantation in several brain injury models has established their therapeutic potential. However, the feasibility of hNSCs transplantation is still not clear for acute subdural hematoma (ASDH) brain injury that needs external decompression. Thus, the aim of this pilot study was to test feasibility using a rat ASDH decompression model with two clinically relevant transplantation methods. Two different methods, *in situ* stereotactic injection and hNSC-embedded matrix seating on the brain surface, were attempted. Athymic rats were randomized to uninjured or ASDH groups (F344/NJcl-rnu/rnu, $n = 7-10/\text{group}$). Animals in injury group were subjected to ASDH, and received decompressive craniectomy and 1-week after decompression surgery were transplanted with green fluorescent protein (GFP)-transduced hNSCs using one of two approaches. Histopathological examinations at 4 and 8 weeks showed that the GFP-positive hNSCs survived in injured brain tissue, extended neurite-like projections resembling neural dendrites. The *in situ* transplantation group had greater engraftment of hNSCs than matrix embedding approach. Immunohistochemistry with doublecortin, NeuN, and GFAP at 8 weeks after transplantation showed that transplanted hNSCs remained as immature neurons and did not differentiate toward to glial cell lines. Motor function was assessed with rotarod, compared to control group ($n = 10$). The latency to fall from the rotarod in hNSC *in situ* transplanted rats was significantly higher than in control rats (median, 113 s in hNSC vs. 69 s in control, $P = 0.02$). This study first demonstrates the robust engraftment of *in situ* transplanted hNSCs in a clinically-relevant ASDH decompression rat model. Further preclinical studies with longer study duration are warranted to verify the effectiveness of hNSC transplantation in amelioration of TBI induced deficits.

Keywords: traumatic brain injury, acute subdural hematoma, transplantation, neural stem cell, treatment

INTRODUCTION

Despite much effort, the prognosis of acute subdural hematoma (ASDH) remains quite poor (1). In the US, approximately 50,000 people die and at least 5.3 million live with disabilities related to traumatic brain injury (TBI) per year, with ASDH significantly contributing to these (2, 3). Further, ASDH is recognized as a leading cause of mortality and morbidity in young adults globally (4–6), while elderly patients with ASDH have the worst outcomes among those with other types of severe TBI (5–7). Thus, ASDH constitutes a heavy burden both on patients and their families.

The pathology of severe TBI has been classically divided as primary and secondary brain injury. Primary brain injury is defined as the one occurring immediately after receiving external force to the cranium. In contrast, secondary brain injury is the additional injury caused by aggravating extracranial and intracranial factors, including hypoxia, hypotension, and hypoperfusion. Since primary injury is unavoidable, the aim of current treatments has been to diminish secondary brain injury. However, several clinical randomized controlled trials have not been able to show efficient reduction in patients' mortality (3, 7–9). The treatment strategies have only focused on preventing secondary brain injury and thus have been limited in clinical situations. Therefore, novel approaches, like regenerating medicine or cell-therapy are needed (10, 11).

In the past decade, a commercially available human neural stem cell (hNSC) line, NSI-566 cells, derived from human 8-week-old fetal spinal cord, has been established for cell-therapy (12). This cell line has been already approved to use in human clinical trials by the US Food and Drug Administration. A recent study showed efficient hNSC transplantation for the amelioration of cognitive function in a rat model of penetrating ballistic-like brain injury (13), as well as in murine models of cortical TBI (14, 15). However, the use of regenerating treatment for more common type TBI (i.e., ASDH) models has not been attempted until now. The aim of this pilot study was to assess the feasibility of hNSC transplantation in a clinically-relevant model of ASDH/decompression in rats.

MATERIALS AND METHODS

Study Design and Animals

Procedures for all animal experiments followed the guidelines established by the Japanese Ministry of Education, Culture, Sports, Science, and Technology Guide for the Care and Use of Laboratory Animals and Animal Research. The protocol of the study was approved by the Nippon Medical School's Institutional Animal Care and Use Committees (NMS IACUC #28-044).

Surgical procedures were performed under aseptic conditions. Athymic rats (F344/NJcl-rnu/rnu, male, 200 g) were used in this study.

Abbreviations: ASDH, acute subdural hematoma; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; hNSCs, human neural stem cells; IQR, interquartile range; TBI, traumatic brain injury; TP, transplantation.

Anesthesia

Anesthesia was induced with isoflurane (1–2%) delivered in a mixture with 30% oxygen. Body temperature was maintained at normothermia ($36 \pm 0.5^\circ\text{C}$) throughout all surgical procedures using a homeothermic heating pad (Harvard Apparatus, South Natick, MA). The tail artery was cannulated with a PE-50 tube in each rat for blood pressure measurement, arterial blood gas monitoring, and for drawing autologous blood for ASDH induction. The pH, PaCO_2 , and PaO_2 were measured using a portable blood gas analyzer (i-STAT 1 analyzer, Abbott Point of Care Inc., Princeton, NJ) before and after ASDH induction.

Induction of Subdural Hematoma

Details for the induction of subdural hematoma and performance of the decompression surgery have been described in our previous reports (16–20). Briefly, the scalp was incised on the midline, and a single burr hole of 3 mm in diameter was drilled 2 mm to the left of the sagittal suture and 3 mm behind the coronal suture (**Figure 1A**). The dura was then incised under a microscope, and a blunt-tipped PE-50 polyethylene tube was inserted into the subdural space. Quick-setting cyanoacrylate glue was used to fix the tube. The burr hole was then sealed with dental cement. The hematoma was induced with the injection of non-heparinized autologous blood (250 μL) into the subdural space over 5 min, allowing it to clot *in situ* (**Figure 1B**). After injection, the induction tube was cut off and sealed.

Thirty minutes after induction of the hematoma, a rectangular craniotomy (15 $\times$ 6 mm) was performed using a dental drill (**Figure 1A**). The subdural hematoma was removed by saline irrigation and forceps, after widely opening the dura. The scalp was then closed over the craniotomy window without replacing the bone, to mimic the clinical practice of decompressive craniotomy. Thus, the ischemic/reperfusion TBI model with large cortical injury ($\sim 6 \text{ mm}^2$) was created (**Figure 1C**; seen by triphenyltetrazolium chloride staining, and **Figure 1D**; macroscopic findings) (21).

Cell Transplantation

hNSC transplantation was performed 7 days after the surgical decompression treatment. Two different methods for the transplantation were attempted as below. In both of transplantation method, rats were anesthetized with 2% isoflurane and secured in a stereotaxic frame; the scalp was reopened along the midline to expose the injured cortical surface.

1. hNSC *in situ* transplantation

A microsyringe was backfilled and flushed with suspension media, then attached to a microsyringe injector and micro4 controller (UMP3-3, World Precision Instruments, Sarasota, FL). The microsyringe was then filled with green fluorescent protein (GFP)-transduced hNSC cells (NSI-566, Neuralstem, Inc. Germantown, Maryland, USA) in suspension media (in a concentration of 100,000 cells/ μL) (22). The cell density was certified by cell counting with 0.4% Trypan blue solution and hemocytometer. The injection was administered at -3 mm AP and $+2 \text{ mm}$ ML from the bregma, ipsilateral to the injury,

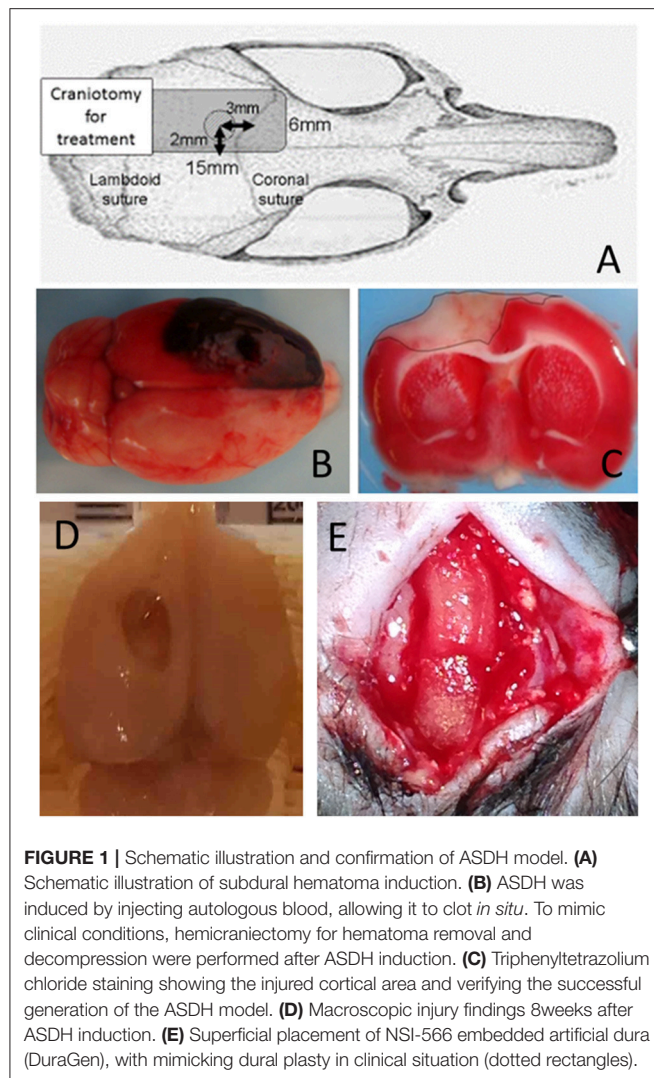


FIGURE 1 | Schematic illustration and confirmation of ASDH model. **(A)** Schematic illustration of subdural hematoma induction. **(B)** ASDH was induced by injecting autologous blood, allowing it to clot *in situ*. To mimic clinical conditions, hemicraniectomy for hematoma removal and decompression were performed after ASDH induction. **(C)** Triphenyltetrazolium chloride staining showing the injured cortical area and verifying the successful generation of the ASDH model. **(D)** Macroscopic injury findings 8 weeks after ASDH induction. **(E)** Superficial placement of NSI-566 embedded artificial dura (DuraGen), with mimicking dural plasty in clinical situation (dotted rectangles).

targeted proximal to the injured motor cortical area. The microsyringe was advanced vertically 4-mm deep into the brain. Using the micro pump, 2 μ L were injected at a rate of 1 μ L per min. The needle was then retracted from the brain. In total, 2×10^5 cells were transplanted in the injured cortex.

2. hNSC transplantation on the cortical surface

For this method, a bovine tendon derived collagen-based dural regeneration matrix (DuraGen, Integra, NJ, USA) was applied. On this matrix, hNSCs (in a concentration of 100,000 cells/ μ L) were embedded. After reopened the scalp, embedded matrix was seated on the injured cortical surface mimicking duralplasty in clinical situation (Figure 1E). The scalp was then re-sutured with aseptic condition.

Behavioral Testing for Assessing Motor Function

Motor function and its recovery were assessed every week for 4 weeks after transplantation using the rotarod performance test (23). The latency to fall from the rotarod was scored

automatically with infrared sensors in Rotamex 5 rotarod (Columbus Inst, Columbus, OH, USA). Each week, three trials were performed for each rat (23, 24), and the best score was retained for the analysis. The acceleration step and time were determined empirically. The speed was increased by 0.5 cm/s every 5 s.

Specimen Collection, Histology, and Imaging

Four to eight weeks after transplantation, rats were transcardially perfused with 0.1 M phosphate buffered saline, followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were dissected (Figure 1D) and post-fixed in the same solution for 12 h and then transferred to a 30% sucrose solution for 24 h. Brains were frozen in embedding matrix using dry ice and stored at -20°C before being sectioned on a cryostat at 40- μ m thickness. Free-floating sections were stored in 0.02% sodium aside in phosphate buffered saline prior to immunohistochemistry. Samples were stained with 4',6-diamidino-2-phenylindole (DAPI) to mark neuronal nuclei and GFP to confirm the presence of transplanted hNSCs. Samples were also assessed with the following primary antibodies: NeuN (Millipore MAB377), DCX (Millipore AB2253), GFAP (Dako Z0334), and IBA-1 (Millipore MABN92). Fluorescent images were observed on a confocal microscope (OLYMPUS BX51, Olympus Optical Co., Ltd., Tokyo, Japan).

Statistical Analysis

Non-parametric data were compared using the Mann–Whitney *U*-test. All non-parametric data are presented as the median and interquartile range (IQR). Rotarod results were compared using two-way repeated measures analysis of variance with Fisher's least significant difference *post-hoc* method. All analyses were performed using StatFlex software (version 6.0; Artech Co. Ltd., Osaka, Japan), and differences were considered statistically significant at a $P < 0.05$.

RESULTS

Baseline Characteristics

We did not find any significant differences between the all transplanted groups and control groups on body weight, body temperature, mean arterial pressure, pH, PaO_2 , and PaCO_2 before and after ASDH induction. Changes in body weights during study period were not significantly different among all treatment groups. All rats, in all groups, survived for at least 4 weeks after injury and craniotomy.

Histological Analysis

In both of transplantation method, the engraftment of NSCs could be seen in the injured cortex and the surface of cortex at least 5 weeks post-transplantation (Figures 2A,B). Several transplanted GFP positive NSI-566 hNSC cells were present in the injured cortex and the hippocampus of the ipsilateral side. However, much robust engraftment of hNSCs were observed in *in situ* transplanted groups. Higher magnification of GFP-positive transplanted hNSCs revealed long processes, resembling

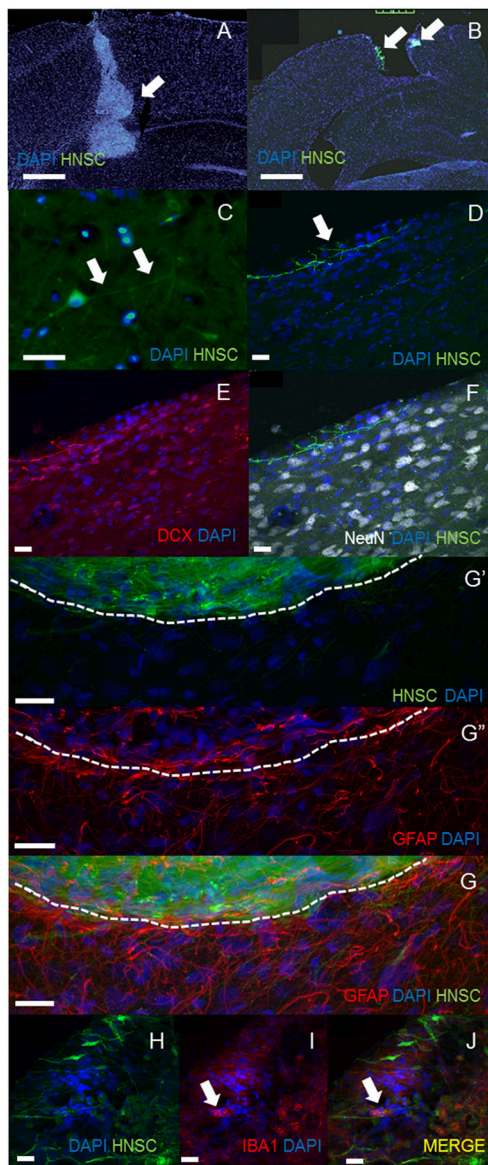


FIGURE 2 | Histological analysis. The engraftment of NSCs could be seen in the injured cortex in *in situ* transplantation (A) and the surface of cortex in superficial transplantation (B) at least 5 weeks post-transplantation (A,B, scale bar = 100 μ m). Much robust engraftment of hNSCs were observed in *in situ* transplanted groups (A). Higher magnification of GFP-positive transplanted hNSCs revealed long processes, resembling neurites [white arrows, (C) in *in situ* transplantation, scale bar = 40 μ m; (D) in surface seeding transplantation] and extending across the injured motor cortex. These cells had firm nuclear and neurites structure which was stained by DCX but not stained by NeuN (D–F, scale bar = 20 μ m). Confocal images of brain sections stained with anti-GFAP antibody also showed absence of GFAP expression in transplanted human hNSC but presence of gliosis at the host-transplant border (dashed white line G,G',G'', scale bar = 20 μ m). Iba-1, a phagocytic markers identifies microglia/infiltrating immune cells (white arrow in H–J, scale bar = 20 μ m).

neurites (white arrows, Figure 2C in *in situ* transplantation, Figure 2D in superficial seeding transplantation) and extending across the injured motor cortex. These cells had firm nuclear and

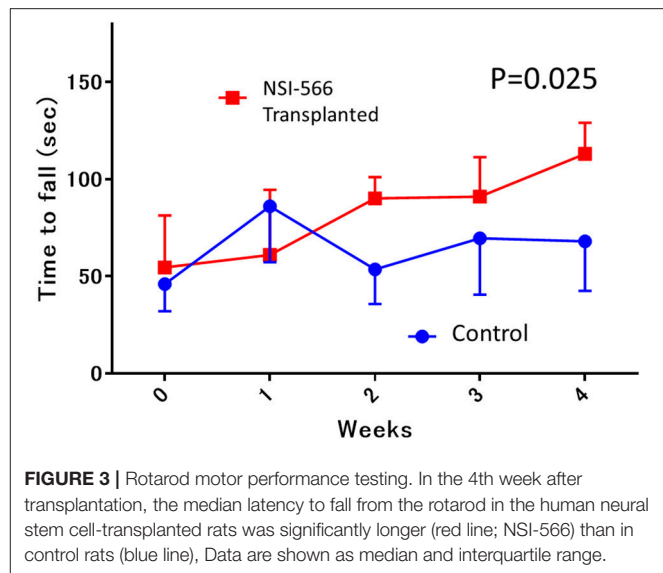


FIGURE 3 | Rotarod motor performance testing. In the 4th week after transplantation, the median latency to fall from the rotarod in the human neural stem cell-transplanted rats was significantly longer (red line; NSI-566) than in control rats (blue line), Data are shown as median and interquartile range.

neurites structure which was stained by DCX but not stained by NeuN (Figures 2E,F). Confocal images of brains sections stained with anti-GFAP antibody also showed absence of GFAP expression in transplanted human hNSC but presence of gliosis at the host-transplant border (dashed white line in Figure 2G). Iba-1, a phagocytic markers identifies microglia/infiltrating immune cells (white arrow in Figures 2H–J).

Rotarod Performance Testing for Motor Function

Rotarod performance testing for motor function was performed in *in situ* transplanted rats (TP group vs. Control group, $n = 10$ each). The median latency to fall from the rotarod in the TP group was significantly superior to that in the control group (median [IQR]: 113 s [76–121] in TP vs. 69 s [43–69] in control, ANOVA, $P = 0.025$; Figure 3).

DISCUSSION

This is the first study demonstrating the feasibility of hNSC transplantation in a clinically-relevant ASDH decompression rat model. In this pilot trial, we found that the transplanted hNSCs were robustly engrafted even until the fourth week after transplantation. GFP-positive hNSCs extended projections resembling neural dendrites. All hNSC transplanted cells were immature but had a tendency to mature toward neuronal cell lines. Rats transplanted with hNSCs survived without weight loss or any adverse effect, similar to rats in the control group. They seemed to perform better behavioral outcome on the rotarod test to control rats.

For the treatment of ASDH associated with intracranial hypertension and cerebral herniation, surgery with craniotomy, to achieve mass evacuation, is recommended as the standard treatment (25). However, the removal of the subdural hematoma itself results in the immediate reversal of global

ischemia and the induction of reperfusion injury (16, 26). Previous experimental and clinical studies have clearly shown that the pathophysiology of ASDH and its removal are synonymous with “ischemic/reperfusion injury” (17, 27). Thus, the pathophysiology of ASDH treated with decompressive craniotomy is quite unique compared to other types of TBI.

To improve the outcome in such patients, several clinical randomized controlled trials have been performed. Recently, the RESCUE-ICP trial clarified that decompressive craniotomy can improve patients’ survival after surgical decompression; however, this trial also demonstrated an increased rate of worse functional outcomes, i.e., persistent vegetable state and severe disability (26). Thus, to improve the functional sequelae in these patients, the novel approach of regenerative medicine is needed.

To date, several preclinical studies have evaluated the efficacy of rodent neural precursor cell transplantation in rodent TBI models (28). The most promising results have been produced by using immunodeficient rodents or host immunosuppression and same-species allografting (29, 30). The exogenous NSCs have been found to integrate into the rodent injured host, aiding endogenous repair and modifying behavior (13).

To study the feasibility of cell transplantation therapy in this unique pathophysiology of ischemic/reperfusion brain injury, we used an ASDH/decompressive craniotomy rat model. We believe that this model shares some characteristics with the simple type ASDH pathophysiology, caused by the slow accumulation of hemorrhage in the subdural space, thus mimicking the clinical disruption of small bridging vessels. Most elderly patients with ASDH have this simple type pathophysiology due to low-impact injuries, such as those caused by tumbling. In Japan, this type of ASDH is actually increasing with the increase in longevity (31). To reflect real-world situations, we considered this model as suitable for our pilot study.

In this pilot study, hNSCs survived and integrated in the ischemic reperfused brain, after surgical decompression. Additionally, transplanted cell morphology changed such that they extended long processes like neurites in the injured cortex and hippocampus. Our IHC data also showed that these hNSCs did not mature but differentiated toward neuronal lineage. Moreover, hNSC transplantation seemed to have better motor functional capacity.

Recently, Inoue et al. showed that rotarod motor performance recovers with the expression of brain derived neurotrophic factor in the motor cortex (32). In our experiment, hNSCs seemed not to be matured yet within 4–8 weeks after transplantation, therefore, in this phase, NSI-566 transplantation may have a potential to support neuronal recovery through secreting neurotrophic factors rather than replenishing neural networks in the motor cortex. Further pathophysiological and electrophysiological examinations with long durations are required to validate our findings.

Our study also demonstrated the safety of hNSC transplantation. Even in immunosuppressed rats, there were no differences in mortality or any side effects compared to control rats. The median body weight did not differ between the TP and control groups, and the transplanted hNSCs did not show malignant proliferation for up to 8 weeks after transplantation.

All rats survived in both groups. These results may serve as a basis for further preclinical animal experiments and clinical trials in the near future.

Limitations

This study has several limitations. First, we used immunosuppressed athymic rats. For translating the results into clinical practice, we should apply this approach to healthy rats without immunosuppression. For this purpose, an appropriate regimen for immunosuppression should be considered. According to a recent publication, intra-peritoneal tacrolimus, methylprednisolone, and mycophenolate mofetil injection were effective for the robust engraftment of hNSCs in a rat model (33). The safety of this regimen should also be confirmed in the ASDH rat model.

Second, we only tracked the engrafted hNSCs for 4–8 weeks after transplantation. In future studies, this observation period will be prolonged. In our recent study, in which we transplanted hNSCs in a rat model of penetrating ballistic-like brain injury, we showed that transplanted cells can survive for up to 16 weeks (13). For the translation of our findings to clinical situations, longer safety and feasibility studies need to be performed.

Third, we only estimated motor function recovery. According to previous literature (14), we considered that rotarod was suitable for motor function testing in the NSC-transplanted animals. To confirm the recovery of spatial learning and memory, other tests should be performed in future studies, like the Y-maze or Morris water maze.

Fourth, our study design did not include sham or naïve surgery. However, since our aim was to clarify the feasibility of NSC transplantation, we only compared injured rats with or without transplantation. For higher accuracy, sham or naïve operated animals should also be examined. These limitations should be taken into consideration in further preclinical studies.

CONCLUSION

In conclusion, our pilot study provides evidence for the feasibility and safety of hNSCs in a rat model of clinical ASDH/decompression. For clinical translatability, future large-scale preclinical studies are warranted.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors to any qualified researcher.

AUTHOR CONTRIBUTIONS

SY, MS, SG, and MB: conception and design. SY and SS: analysis and interpretation of data. SY drafting the article. All authors: critically revising the article. All authors reviewed submitted version of manuscript. SY approved the final version of the manuscript on behalf of all authors. TH and KJ: administrative/technical/material support. MB and HY: study supervision.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Comprehensive Profile of Acute Mitochondrial Dysfunction in a Preclinical Model of Severe Penetrating TBI

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Mitochondria constitute a central role in brain energy metabolism, and play a pivotal role in the development of secondary pathophysiology and subsequent neuronal cell death following traumatic brain injury (TBI). Under normal circumstances, the brain consumes glucose as the preferred energy source for adenosine triphosphate (ATP) production over ketones. To understand the comprehensive picture of substrate-specific mitochondrial bioenergetics responses following TBI, adult male rats were subjected to either 10% unilateral penetrating ballistic-like brain injury (PBBI) or sham craniectomy ($n = 5$ animals per group). At 24 h post-injury, mitochondria were isolated from pooled brain regions (frontal cortex and striatum) of the ipsilateral hemisphere. Mitochondrial bioenergetics parameters were measured *ex vivo* in the presence of four sets of metabolic substrates: pyruvate+malate (PM), glutamate+malate (GM), succinate (Succ), and β -hydroxybutyrate+malate (BHBM). Additionally, mitochondrial matrix dehydrogenase activities [i.e., pyruvate dehydrogenase complex (PDHC), α -ketoglutarate dehydrogenase complex (α -KGDHC), and glutamate dehydrogenase (GDH)] and mitochondrial membrane-bound dehydrogenase activities [i.e., electron transport chain (ETC) Complex I, II, and IV] were compared between PBBI and sham groups. Furthermore, mitochondrial coenzyme contents, including NAD_(t) and FAD_(t), were quantitatively measured in both groups. Collectively, PBBI led to an overall significant decline in the ATP synthesis rates (43–50%; $*p < 0.05$ vs. sham) when measured using each of the four sets of substrates. The PDHC and GDH activities were significantly reduced in the PBBI group (42–53%; $*p < 0.05$ vs. sham), whereas no significant differences were noted in α -KGDHC activity between groups. Both Complex I and Complex IV activities were significantly reduced following PBBI (47–81%; $*p < 0.05$ vs. sham), whereas, Complex II activity was comparable between groups. The NAD_(t) and FAD_(t) contents were significantly decreased in the PBBI group (27–35%; $*p < 0.05$ vs. sham).

The decreased ATP synthesis rates may be due to the significant reductions in brain mitochondrial dehydrogenase activities and coenzyme contents observed acutely following PBBI. These results provide a basis for the use of “alternative biofuels” for achieving higher ATP production following severe penetrating brain trauma.

Keywords: traumatic brain injury (TBI), penetrating ballistic-like brain injury (PBBI), alternative biofuels, brain energy metabolism, energy crisis, mitochondria preferred substrates, dehydrogenase activities, therapeutics

HIGHLIGHTS

- Metabolic substrates mediated decline in ATP synthesis following TBI.
- Decreased brain mitochondrial dehydrogenase activities following TBI.
- Decreased brain mitochondrial NAD_(t) and FAD_(t) contents following TBI.
- Alternative biofuels are recommended to overcome energy crisis after TBI.

INTRODUCTION

There is a growing interest in further understanding of the brain metabolic responses following traumatic brain injury (TBI) to facilitate acute critical care management of TBI patients; and develop novel targeted therapeutic interventions for TBI. TBI produces immediate hypo- or hyper-metabolic responses, which are indicated by the changes in the cerebral metabolic rate of glucose (CMR_{glc}), blood flow and oxygenation. Although the magnitude and duration of CMR_{glc} metabolic pattern varies with the injury severity, species, and the type of brain injury models, all studies consistently showed transient hyper-function followed by prolonged depression during acute to sub-acute phase of secondary injury in both pre-clinical injury models and in TBI patients (1–4). This metabolic shift has been observed in different experimental injury models and clinically over the acute and sub-acute phases following TBI (5–8). During this period, concomitant increases in anaerobic lactate accumulation due to glycolysis was evident in both tissue and extracellular space (3, 5, 9–12). Therefore, a decline in brain energy resulting from TBI is a significant issue that remains to be addressed in the management of TBI (13). In fact, several clinical reviews have recommended early intervention of supplying energy intermediate substrates for improvements in mortality and neurological outcomes following TBI (13–15).

Mitochondria play a pivotal role in maintaining cellular energy homeostasis critical to neuronal cell survival. Previous studies have implicated mitochondrial dysfunction as a

prominent feature of TBI. Abnormal mitochondrial functions include reduced respiratory rates, depleted energy stores (i.e., adenosine triphosphate, ATP), increased free radical production, mitochondrial calcium overload, and early opening of mitochondrial permeability transition pore (mPTP) (16–18). The increased production of free radicals (i.e., reactive oxygen and nitrogen species, ROS/RNS) overwhelms endogenous antioxidants resulting in oxidative stress (19, 20). Additionally, mitochondria release many pro-inflammatory, apoptotic and necrotic signaling factors contributing to cell death overtime (17, 21–23).

In the current study, we hypothesized that TBI leads to altered utilization of glucose and ketone intermediates for brain energy production. We compared mitochondrial ATP synthesis rates for glucose and ketone intermediate substrates (i.e., pyruvate, glutamate, succinate, and β -hydroxybutyrate), each using an identical *ex vivo* condition. Additionally, mitochondrial dehydrogenase activities, and coenzyme contents were quantified following PBBI vs. sham. Collectively, our results provide a basis for the use of therapeutic drugs and nutraceuticals as “alternative biofuels” for the management of energy crisis following brain trauma.

MATERIALS AND METHODS

Materials

Mannitol, sucrose, bovine serum albumin (BSA), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) potassium salt, potassium phosphate monobasic anhydrous (KH₂PO₄), magnesium chloride (MgCl₂), ethylene-diamine-tetra-acetic acid (EDTA), ethylene-glycol-tetra-acetic acid (EGTA), pyruvate, malate, glutamate, succinate, β -hydroxybutyrate, α -ketoglutarate, adenosine-5'-diphosphate (ADP), oligomycin A, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), rotenone, succinate, dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). BCA protein assay kit was purchased from Pierce (Rockford, IL). Both NAD_(t) and FAD_(t) content assessments kits were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mitochondrial substrates and inhibitors stock solutions were prepared and aliquots stored at -80°C .

Animals

Adult male Sprague-Dawley rats (280–350 g, Charles River Laboratories, Raleigh, VA) were used for this study. Animals were housed individually under a normal 12 h light/dark cycle (lights on at 0600 EST) in a well-ventilated facility accredited by the Association for Assessment and Accreditation of Laboratory

Abbreviations: ATP, Adenosine 5'-Triphosphate; α -KGDHC, α -Ketoglutarate Dehydrogenase Complex; ETC, Electron Transport Chain; FAD, Flavin Adenine Dinucleotide (oxidized); FADH₂, Flavin Adenine Dinucleotide (reduced); FAD_(t), Flavin Adenine Dinucleotide (total content); GDH, Glutamate Dehydrogenase; NAD, Nicotinamide Adenine Dinucleotide (oxidized); NADH, Nicotinamide Adenine Dinucleotide (reduced); NAD_(t), Nicotinamide Adenine Dinucleotide (total content); OXPHOS, Oxidative Phosphorylation; PBBI, Penetrating Ballistic-Like Brain Injury; PDHC, Pyruvate Dehydrogenase Complex; TBI, Traumatic Brain Injury; TCA, Tricarboxylic Acid Cycle.

Animal Care (AAALAC), and allowed 7 d for acclimation to the housing facility before any procedures were performed. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Walter Reed Army Institute of Research (WRAIR). Animal studies were conducted in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals (National Research Council), and other federal statutes and regulations relating to animals and experiments involving animals.

Penetrating Ballistic-Like Brain Injury (PBBI) Model

All surgical procedures were performed under isoflurane anesthesia (3–5% for induction and 2% for maintenance) and aseptic conditions with careful monitoring of physiological vital signs. The PBBI surgery was performed as described previously (24–26). The PBBI apparatus consists of a specifically designed probe (Kadence Science, Lake Success, NY), a stereotaxic frame (Kopf, Tujunga, CA) and a hydraulic pressure-pulse generator (4B080; MITRE, MA). The probe was made of a 20G stainless steel tube with fixed perforations along one end which was sealed by a piece of airtight elastic tubing. The probe was secured on the probe holder with the un-perforated end attached to the pulse generator, angled at 50° from the vertical axis and 25° counter clockwise from the midline. Core body temperature was maintained normothermic (~37°C) using a heating blanket (Harvard Apparatus, South Natick, MA). Under isoflurane anesthesia (2%; in air/oxygen mixture), rat head was secured in the stereotaxic device for insertion of the PBBI probe. After a midline scalp incision, a right frontal cranial window (diameter = 4 mm) was created using a dental drill to expose the right frontal pole (+4.5 mm AP, +2 mm ML to bregma). The PBBI probe was then advanced through the cranial window into the right hemisphere to a depth of 1.2 cm from the surface of the brain. Once the probe was in place, the pulse generator was activated by a computer to release a pressure pulse calibrated to produce a rapid expansion of the water-filled elastic tubing to create an elliptical shaped balloon (diameter = 0.633 mm) to a volume equal to 10% of the total brain volume. This rapid inflation/deflation (duration = 40 ms) produced a temporary cavity in the brain. After deflation, the probe was immediately retracted from the brain and the cranial opening was sealed with sterile bone wax, and the skin incision was closed with wound clips. All sham animals underwent craniectomy only with no insertion of the PBBI probe.

Mitochondrial Isolation

At 24 h post-PBBI, animals were asphyxiated with CO₂, rapidly decapitated, and the brains were removed and placed in mitochondrial isolation buffer at 4°C. From ipsilateral hemisphere of PBBI and sham injured brains, the frontal cortex and striatum regions were isolated and pooled together for mitochondrial isolation. The pooled regions represent the injury core and perilesional area of the injury, where extensive cell death may occur due to the initial mechanical force (primary injury) and excitotoxicity (secondary injury). Mitochondria were isolated under identical experimental conditions using

an established Ficoll-based mitochondrial ultra-purification procedure (27–29). All isolation steps were performed at 4°C using mitochondrial isolation buffer (MIB) composed of 215 mM Mannitol, 75 mM Sucrose, 0.1% BSA, 1 mM EGTA, and 20 mM HEPES at pH 7.2. Tissue homogenates were centrifuged at 1,300 × g for 3 min to remove cell debris and nuclei, and collected supernatants were then centrifuged at 13,000 × g for 10 min to pellet crude mitochondria. The resultant crude mitochondrial samples were re-suspended in 500 μl of MIB and kept in a nitrogen cell decompression bomb (model 4,639, Parr Instrument Co., Moline, IL, USA) at 1,200 psi for 10 min at 4°C to release mitochondria from synaptosomes. The mitochondrial samples were then placed on top of a discontinuous Ficoll gradient (layered 2 ml of 7.5% Ficoll solution on top of 2 ml of 10% Ficoll solution) and centrifuged at 100,000 × g for 30 min at 4°C using a Beckman ultracentrifuge with SW55Ti rotor (Beckman Coulter, IN, USA). Ultrapure mitochondrial pellets formed at the bottom were carefully collected, avoiding contamination from ruptured synaptic plasma membranes isolated at the interface. These pure mitochondrial pellets were resuspended in 2 ml MIB[−] (without 1 mM EGTA buffer) and centrifuged at 10,000 × g for 10 min. The washed mitochondrial pellets were finally re-suspended in MIB[−] to achieve desirable mitochondrial protein concentration (~10 mg/ml) for mitochondrial function assessments. Mitochondrial protein concentration was determined using the BCA protein assay kit measuring absorbance at 562 nm with BioTek Synergy HT plate reader (Winooski, VT, USA). Following the completion of mitochondrial isolation, mitochondrial respiration was measured within ~3 h post-isolation; whereas remaining samples were stored at −80°C for dehydrogenase activities and coenzyme content assessments.

Mitochondrial Respiration

The real-time mitochondrial respiration was assessed by the Clark-type oxygen electrode in a continuously stirred, sealed chamber thermostatically maintained at 37°C (Oxytherm System, Hansatech Instruments Ltd.), as described previously (16, 27, 29). Approximately 50 μg of mitochondrial protein was added into the chamber containing 250 μl of KCl-based respiration buffer (125 mM KCl, 2 mM MgCl₂, 2.5 mM KH₂PO₄, 0.1% BSA, 20 mM HEPES, pH 7.2). After 1 min equilibration, the State II respiration was initiated by the addition of either one of the four separate sets of metabolic substrates [e.g., pyruvate+malate (PM), glutamate+malate (GM), succinate (Succ), or β-hydroxybutyrate+malate (BHBM)] in the respiration chamber. The State III respiration rate was measured subsequently after addition of two boluses of 150 μM ADP followed by State IV respiration rates that were measured by the addition of 1 μM oligomycin. The mitochondrial uncoupler FCCP (1 μM) was added at last to measure uncoupling respiration rates (State V) in the chamber. For each mitochondrial sample, all four substrates driven mitochondrial respiration were individually performed; and their respiration rates (i.e., from State II to State V) were recorded to calculate oxygen consumption rates (nmols O₂ consumed/mg protein).

Mitochondrial Dehydrogenase Activity Assessments

All remaining -80°C stored mitochondrial samples were freeze-thawed and sonicated together three times before measuring enzyme activities at 37°C . Mitochondrial matrix dehydrogenase activities [i.e., pyruvate dehydrogenase complex (PDHC), alpha-ketoglutarate dehydrogenase complex (α -KGDHC), and glutamate dehydrogenase (GDH)] and membrane-bound dehydrogenase activities (i.e., electron transport chain (ETC) Complex I, II, and IV) were performed by either absorbance or fluorescence based assays using an automated 96-well microplate reader (BioTek Instruments, INC, Winooski, VT, USA) as described below (27, 30–34).

Pyruvate Dehydrogenase Complex (PDHC) Enzyme Activity

The mitochondrial gate keeper enzyme PDHC catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA and thereby links the glycolysis pathway to TCA cycle. It provides intermediate acetyl-CoA substrates to TCA cycle for energy metabolism. The first subunit of the PDHC i.e., pyruvate dehydrogenase (PDH) enzyme activity was measured using previously described methods with slight modification. The assay was performed using a substrates, inhibitor and cofactor mixtures in a buffer containing 50 mM KCl, 10 mM HEPES pH 7.4, 0.3 mM thiamine pyrophosphate (TPP), $10\text{ }\mu\text{M}$ CaCl_2 , 0.2 mM MgCl_2 , 5 mM pyruvate, $1\text{ }\mu\text{M}$ rotenone, and 0.2 mM NAD. Ficoll-purified mitochondrial protein ($8\text{ }\mu\text{g}$ / well) was assayed in triplicate for each sample. The enzyme reaction was started by the addition of 0.14 mM coenzyme A. The fluorescence based enzyme activity assay was performed (Ex λ 340 nm, Em λ 460 nm), and increment of NADH fluorescence was measured per minute interval. The PDHC activity was calculated and expressed as % change in PBBI vs. sham.

Glutamate Dehydrogenase (GDH) Enzyme Activity

The mitochondrial enzyme GDH catalyzes the reversible oxidative deamination of glutamate to α -ketoglutarate and serves as a key link between anabolic and catabolic pathways. The GDH enzyme activity ($10\text{ }\mu\text{g}/\text{well}$) assay was determined (i.e., absorbance 450 nm) by a coupled enzyme colorimetric assay ($100\text{ }\mu\text{l}$ assay volume) in which glutamate was consumed by GDH generating NADH, which in turn reacts with a probe proportional to the GDH activity present. One unit of GDH is the amount of enzyme that generates 1 mmols of NADH per minute at pH 7.6 at 37°C . The GDH assay was performed using commercially available assay kit (Sigma-Aldrich, USA) and the data was expressed as % change in PBBI vs. sham.

α -Ketoglutarate Dehydrogenase Complex (α -KGDHC) Enzyme Activity

The first subunit form of the mitochondrial enzyme α -KGDHC, 2-oxoglutarate dehydrogenase complex catalyzes the conversion of 2-oxoglutarate to succinyl-CoA and CO_2 in the TCA cycle. The first subunit of the α -KGDHC i.e., α -ketoglutarate

dehydrogenase (α -KGDH) activity ($10\text{ }\mu\text{g}/\text{well}$) assay was determined (i.e., absorbance 450 nm) by a coupled enzyme colorimetric assay ($100\text{ }\mu\text{l}$ assay volume) in which 2-oxoglutarate was consumed by α -KGDH generating NADH, which in turn reacts with a probe proportional to the α -KGDH activity present. One unit of α -ketoglutarate dehydrogenase activity is the amount of enzyme that generates 1 mmols of NADH per minute at pH 7.5 at 37°C . The α -KGDH assay was performed by commercially available assay kit (Sigma-Aldrich, USA) and the data was expressed as % change in PBBI vs. sham.

Complex I (NADH Dehydrogenase) Enzyme Activity

Complex I, or NADH dehydrogenase, is the first enzyme of the mitochondrial ETC. It catalyzes the transfer of electrons from NADH to coenzyme Q. The Complex I activity assay was performed in 25 mM KH_2PO_4 buffer (pH 7.2) containing 5 mM MgCl_2 , 1 mM KCN, 1 mg/ml BSA and $150\text{ }\mu\text{M}$ NADH. Mitochondrial protein ($6\text{ }\mu\text{g}/\text{well}$) was added to the reaction buffer, and the assay was performed in the absence or presence of rotenone ($10\text{ }\mu\text{M}$) to determine rotenone-insensitive and rotenone-sensitive enzyme activities. The reaction was started by the addition $50\text{ }\mu\text{M}$ of coenzyme Q_1 . The fluorescence based enzyme activity assay was performed (Ex λ 340 nm, Em λ 460 nm), and decrease in fluorescence of NADH was measured per 1 min intervals. The Complex I activity was calculated and expressed as % change in PBBI vs. sham.

Complex II (Succinate Dehydrogenase) Enzyme Activity

Complex II, or succinate dehydrogenase, is the only membrane-bound enzyme of the TCA cycle that participates additionally in the ETC activities. The FAD containing enzyme catalyzes the oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol. The enzyme activity was determined by coupled reactions measuring the change in absorbance of a dye, 2,6-Dichlorophenolindophenol (DCIP), at 600 nm using a BioTek Synergy HT plate reader (BioTek, Winooski, VT, USA). Briefly, the mitochondrial protein ($8\text{ }\mu\text{g}/\text{well}$) was added to 200 mM KH_2PO_4 (pH 7.0) assay buffer containing 20 mM K-succinate as substrate for Complex II, $10\text{ }\mu\text{M}$ EDTA, 0.01% Triton X-100, $1\text{ }\mu\text{g}$ coenzyme Q_{10} in a total assay volume of $100\text{ }\mu\text{l}/\text{well}$. The Complex I inhibitor rotenone was not included in the assay system. The $30\text{ }\mu\text{l}$ of concentrated DCIP dye (20 mg%) was added to each well, giving initial absorbance readings near 0.9–1.0. In the reaction, succinate is oxidized to fumarate and reduces DCIP dye (blue color) into DCIPH₂ (colorless) compound. During this succinate-DCIP oxidation-reduction, coenzyme Q_{10} acts as an intermediate compound that transiently accepts electrons and further donates to DCIP. The decrease in DCIP absorbance was measured per minute based on nmols of succinate oxidized; and expressed as % change in PBBI vs. sham.

Complex IV (Cytochrome C Oxidase) Enzyme Activity

Complex IV is the terminal electron acceptor enzyme in the mitochondrial ETC complexes. It receives an electron

from four cytochrome c molecules and transfers them to one oxygen molecule to convert molecular oxygen; together with translocating four protons into intermembrane space from mitochondrial matrix to establish a transmembrane electrochemical potential ($\Delta\Psi$ m). The cytochrome c oxidase activity was measured in 10 mM KH_2PO_4 buffer by co-incubating mitochondrial protein (6 $\mu\text{g}/\text{well}$). The reaction was initiated by adding 50 μM reduced cytochrome c. The rate of oxidation of cytochrome c was measured by detecting decrease in absorbance of reduced cytochrome c at 550 nm per 1 min intervals. The Complex IV activity was calculated and expressed as % change in PBBI vs. sham.

Mitochondrial Coenzyme Contents

NAD_(t) Quantification

Nicotinamide adenine dinucleotide (NAD) is an enzymatic cofactor involved in many redox reactions. NAD functions as an electron carrier, cycling between the oxidized (NAD) and reduced (NADH) forms. In addition to its role in redox reactions, NAD plays critical roles in several cellular metabolic reactions. In a colorimetric assay (i.e., absorbance 450 nm), the total form of NAD or NAD_(t) (i.e., the combined form of both NAD and NADH) was quantified using commercially available NAD quantification kit (Sigma-Aldrich, USA). This assay is specific for NAD_(t) and does not detect NADP nor NADPH. Briefly, mitochondrial proteins (100 μg) were re-suspended in 100 μl of NAD extraction buffer. Samples were vortexed and freeze-thawed three times, and any remaining insoluble debris was finally pelleted at $13,000 \times g$ for 10 min and discarded. The extracted supernatant was transferred into a new tube and stored for measurement (50 $\mu\text{g}/\text{well}$). The NAD_(t) concentration was measured as pmoles per mg protein based on a standard curve and expressed as % change in PBBI vs. sham.

FAD_(t) Quantification

Flavin Adenine Dinucleotide (FAD) is a coenzyme, synthesized from riboflavin, which plays critical roles in many metabolic pathways. FAD functions as an electron carrier in multiple redox reactions, cycling between FAD and FADH_2 . The primary sources of reduced FAD in eukaryotic metabolism are the TCA cycle and the beta oxidation reaction pathways. For total FAD or FAD_(t) (i.e., the combined form of both FAD and FADH_2) content measurement, mitochondrial proteins (100 μg) were re-suspended in 100 μl of FAD extraction buffer and deproteinized with 8% perchloric acid solution. Briefly vortex and incubate the precipitate mixture for 5 min. The mixture was centrifuged at $1,500 \times g$ for 10 min and then transferred into a new tube for measurement (50 $\mu\text{g}/\text{well}$). The FAD_(t) concentration was measured using fluorescence-based assay (Ex λ 535 and Em λ 587 nm) using commercially available FAD quantification kit (Sigma-Aldrich, USA). The FAD_(t) concentration were measured as pmoles per mg protein based on a standard curve and expressed as % change in PBBI vs. sham.

Statistical Analysis

A *priori* power analyses were conducted to determine the sample size within individual experiments using G*Power 3

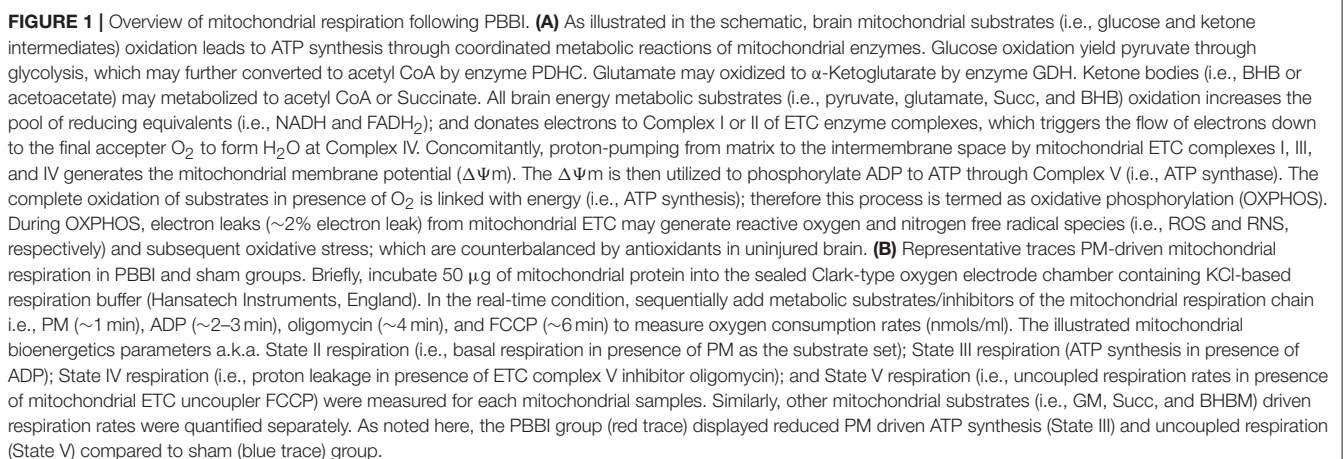
(Germany) statistical program. The power analyses based on previous mitochondrial bioenergetics data indicated that an $n = 5$ sample size/group, is sufficient to detect a 20% change as statistically significant ($p < 0.05$) with power of 0.8 compared to control. The experimental data are presented as bar graphs with error bars represented by $\pm$ standard error of the mean (SEM). All mitochondrial samples were prepared from individual animals ($n = 5/\text{group}$) using brain tissue derived from the cortex/striatum in the injured hemisphere. All samples were evaluated as triplicates in each experiment. An unpaired *t*-test was used for between-group comparisons (2 groups) whereas multiple group (> 2 groups) comparisons were conducted using analysis of variance (ANOVA) followed by a Fisher protected least squared differences (PLSD) *post-hoc* test (GraphPad Prism 6 software package, GraphPad Software, Inc. La Jolla, CA). Statistical significance was defined at $*p < 0.05$.

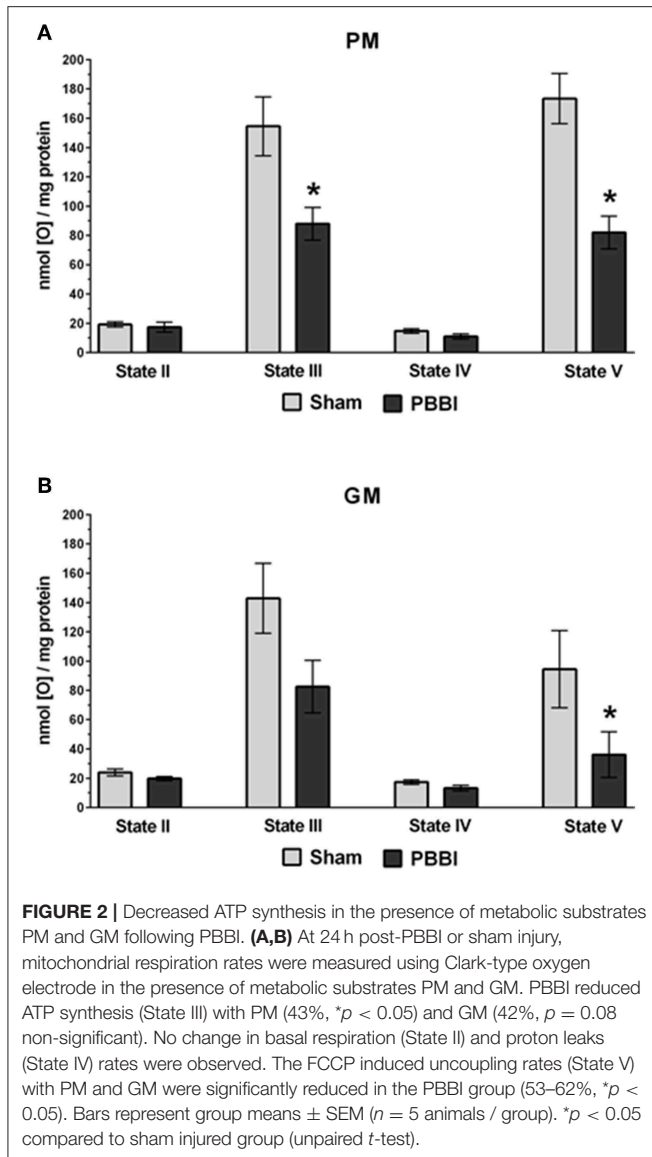
RESULTS

Mitochondrial bioenergetics parameters were evaluated in brain samples that were pooled from the frontal cortex and striatum of the injured hemisphere. At 24 h post-PBBI, mitochondrial bioenergetics parameters (i.e., State II to State V respiration), mitochondrial dehydrogenase activities, and coenzyme contents were measured in the sham and PBBI groups. The schematic overview of brain mitochondrial metabolic reactions and bioenergetics (Figure 1A), and the real-time tracing of PM-driven mitochondrial respiration in PBBI vs. sham group were represented as illustrated (Figure 1B).

Global Mitochondrial Bioenergetics Depression in PBBI

At 24 h post-PBBI or sham injury, mitochondrial respiration rates were measured in the presence of glucose intermediate substrate sets: pyruvate+malate (PM) and glutamate+malate (GM) (Figure 2). Following PBBI, mitochondrial respiration with PM showed a significant reduction in both State III (43%, $*p < 0.05$) and State V (53%, $*p < 0.05$) respiration. No change in PM driven basal respiration (State II) and proton leaks (State IV) were observed between the PBBI vs. sham groups (Figure 2A). Substrates GM showed similar trend in the PBBI group. Comparing to sham, the mitochondrial respiration with GM showed a trend toward altered State III respiration (42%, $p = 0.08$, non-significant), whereas State V respiration was reduced significantly (62%, $*p < 0.05$) in the PBBI group. No injury-induced changes were detected in GM driven basal respiration (State II) and proton leaks (State IV) (Figure 2B). Similarly, we evaluated mitochondrial bioenergetics parameters in the presence of two sets of additional glucose and ketone intermediates: succinate (Succ) and β -hydroxybutyrate+malate (BHBM) (Figure 3). When Succ was used as the metabolic substrate, both State III (50%, $*p < 0.05$) and State V (40%, $*p < 0.05$) respiration were significantly decreased in PBBI compared to sham. No change in Succ driven basal respiration (State II) and proton leaks (State IV) rates were observed between two groups (Figure 3A). With BHBM as substrates, the State III respiration

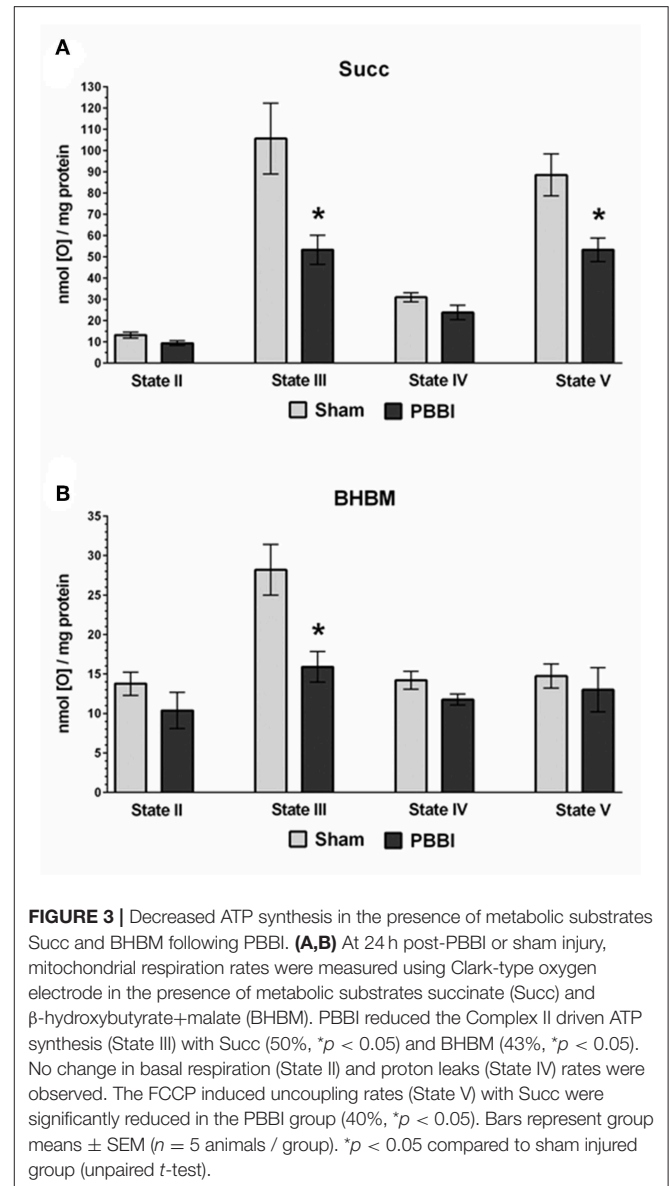




rate was reduced significantly (43%, $*p < 0.05$) in PBBI; whereas BHBM driven basal respiration (State II), proton leaks (State IV), and uncoupling rate (State V) were comparable between the sham and PBBI groups (Figure 3B).

Substrate Preference for ATP Synthesis

To evaluate within group substrate preferences, the ATP synthesis rates of the four substrates were compared under either sham or PBBI condition (Figure 4). In the sham group, the PM, GM, and Succ substrate driven ATP synthesis (State III) were comparable; whereas the BHBM driven ATP synthesis was significantly lower compared to other substrates (PM=GM=Succ>BHBM, $*p < 0.05$). In the PBBI group, the pattern of substrate utilization for ATP synthesis remained identical, but at a lower magnitude compared to the sham group. In the PBBI group, both PM and GM



driven ATP synthesis remained higher compared to that driven by Succ and BHBM. The ATP synthesis between PM, Succ, and BHBM were significantly distinct amongst each other (PM=GM>Succ>BHBM, $*p < 0.05$).

Mitochondrial Dehydrogenase Enzyme Activities in PBBI

Mitochondrial matrix dehydrogenases, PDHC and GDH activities were significantly decreased compared to the sham group (42 and 53%, respectively, $*p < 0.05$). No injury-specific differences were detected in the α -KGDHC activity between PBBI and sham groups (Figure 5A). In the PBBI group, mitochondrial membrane-bound dehydrogenases, Complex I and Complex IV enzyme activities were significantly decreased compared to the sham group (47 and 81%, respectively, $*p < 0.05$);

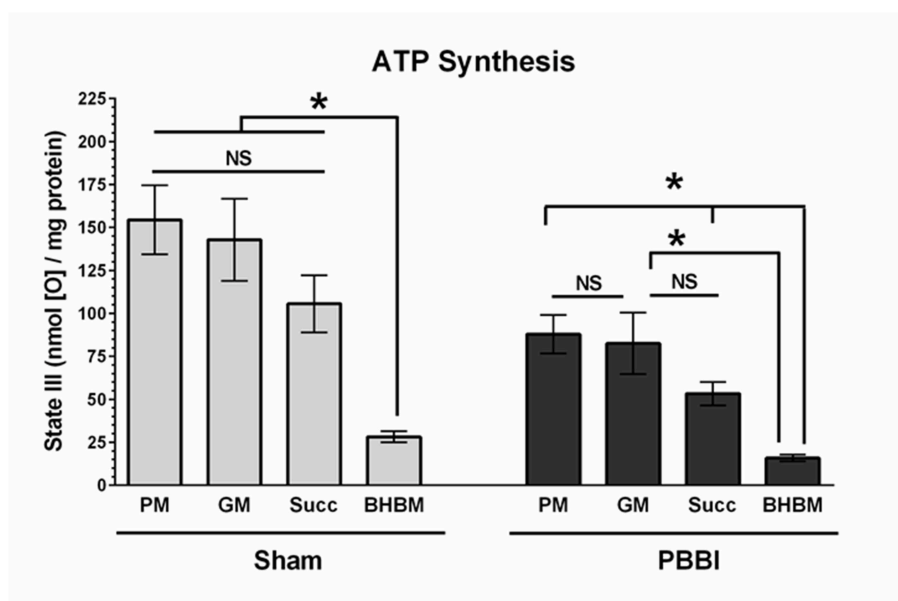


FIGURE 4 | Comparison of ATP synthesis rates within the experimental group. The ATP synthesis rates of the four substrates (i.e., PM, GM, Succ, and BHBM) were compared within sham or PBBI group. In the sham group, the BHBM showed significantly less ATP synthesis than any other substrates (PM=GM=Succ>BHBM, $*p < 0.05$). In the PBBI group, the ATP synthesis of PM, Succ, and BHBM were significantly distinct amongst each other (PM=GM>Succ>BHBM, $*p < 0.05$). Bars represent group means $\pm$ SEM ($n = 5$ animals / group). $*p < 0.05$ compared within four substrates groups of sham or PBBI (ANOVA, Fisher *post-hoc* test).

whereas Complex II activity was comparable to that in the sham group (Figure 5B).

Decreased Coenzyme Contents in PBBI

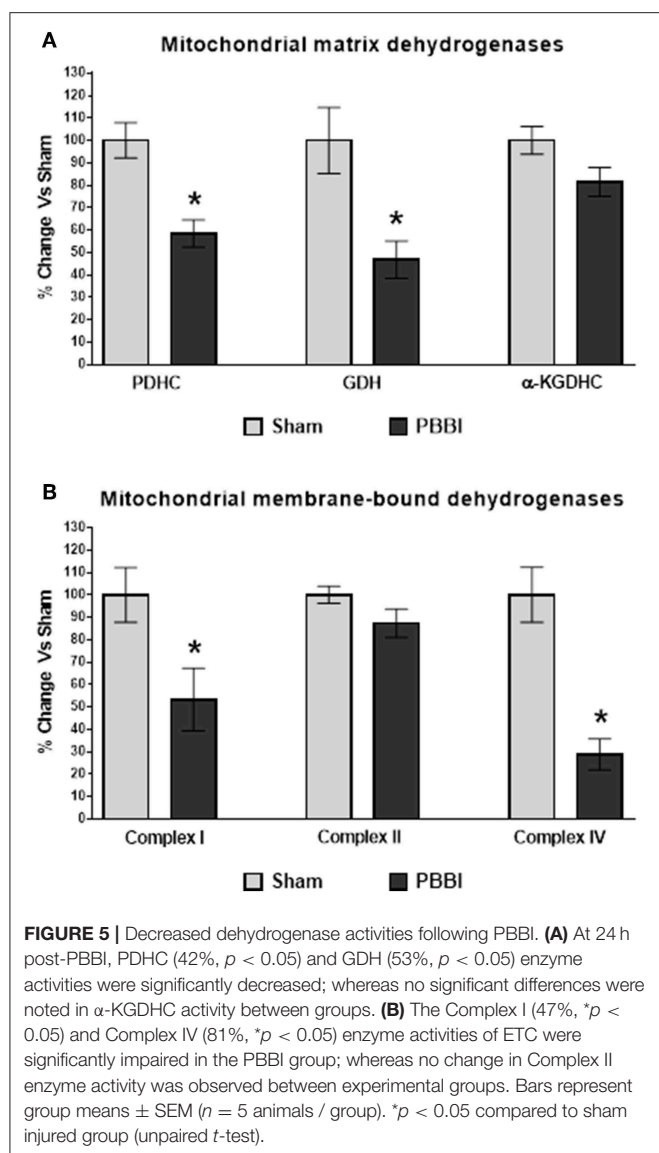
Mitochondrial coenzyme contents (i.e., NAD_(t) and FAD_(t)) were quantitatively measured in PBBI and sham groups. In the PBBI group, both NAD_(t) (35%, $*p < 0.05$) and FAD_(t) contents (27%, $*p < 0.05$) were significantly decreased compared to the sham group (Figure 6).

DISCUSSION

The uninjured healthy brain preferentially utilizes glucose intermediates (vs. ketones) as the primary and obligated source of energy-rich metabolic fuels (35). Our previous research revealed significant alterations in metabolic responses between 30 min and 7 d post-PBBI, including osmotic stress, neurotransmitters imbalance, hyper-glycolysis, decreased TCA cycle anaplerotic metabolism, depletion of creatine stores, and enhanced complex lipid hydrolysis (36). We also detected altered glucose uptake and oxygen consumption in the injured cortex, including both the injury core and the peri-lesional area, at 2.5 h post-PBBI (37). In addition, using intracerebral microdialysis, we have reported an early energy metabolic dysfunction and the metabolic shift toward anaerobic glycolysis (indicated by high lactate to pyruvate ratio) within 3 h following PBBI (38).

The current study was designed to examine the acute brain mitochondrial bioenergetics dysfunction following PBBI based on our previous observations of the cerebral metabolomics

alterations in this brain injury model. Results of the current study demonstrate mitochondrial bioenergetics failure evident at 24 h post-PBBI, which was likely due to a global declines in substrate utilization for energy production, including altered mitochondrial dehydrogenase activities and coenzyme contents. These results correspond with and build on previous work conducted in the CCI model of TBI (16, 34, 39–44). In the CCI model, the majority of published studies have utilized only pyruvate+malate (PM) as the energy substrates to evaluate Complex I driven mitochondrial respiration, in which the PM dependent mitochondrial ATP synthesis rates were significantly reduced within 30 min post-injury (39) and remain compromised up to 2–5 d post-injury (40–42). However, Xiong et al. reported that glutamate+malate (GM) dependent Complex I driven mitochondrial ATP synthesis was decreased between 1 h and 14 d post-injury (43). In the current study, we evaluated both PM and GM as metabolic substrates for complex I driven mitochondrial respiration in the PBBI model. Our results showed a reduction in both complex I driven ATP synthesis rates (State III) and uncoupling rates (State V) following PBBI (Figure 2). Notably, we had previously hypothesized that the reduction in PM driven ATP synthesis may be counterbalanced by the use of GM as an alternative metabolic substrate to produce ATP following PBBI, based early evidence that showed elevated extracellular glutamate levels following TBI (45–47). However, results from the current study showed a GM-dependent non-significant decrease trend in ATP synthesis following PBBI, which indirectly suggests that the glutamate substrate may not be an efficient alternative fuel for energy replenishment following PBBI.



We used two additional glucose and ketone intermediate substrates, succinate (Succ), and beta-hydroxybutyrate+malate (BHBM), to evaluate their bioenergetics capacity in the PBBI model (Figure 3). Both of these substrates support Complex II driven mitochondrial respiration for ATP synthesis, and thereby may bypass the Complex I driven respiration and partially circumvent the dependence on both PDHC and Complex I enzyme functions. It was reported that both PDHC and Complex I enzyme activities were significantly decreased at 3 h post-injury following CCI (34). To evaluate whether the Complex II driven respiration is also compromised, the Succ and BHBM dependent mitochondrial ATP synthesis rates were measured in isolated mitochondria following TBI. The results showed that both Succ and BHBM dependent ATP synthesis were significantly decreased following PBBI, which indicates that Complex II driven energy production were significantly compromised. Together, a significant decline in both Complex

I and II driven ATP synthesis rates indicated global metabolic depression following PBBI. As such, this global energy crisis might potentially be mitigated using energy enhancers as a therapeutic treatment for acute TBI.

In support of this hypothesis, recent efforts were carried out to potentiate brain energy metabolism using alternative energy substrates in TBI patients. In one such study, Jalloh et al. reported that the ^{13}C -labeled succinate (12 mmol/L for 24 h) perfused via intracerebral catheter decreased the lactate/pyruvate ratio measured in microdialysate samples, and improved glucose and glutamate utilization, thereby favoring aerobic glycolysis through metabolic flux into the TCA cycle in nine TBI patients with Glasgow Coma Scale ≤ 8 (48). These results provide support for the use of TCA cycle products or alternative energy substrates for enhancing brain mitochondrial metabolism in TBI patients as previously suggested (49–56).

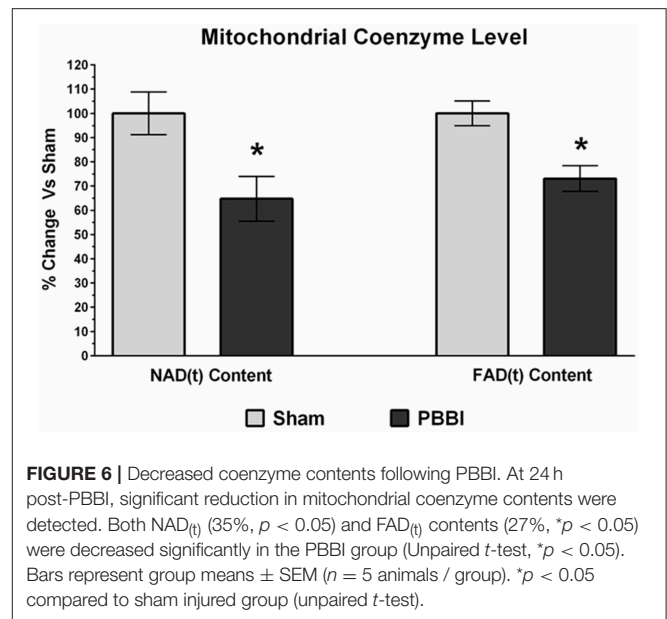
In the current study, substrate dependent ATP synthesis rates were compared among major energy intermediates (i.e., pyruvate, glutamate, succinate and β -hydroxybutyrate) to identify the mitochondria preferred energy substrates at 24 h following either sham or PBBI procedure (Figure 4). Under the sham condition, the ATP synthesis rate of PM, GM, and Succ were unchanged amongst each other and were all significantly higher than that of BHBM (PM=GM=Succ>BHBM). Similarly, Berg et al. observed that the uninjured healthy brain mitochondria preferred glucose intermediates over ketones as energy substitutes and the metabolic responses were tissue-specific (35). In the current study, it was postulated that the metabolic substrates preference for energy production may have changed due to incurred metabolic stress following PBBI. Interestingly, our results showed that under the PBBI condition, PM and GM remained as preferred substrates for the injured brain, while the ketone BHBM utilization remained as the lowest compared to other glucose intermediates (PM=GM>Succ>BHBM). This is the first time that global metabolic substrate utilization pattern for ATP synthesis was evaluated in *ex vivo* condition following TBI. Overall, our results showed that glucose intermediate substrate utilization was declined following injury and ketone utilization remained the lowest when tested in *ex vivo* condition using isolated mitochondria from the PBBI brains.

All four metabolic substrates tested here for ATP synthesis showed energy deficits at 24 h post-PBBI, suggesting that therapeutic treatments should be initiated as early as possible to mitigate secondary injury responses of energy crisis following PBBI. Additionally, it may possible that these mitochondrial energy dysfunctions persist upto 2–5 d post-PBBI, as observed in the CCI model (40, 41). Similar to the current energy deficits observed at the injury core and peri-lesional brain regions (i.e., frontal cortex and striatum), the other brain regions which are distant to the injury site may show mitochondrial bioenergetics impairments. Therefore, a comprehensive post-injury time-course analysis of mitochondrial bioenergetics from different brain regions are warranted to better understand the brain region-specific injury responses following PBBI.

Several mitochondrial proteins involved in the cellular bioenergetics displayed oxidative modification following TBI.

A proteomics study carried out by Opii et al. observed that mitochondrial dehydrogenase activities were reduced at 3 h post-CCI and several mitochondrial proteins displayed oxidative damage following TBI (34). In the current study, we measured mitochondrial dehydrogenase activities at 24 h post-PBBI (**Figure 5**). The mitochondrial matrix dehydrogenase (i.e., PDHC and GDH) activities were significantly reduced following PBBI, whereas the α -KGDHC activity was comparable between the sham and PBBI groups. The reduced PDHC and GDH enzyme activities may affect the PM and GM dependent ATP synthesis rates in the PBBI group as discussed previously. Moreover, the mitochondrial membrane-bound dehydrogenase Complex I and IV activities were significantly decreased after PBBI. In contrast, Complex II enzyme activity was not affected following PBBI. Originally, it was postulated that mitochondrial dehydrogenase activities would drop significantly due to the observed global decline in energy metabolism indicated by ATP synthesis and coenzyme (NAD_t and FAD_t) contents following PBBI. However, it is counterintuitive that both α -KGDHC and Complex II enzyme activities remained unchanged despite of their cofactors' levels were significantly lower following PBBI. Overall, these data suggest the differential susceptibility of mitochondrial dehydrogenase activities to secondary injury, possibly due to the divergent redox-state sensitivity within their structures, ultimately leading to altered substrates oxidation, metabolic suppression, and energy depletion following injury. However, this hypothesis warrants further evaluation to confirm the causative effects of mitochondrial enzyme oxidation on the post-injury energy metabolism.

The use of Complex II driven energy substrates such as succinate or ketones, which oxidation feed electrons through Complex II, would be a good choice as “alternative biofuels,” given that they bypass the enzymatic dysfunction of both PDHC and Complex I in OXPHOS following PBBI. In the literature, pro-drugs that serve as alternative-energy substrates have been evaluated, glyceryl triacetate, or acetyl L-carnitine, which may bypass mitochondrial PDHC deficiency following brain and spinal cord injuries (12, 54, 55, 57–59). Similarly, decreases in PDHC and Complex I enzyme activities following PBBI observed here may be bypassed by the utilization of complex II driven alternative energy fuels for severe penetrating brain trauma. Note that the Complex IV enzyme activity was severely reduced following PBBI, consistent with previous findings in the CCI model (34). As Complex IV is the terminal enzyme complex of the ETC oxidative phosphorylation, any therapeutic intervention discussed above designed to bypass PDHC and Complex I enzymes may not be exclusively efficient to overcome complex IV deficiency. Therefore, drug intervention that can prevent or delay damage to Complex IV enzyme may prove beneficial for therapeutic purposes in combination with alternative biofuels/drugs to increase ATP synthesis following injury. Comprehensively, the current study has evaluated several mitochondrial targets, which can be bypassed / protected individually or in combination in the future using therapeutic interventions to alleviate metabolic depression following severe penetrating TBI.



In the current study, we measured both NAD_t and FAD_t contents using biochemical assays. These cofactors play essential roles in many cellular and mitochondria specific metabolic reactions by acting as electron (e^-) and proton (H^+) donors for substrate oxidation. In the mitochondrial ETC chain, the NADH transfers electrons to Complex I, whereas the FAD is a prosthetic group of Complex II which receives electrons from succinate, thereby bypassing Complex I for ATP synthesis. Our data presented here (**Figure 6**) showed a significant decline in both NAD_t and FAD_t contents following PBBI, which indicated that the capability to carry out efficient electron and proton transfers for ATP synthesis remained limited. Therefore, therapeutics that target replenishment of NAD_t and FAD_t may be useful in combination with Complex IV agonists to enhance energy efficiency following severe TBI. Efforts toward evaluating NAD and its precursors, nicotinamide and nicotinic acid, as neuroprotective agents for TBI and ischemic brain injury have shown some improvement in behavioral functions (60–68). However, more rigorous efforts are needed to validate bioenergetics and neuroprotective efficacy of both NAD and FAD precursors as a treatment for TBI.

In summary, our study provided a comprehensive evaluation of mitochondrial dysfunction at 24 h following PBBI. We observed glucose or ketone intermediate substrates mediated decline in ATP synthesis following PBBI. Additionally, mitochondrial dehydrogenase activities and coenzyme contents were significantly decreased following PBBI. While additional experiments are warranted to provide a comprehensive time-course and injury severity profile of mitochondrial bioenergetics in the PBBI model, the results of the current study provide a basis for the use of “alternative biofuels” for achieving higher ATP production following severe penetrating brain trauma.

ETHICS STATEMENT

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Walter Reed Army Institute of Research (WRAIR). Animal studies were conducted in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals (National Research Council), and other federal statutes and regulations relating to animals and experiments involving animals.

AUTHOR CONTRIBUTIONS

JP contributed in literature review, research experiments, data analysis, and manuscript writing. XY and

WF provided technical assistance. LL, YD-B, JG, and DS participated in experimental design and manuscript writing.

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Chronic Cognitive Deficits and Associated Histopathology Following Closed-Head Concussive Injury in Rats

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Close-head concussive injury, as one of the most common forms of traumatic brain injury (TBI), has been shown to induce cognitive deficits that are long lasting. A concussive impact model was previously established in our lab that produces clinically relevant signs of concussion and induced acute pathological changes in rats. To evaluate the long-term effects of repeated concussions in this model, we utilized a comprehensive Morris water maze (MWM) paradigm for cognitive assessments at 1 and 6 months following repeated concussive impacts in rats. As such, adult Sprague-Dawley rats received either anesthesia (sham) or repeated concussive impacts (4 consecutive impacts at 1 h interval). At 1 month post-injury, results of the spatial learning task showed that the average latencies to locate the hidden “escape” platform were significantly longer in the injured rats over the last 2 days of the MWM testing compared to sham controls ($p < 0.05$). In the memory retention task, rats subjected to repeated concussive impacts also spent significantly less time in the platform zone searching for the missing platform during the probe trial ($p < 0.05$). On the working memory task, the injured rats showed a trend toward worse performance, but this failed to reach statistical significance compared to sham controls ($p = 0.07$). At 6 months post-injury, no differences were detected between the injured group and sham controls in either the spatial learning or probe trials. However, rats with repeated concussive impacts exhibited significantly worsened working memory performance compared to sham controls ($p < 0.05$). In addition, histopathological assessments for axonal neurodegeneration using silver stain showed that repeated concussive impacts induced significantly more axonal degeneration in the corpus callosum compared to sham controls ($p < 0.05$) at 1 month post-injury, whereas such difference was not observed at 6 months post-injury. Overall, the results show that repeated concussive impacts in our model produced significant cognitive deficits in both spatial learning abilities and in working memory abilities in a time-dependent fashion that may be indicative of progressive pathology and warrant further investigation.

Keywords: traumatic brain injury, concussion, cognition, neurobehavior, neurodegeneration

INTRODUCTION

Mild traumatic brain injury (mild TBI) or concussion has been reported to occur to an estimated 42 million people worldwide annually (1). While many patients showed improvements and returned to work within days or months after the injury, reportedly 22–36% suffered from prolonged cognitive impairments that lasted months and years beyond the initial injury (2). As such, there have been empirical data suggesting that specialized treatment is needed to improve the long-term outcomes of mild TBI patients (3). However, it remains challenging to delineate contributing factors that underlie various post-concussion symptoms and explore targeted treatments (4).

To better understand the neurobiology of concussion, we have previously developed and refined a closed-head concussive rat model that simulates a projectile impact concussion in humans and produces concussion-like clinical symptoms, such as loss of consciousness (LOC) (5). Compared to other existing mild TBI animal models (6–8), the WRAIR projectile concussive impact (PCI) model does not require scalp incision or craniotomy, which more closely resembles the real scenario of a close-head concussion. In addition, the PCI device produces a projectile impact at the rat head and the rat's head and cervical spine are allowed to move freely upon the impact, which commonly occurs in a true concussive event. Biomechanical calibration on model parameters, such as projectile mass, impact energy, and head movement kinetics, indicates that this model produces highly consistent and reproducible close-head concussive impacts in rats, making it an optimal exploratory platform for studying concussion preclinically (5).

Using the PCI model, we have previously conducted preliminary neurobiological assessments following a single vs. repeated concussive impacts for up to four impacts at 1 h intervals (5). The duration of loss of righting reflex, which indicates LOC, increases as the number of repeated impacts increases, suggesting increases in injury severity. It is noted that four consecutive concussive impacts produced more severe symptoms than a single impact did but remained to be a mild TBI, indicated by the duration of loss of righting reflex at <15 min (9). Anatomical examination of the brain tissues after four consecutive impacts in the PCI model showed that they were free of gross pathology, also indicating a mild TBI. Given concussive events, such as sports concussions, could occur repeatedly within a short time frame, a repetitive concussive model that results in a mild TBI would have important clinical relevance and more likely to lead to long lasting, rather than transient, cognitive deficits. Therefore, we examined the chronic cognitive deficits in rats subjected to repeated concussive impacts in the PCI model using Morris water maze (MWM).

The MWM is commonly used in animal models for assessing neurobehavior, especially spatial learning ability and memory function (10). It has a wide range of applications in understanding cognitive dysfunctions in neurodegenerative diseases, such as aging and neurotrauma. Different testing paradigms in the MWM have been designed to evaluate various aspects of cognitive dysfunctions in animal models of TBI. For

example, retrograde memory loss was detected using MWM memory retention tests in rats that received a lateral fluid percussion injury (FPI), and such memory dysfunction was correlated to ipsilateral hippocampal cell loss (11). When the impact location was changed to the sagittal suture to generate a central FPI, rats performed worse than the controls in the MWM working memory test (12). In another study, the long-term MWM spatial learning disability in rats that sustained contusive impacts to the medial prefrontal cortex was suggested to be the result of attentional deficits due to TBI (13). It has also been reported that rats' inability to initiate search strategies in the MWM spatial learning task was linked to prefrontal cortical lesions as a result of penetrating injuries to the brain (14). More recently, methods in cognitive evaluation using MWM in neurotrauma have been summarized based on data produced in our lab (15). In the current study, we have re-designed some of the testing parameters for a more sensitive measurement of the spatial acquisition ability, memory retention, and working memory at chronic time points following repeated concussive impacts. Additionally, histological assessment for axonal neurodegeneration was conducted in rats that have completed the behavioral testing to explore possible pathological mechanisms that underlie long-term behavioral changes.

METHODS

Subjects

Male adult Sprague-Dawley rats (48 rats total; 280–320 g; Charles River Labs, Raleigh, NC, USA) were used in these experiments. All procedures involving animal use were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Walter Reed Army Institute of Research. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition. Animals were housed individually under a 12 h light/dark cycle in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALACI).

PCI Model

The PCI-induced concussive injury was described previously (5). The PCI apparatus consists of an elevated platform and a computer-controlled electro-pneumatic pressure release system used to launch a small projectile (i.e., a 3.52 g stainless steel sphere) targeted at the rat's head. Following anesthetization with 4% isoflurane, a custom-designed helmet (Army Research Lab, Aberdeen Proving Ground, MD) was securely fastened onto the rat's head. The anesthetized rat was placed on the elevated platform with its head positioned above an oval opening in the elevated platform such that the helmet-protected head was exposed to the projectile. A computer program was used to trigger the targeted release of the projectile at an operating pressure of 80 psi and produce a concussion targeting the right frontal region of the rat brain. Immediately following the concussive impact, the helmet was removed, and the rat was

returned to its home cage. Rats were subjected to 4 repeated concussive impacts, spaced at 1 h apart, to produce more severe concussive symptoms, yet remaining within the limits of the mild TBI spectrum (5). Prior to each impact, rats were anesthetized, and the time of anesthesia was kept constant. The sham control rats received the same procedures (4 times anesthesia and air puffs, 1 h apart) except the projectile impact.

Morris Water Maze (MWM) Task

Cognitive abilities were assessed in the MWM (Noldus EthoVision XT, VA) at 1 and 6 months in the same animals following repeated concussive impacts or sham procedures (Figure 1).

The MWM apparatus consists of a circular basin (75 cm deep; 175 cm diameter) filled with clear water (22°C room temperature) to a depth of 60 cm placed in a dark room with visual cues. A clear, plexiglas platform was submerged to a depth of 2.5 cm from the water surface and placed in the center of the northwest quadrant of the pool. The platform position remained constant during the spatial acquisition testing paradigm. Rats were placed in the pool at one of the equally spaced starting positions (north, south, east, and west). The starting position was pseudo-randomly determined for each trial within a day, alternating between short- and long-arms in reference to the platform. Each rat was allowed to swim freely to find the hidden platform or until 60 s elapsed. Rats were given four trials per day (5 min inter-trial interval) for 4 consecutive days. Mean latency to find the hidden platform were recorded on each day. A probe trial for testing memory retention was given on the last day immediately following the last trial of the spatial acquisition test. Each rat was allowed to swim freely until 60 s elapsed. Percent time spent in the platform zone searching for the missing platform during the probe trial was recorded. On testing day five, rats were given two sets of “one-trial learning” working memory test. Each set of the trials consist of two trials with 4 min inter-trial interval, in which the rats were given two opportunities to swim freely to search for the hidden platform or until 60 s elapsed. Platform location and starting positions for each trial set were determined pseudo-randomly. Latency difference (delta) to locate the platform between two trials within a trial set during the working memory test was recorded.

Histology

At 1 month following repeated concussive impacts or sham procedures, a subgroup of animals ($n = 12$ /time point/group) were euthanized after completing the MWM tasks, while the remaining animals were euthanized after they were tested again in the MWM at 6 months post-injury ($n = 12$ /time point/group). For histological studies, rats were transcardially perfused with phosphate-buffered saline (PBS), followed by 4% cold paraformaldehyde under deep anesthesia. Coronal brain sections (40 μ m) were cut from +3.72 to -6.84 mm anteroposterior from Bregma. Four sets of serial sections were collected at 960- μ m intervals. All the samples were processed at FD NeuroTechnologies (Ellicott City, MD). The first set was processed for the detection of neurodegeneration with FD NeuroSilver™ Kit II (FD Neurotechnologies, Ellicott City, MD)

according to the manufacturer's instructions. The remaining brain sections were stored for other histopathology assessments. The sections were mounted on microscope slides and cover-slipped with Permount (Fisher Scientific, Fair Lawn, NJ). Investigators blinded to the injury conditions took images of the sections using an Olympus VS120 Whole Slide Scanning System (Olympus Corporation of the Americas, Waltham, MA) at uniform criteria for sensitivity and exposure time. Silver positively stained cells were quantified using threshold analysis in the corpus callosum to evaluate neurodegeneration. The threshold value was set to consistently detect maximal positive staining of silver. To ensure objective quantification, the same threshold value was applied to all brain sections. All quantification was performed by an investigator blinded to the groups using Image J (NIH, Version 1.49).

Statistical Analysis

Statistical analysis was performed using SAS software v9.1 (SAS Institute, NC) and SigmaPlot 12.0 (Systat Software Inc., CA). Two-way repeated measures analysis of variance (ANOVA) was used to analyze the behavioral data. One-way ANOVA was used to compare the data obtained from the probe trial of Morris water maze. Student's *t*-test was used to determine the between-group difference in histology data. The significance criterion of all the statistical tests was set at $p < 0.05$. Data are presented as the mean $\pm$ standard error of means (SEM).

RESULTS

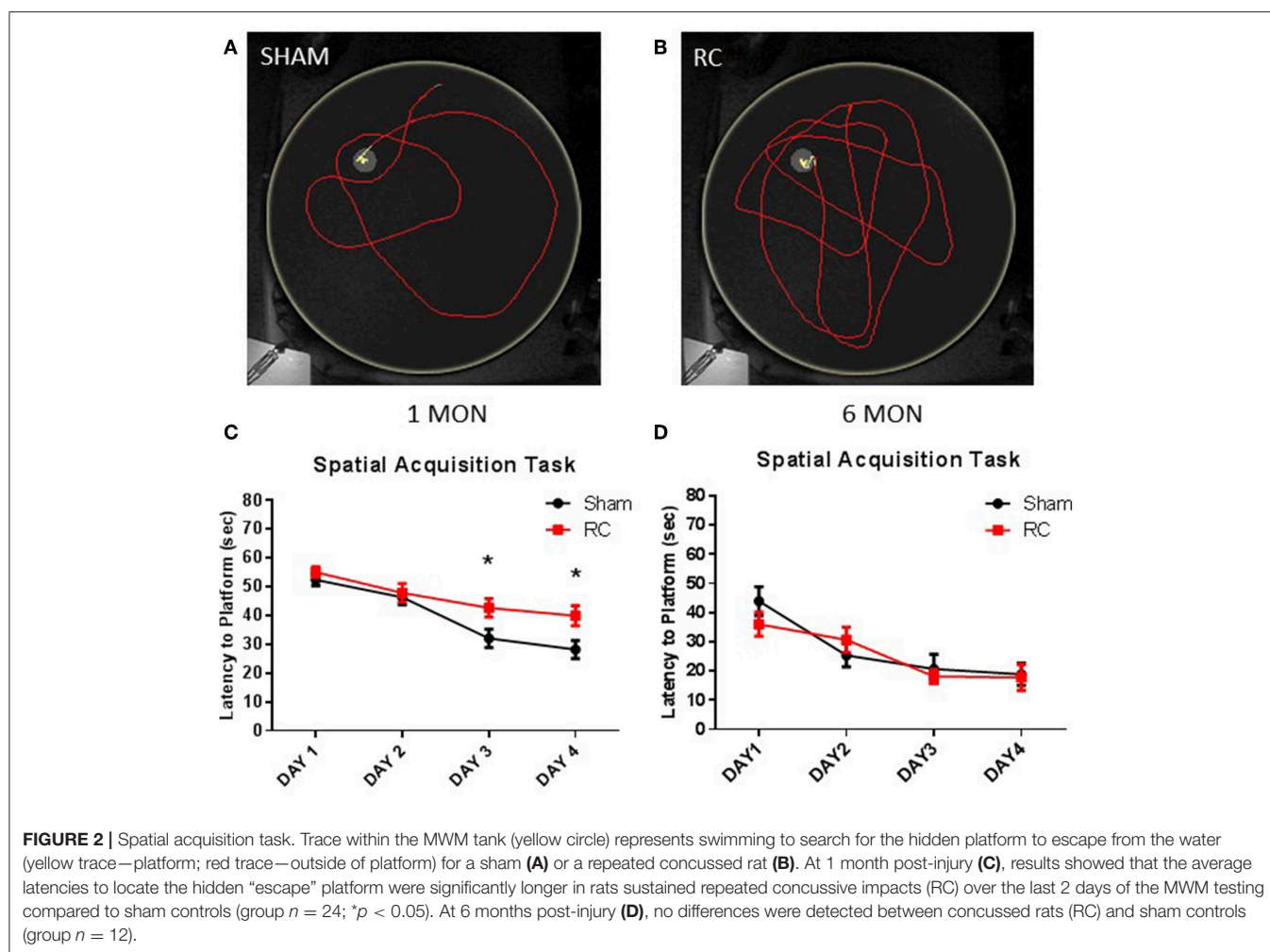
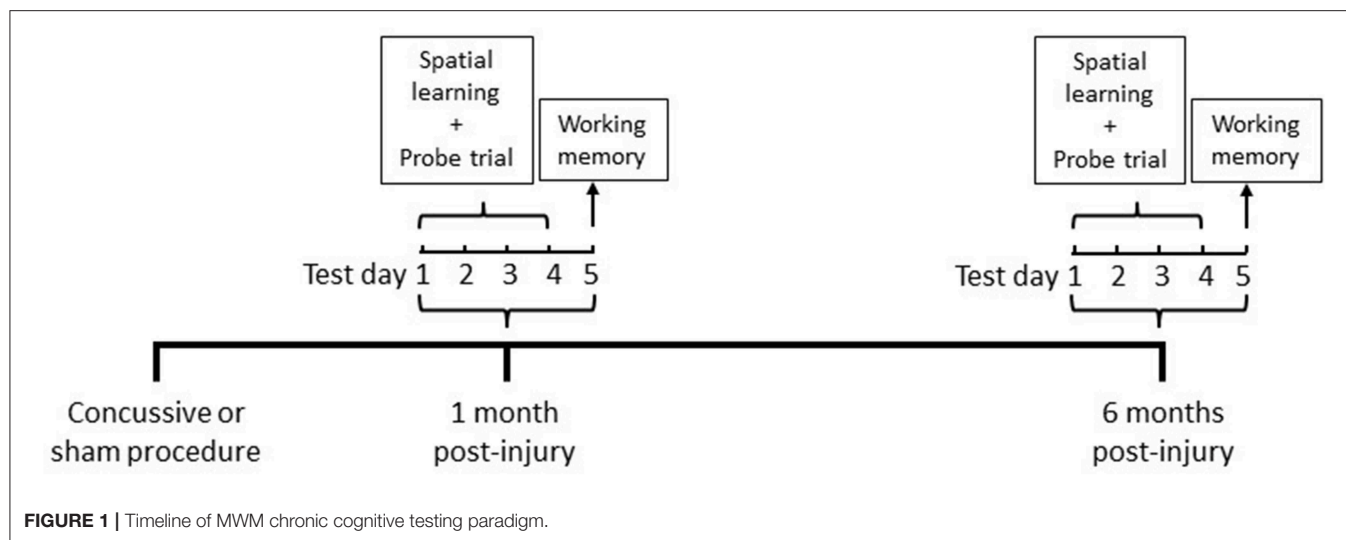
Spatial Acquisition Task

Spatial acquisition learning ability was assessed in the MWM at 1 and 6 months following sham procedure or repeated concussive impacts (Figure 2). In this task, the rats were allowed to swim freely to locate the platform and escape from the water using only spatial cues in a dark room. Representative trace images showed that a sham control rat (Figure 2A) quickly located the platform using visual cues, whereas rats that sustained repeated concussive impacts (Figure 2B) swam longer distance in searching for the hidden platform relatively aimlessly. Group average of mean latency (sec) to locate the platform per day was calculated. At 1 month post-injury, the sham controls showed better ability to navigate and spent significantly less amount of time to locate the platform on the 3rd and 4th day of the spatial acquisition task compared to the concussed rats ($p < 0.05$; two-way repeated ANOVA with S-N-K *post-hoc* test) (Figure 2C). At 6 months post-injury, the earlier observed superior searching ability in the sham control rats was lost and they exhibited similar spatial learning ability as the rats sustained concussive impacts (Figure 2D).

Memory Retention Task

Memory retention ability was evaluated during a probe trial in the MWM at 1 and 6 months following sham procedure or repeated concussive impacts (Figure 3).

At the end of the spatial acquisition assessment, the hidden platform was removed from the water tank, and rats were put back into the maze to search for the missing platform based on



their recent memory. Representative trace images indicate that a sham control rat (**Figure 3A**) exhibited better memory retention and spent more time circling in the platform area to escape

from the water, whereas a concussed rat (**Figure 3B**) distributed its search for the platform evenly throughout the water maze. Group average of percent (%) time out of 60 s that the rats spent

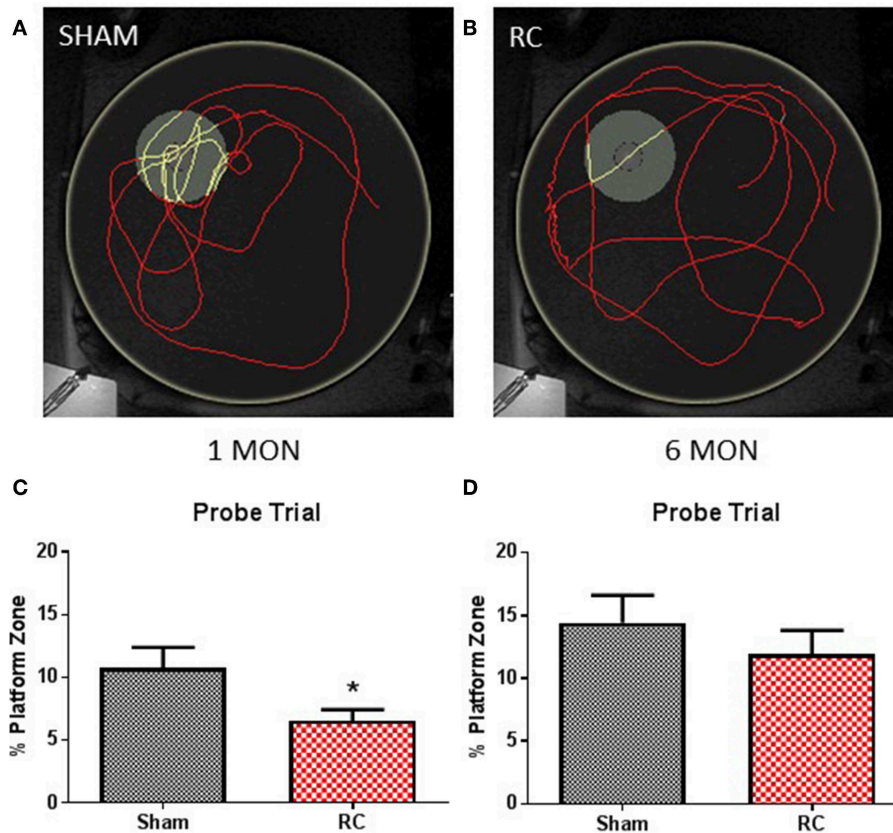


FIGURE 3 | Memory retention task. Trace within the MWM tank (yellow circle) represents swimming to search for the missing platform during the probe trial (yellow trace—platform; red trace—outside of platform) for a sham (A) or a repeated concussed rat (B). At 1 month post-injury (C), rats sustained repeated concussive impacts (RC) spent significantly less time in the platform zone searching for the missing platform during the probe trial in the memory retention task (group $n = 12$; $*p < 0.05$). At 6 months post-injury (D), no differences were detected between the concussed rats (RC) and sham controls (group $n = 12$).

in the platform zone was recorded and calculated. At 1 month post-injury, the concussed rats spent a significant less percentage of time in the platform zone compared to sham controls ($p < 0.05$; Student's t -test) (Figure 3C). At 6 months post-injury, both sham and rats subjected to repeated concussive impacts exhibited similar performance in the probe trial (Figure 3D).

Working Memory Task

Working memory function was assessed using a one-trial learning test in the MWM at 1 and 6 months following sham procedure or repeated concussive impacts (Figure 4).

In this test, paired trials (Trial 1 and Trial 2) with the same starting location and platform location was presented to the rats, in which the difference in time for the rats to locate the platform between the two trials was calculated. The latency different ($\text{Time}_{\text{Trial1}} - \text{Time}_{\text{Trial2}}$) between two trials within a trial pair indicates the working memory ability, such that the larger the latency difference the better working memory function. The test was then repeated with a different pair of trials that have a different set of starting location and platform location from the previous trial pair. Representative trace images demonstrate that a rat spent more time in the first trial (Trial 1; Figure 4A)

to locate the platform, while it spent less time when the trial (Trial 2; Figure 4B) was repeated immediately. Group average of the mean latency difference was calculated. At 1 month post-injury, concussed rats exhibited a trend toward worsened working memory function compared to sham controls ($p = 0.07$; Student's t -test) (Figure 4C). At 6 months post-injury, rats with repeated concussive impacts showed a significantly worse performance in the working memory task compared to sham controls ($p < 0.05$; Student's t -test) (Figure 4D).

Histology

Silver staining for axonal neurodegeneration in the corpus callosum (Supplement Figure 1) was quantified at 1 and 6 months following sham procedure or repeated concussive impacts in rats ($n = 12/\text{time point/group}$) that completed the cognitive assessments (Figure 5).

Representative coronal brain sections showed an example of the intensity of the silver staining in sham (Figure 5B) vs. rats subjected to concussive impacts (Figure 5C). Area fraction of the silver staining in both ipsilateral and contralateral corpus callosum region was presented in the bar graph (Figure 5A). At 1 month post-injury, the results showed that silver staining

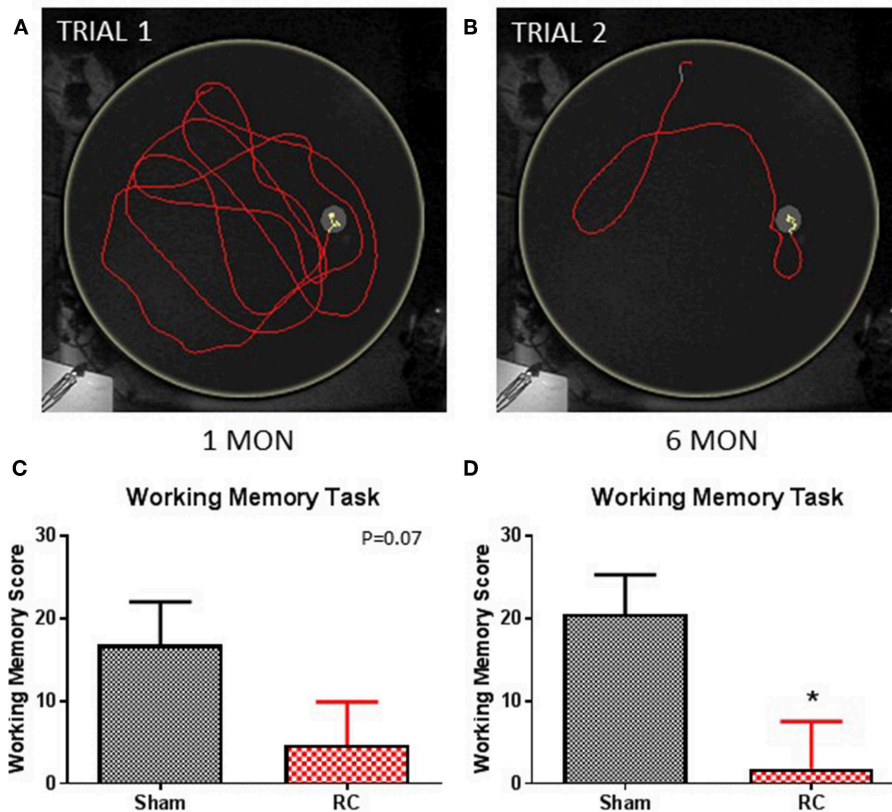


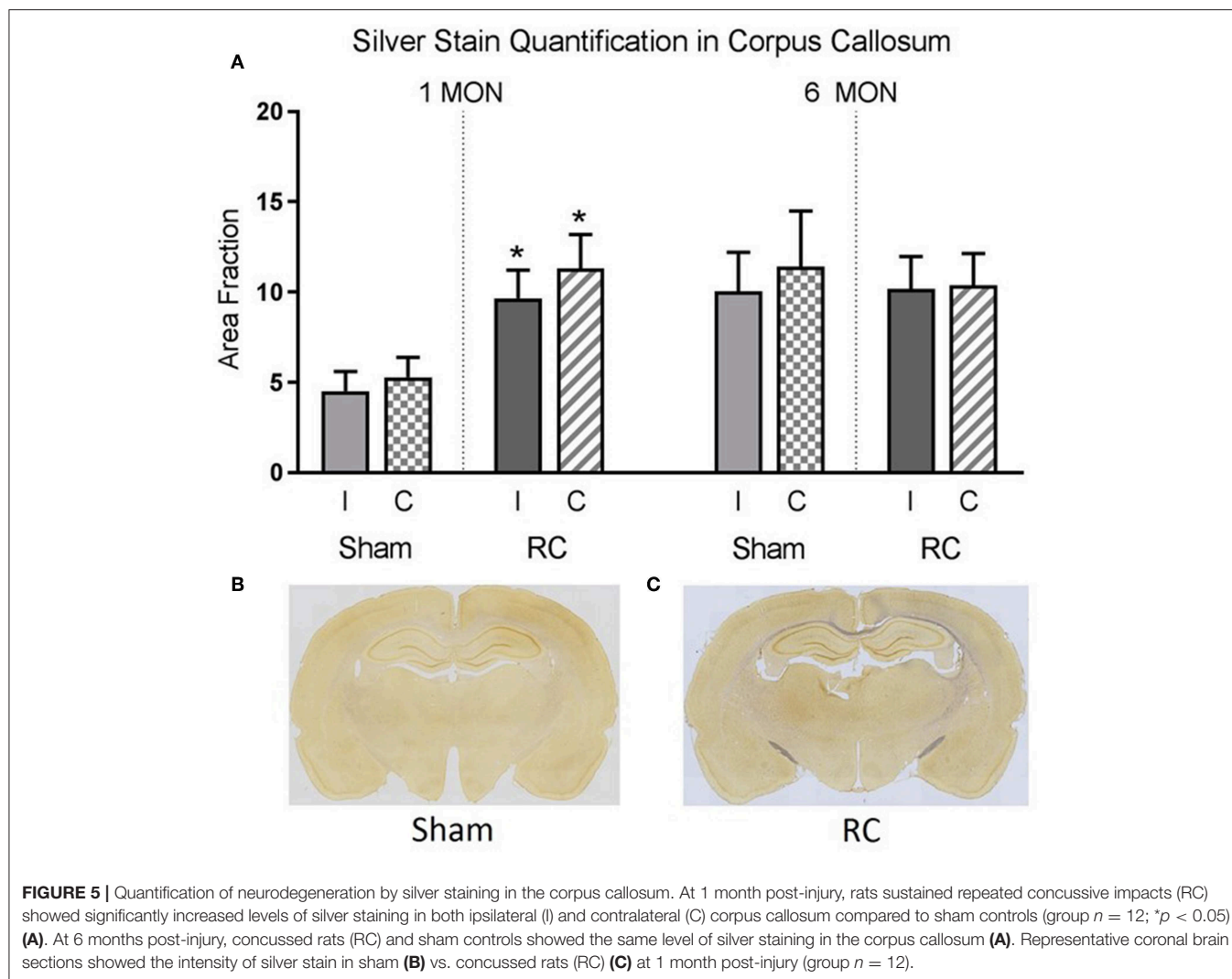
FIGURE 4 | Working memory task. Trace within the MWM tank (yellow circle) represents swimming to search for the hidden platform in a set of trials: trial 1 (A) and trial 2 (B) (yellow trace—platform; red trace—outside of platform) for a sham rat. At 1 month post-injury (C), rats sustained repeated concussive impacts (RC) showed a trend toward worse performance, but this failed to reach statistical significance compared to sham controls on the working memory task (group $n = 12$; $p = 0.07$). At 6 months post-injury (D), the concussed rats (RC) exhibited significantly worsened working memory performance compared to sham controls (group $n = 12$; $*p < 0.05$).

was significantly increased in the concussed rats compare to the sham controls in both ipsilateral and contralateral corpus callosum ($p < 0.05$; Student's t -test). At 6 months post-injury, the silver staining levels in sham control rats elevated and showed no difference from that detected in the concussed rats.

DISCUSSION

The current study demonstrates that repeated concussive impacts produced by the PCI device resulted in long-term cognitive deficits in rats that are detectable using MWM. While the repeated impacts produced a mild TBI, the MWM paradigms designed in this study were shown to be sensitive for measuring small yet significant long-term cognitive differences between concussed rats and sham controls. In addition, rats sustained repeated concussive impacts demonstrated cognitive dysfunction in spatial learning at 1 month post-injury, while it progressed to working memory deficits at 6 months post-injury. Histopathological evaluation revealed that axonal degeneration in the corpus callosum may be related to cognitive dysfunction after repeated concussive impacts.

Cognitive deficits are often experienced by mild TBI patients during the acute phase following injuries and in many cases progress to long-term problems. Mild TBI patients have reported to experience numerous cognitive impairments, including having difficulty in learning and memory, and attention and information processing speed (16). In this study, results showed that rats that received repeated concussive impacts performed significantly worse compared to sham controls in the MWM spatial acquisition task and the probe trial at 1 month post-injury. In experimental models, spatial acquisition is thought to largely depend on hippocampal function for retaining spatial memory during the MWM trials (17). In support of that, the current study showed that the concussed rats' ability to retain memory during the probe trial at 1 month post-injury was also significantly impaired compared to sham controls. Additionally, it has been indicated that dorsal hippocampus is more susceptible to a blunt head impact and thus plays a more important role on spatial learning ability than the ventral side (18). However, the ability to acquire spatial learning strategy may not be limited to mnemonic function, but involves other aspects of the brain function, such as search strategies and attention. For example, lesions to medial



thalamus in animal models revealed its important role in search strategies and swimming behavior (e.g., thigmotaxic swimming) in the MWM (19). In addition, frontal lobe injuries resulted in rats spending more time and swimming longer distance to find the hidden platform, indicating their inability to initiate search strategies in the MWM (20). Although repeated concussive impacts in our model produces a mild injury, it is possible that the high velocity projectile impact and the rapid rotation of the head led to impairment of brain functions that depend on frontal lobe and related circuitry. Consistent with that, clinical evidence has shown that frontal lobes and subcortical structures that support executive functions, such as planning, aspects of attention, and purposeful behavior, are vulnerable to injuries, leading to cognitive dysfunctions in mild TBI patients (21).

The MWM one-trial learning test showed that the concussed rats' performance trended worse compared to sham controls at 1 month and the effect reached statistical significance at 6 months post-injury. In this test, working memory function is required to store and process the trial-specific information

for a short term in order to guide the navigation to the platform in the immediately repeated trial. Working memory process is thought to be largely involved with the prefrontal cortical region (22). As such, repeated impacts to the frontal lobe and related circuitry can also lead to working memory deficits, given that these areas appear to be susceptible to injuries in the PCI model as discussed above. Working memory function is essential for complex cognitive activities, including executive functions such as learning and reasoning (23). The cognitive processes that are used in the spatial acquisition task, such as planning, memory, and attention, involve some degree of working memory function. As such, working memory deficits may have contributed partially to the worse performance in the spatial acquisition task in rats that received repeated concussive impacts compared to sham controls. This explains the concurrent deficits in both spatial learning ability and working memory function observed at 1 month following repeated concussive impacts in the current study. Consistent with the observation in the rat

PCI model, functional imaging data have shown that working memory capacity was impaired in mild TBI patients (24). It is noted that persistent working memory deficits can present without observable brain structural damage. Therefore, changes in neurotransmitters and receptors, along with the possible abnormality in cellular structures that develops over time, are suggested to be the underlying mechanisms for prolonged working memory dysfunction following TBI (25).

In the present study, MWM tasks were repeated in the same rats at 6 months post-injury in order to evaluate the progression of cognitive dysfunction following repeated concussive impacts. Notably, spatial learning and memory retention deficits observed in the concussed rats at 1 month post-injury was not detected at 6 months post-injury, while working memory dysfunction persisted through 6 months. This suggests cognitive recovery in the time spent in MWM to locate the hidden platform, while other aspects of cognitive deficits worsened. Similarly, Darwish et al. reported that rats showed normal latency to locate the platform in MWM, while impairments in search strategies and memory retention remained within 1 month following a mild TBI (26). Clinically, long-term effects of a mild TBI on cognitive performance are more complex. For example, cognitive dysfunction following sports-related concussion in some patients reverted to normalcy based on neurocognitive testing at around 2–3 months' time post-injury and yet self-reported symptoms persisted (27). While these self-reported chronic cognitive symptoms are not always reflected by the neurocognitive testing results, mild TBI patients with persistent symptoms showed significantly worse performance in working memory and information processing speed tasks than non-symptomatic mild TBI patients beyond 1 year after injury (28). Consistent with that, experimental TBI models also showed chronic deficits in working memory function that plays a key role in many high-level cognitive activities, including information processing speed and attention. However, working memory dysfunction can persist without overt brain structural damage during chronic phase following TBI. It is suggested that such dysfunction may be, in part, explained by the cellular and molecular mechanisms, such as the increased GABA-mediated inhibition of prefrontal neuronal activity (29). However, neurotransmission inhibition appeared to be acute and resolved in about 1 month post-injury. Alternatively, increased dendritic spine density and altered catecholamine signaling were purported to be responsible for working memory deficits during chronic phase following TBI (25). In this study, repeated concussive injury resulted in a time-dependent change in cognitive function that was detectable using different MWM tasks, each designed to evaluate specific cognitive functions including spatial learning ability and working memory function. As such, the PCI model may serve as an ideal platform for exploring mechanisms that underlie acute and chronic cognitive dysfunctions following close-head concussions.

Silver staining showed that neurodegeneration significantly increased in the corpus callosum in the concussed rats compared to sham controls at 1 month and this level of silver staining sustained out to 6 months following injury. There have been clinical evidence indicating white matter track abnormalities

following sports-related concussions in retired athletes, which was purported to associate with cognitive deficits (30). In addition, pronounced atrophy was detected in the corpus callosum in TBI patients who survived the injury after 1 year (31). However, the link between white matter track abnormalities and chronic cognitive deficits remains unclear. It has been purported that axonal demyelination occurs in the corpus callosum, or there have been anterograde degeneration through corpus callosum from ipsilateral degenerating neurons. In addition, chronic inflammation was observed within the corpus callosum in mild TBI models (32, 33). Thus, one of the limitations of this study is that neuroinflammatory responses, such as microglial reactivity, were not investigated. Another limitation would be that cellular mechanisms underlying potential axonal demyelination of the commissural fibers within the corpus callosum were not examined. It should be noted that the silver staining in the corpus callosum in sham controls increased at 6 months compared to 1 month after sham procedure, reaching similar levels as that in rats subjected to repeated concussive impacts measured at 6 months after injury. Similarly, Onyszchuk et al. also reported increased neurodegeneration in the aged sham brains detected by silver staining (34). This study suggests that a more pronounced blood-brain barrier opening in the aged sham animals may have contributed to the increased neurodegeneration, which also positioned the aged brains more vulnerable to injuries (34). In the current study, an earlier onset (1 month) of increased neurodegeneration was detected in the concussed brains compare to the sham controls, but this did not progress when assessed at 6 months post-injury. Given that in this study all the animals received repeated concussive impacts or sham procedure were at the same age (i.e., adults), the mild injuries that the concussed rats sustained may have only expedited the onset of the neurodegeneration process that would have occurred later due to normal aging. However, further studies are warranted to confirm this hypothesis. For the increased axonal degeneration detected by silver staining in the corpus callosum area, there have been reports suggest that damage to callosal fibers can cause visual disturbances in animals (35, 36). Thus, the rats' MWM performance could be affected by potential impairments to the visual center of the brain, and not limited to memory deficits, indicating a possible limitation of this study. However, MWM data at 6 months post-injury showed that the sham controls and the concussed rats performed similarly in both spatial acquisition task and memory retention task, suggesting that any potential visual center damage may not have induced detectable differences in the MWM performance. In support of that, it has been reported that axonal damage in optic nerve and optic track areas did not induce noticeable effects on cognitive and motor outcomes (6). Nonetheless, given the higher level of silver staining in the corpus callosum, future studies are warranted to test visual center impairment following concussive injuries.

Previously, we have demonstrated that the PCI model produces clinically relevant concussion symptoms and acute pathological changes in rats. The current study showed that the PCI model also generates long lasting cognitive

deficits that may reflect chronic cognitive impairments seen in mild TBI patients. More importantly, the PCI-induced cognitive deficits in spatial learning ability and working memory developed over time, which may indicate progressive changes in cellular and molecular mechanisms despite the lack of gross pathology. Further studies using this model would be informative with regard to investigating mechanistic factors that underlie chronic clinical symptoms following concussions.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Walter Reed Army Institute of Research.

AUTHOR CONTRIBUTIONS

YD-B wrote the manuscript and designed the study. YD-B, LL, SM, JF, FY, WY, and DS contributed to data collection, data

analysis, or interpretation. JG and DS reviewed the study design and data analysis, and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fneur.2019.00699/full#supplementary-material>

Supplement Figure 1 | Higher magnification of silver staining in the corpus callosum. Silver staining indicates elevated levels of axonal degeneration in the corpus callosum region in rats received projectile concussive impact (PCI) than the sham controls at 6 months post-injury.

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COX-2 Inhibition by Diclofenac Is Associated With Decreased Apoptosis and Lesion Area After Experimental Focal Penetrating Traumatic Brain Injury in Rats

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Traumatic brain injury (TBI) is followed by a secondary inflammation in the brain. The inflammatory response includes prostanoïd synthesis by the inducible enzyme cyclooxygenase-2 (COX-2). Inhibition of COX-2 is associated with improved functional outcome in experimental TBI models, although central nervous system-specific effects are not fully understood. Animal studies report better outcomes in females than males. The exact mechanisms for this gender dichotomy remain unknown. In an initial study we reported increased COX-2 expression in male rats, compared to female, following experimental TBI. It is possible that COX-2 induction is directly associated with increased cell death after TBI. Therefore, we designed a sequential study to investigate the blocking of COX-2 specifically, using the established COX-2 inhibitor diclofenac. Male Sprague-Dawley rats weighing between 250 and 350 g were exposed to focal penetrating TBI and randomly selected for diclofenac treatment (5 µg intrasessionally, immediately following TBI) ($n = 8$), controls ($n = 8$), sham operation ($n = 8$), and normal (no manipulation) ($n = 4$). After 24 h, brains were removed, fresh frozen, cut into 14 µm coronal sections and subjected to COX-2 immunofluorescence, Fluoro Jade, TUNEL, and lesion area analyses. Diclofenac treatment decreased TUNEL staining indicative of apoptosis with a mean change of 54% ($p < 0.05$) and lesion area with a mean change of 55% ($p < 0.005$). Neuronal degeneration measured by Fluoro Jade and COX-2 protein expression levels were not affected. In conclusion, COX-2 inhibition by diclofenac was associated with decreased apoptosis and lesion area after focal penetrating TBI and may be of interest for further studies of clinical applications.

Keywords: cyclooxygenase-2, diclofenac, focal penetrating TBI, NSAID, traumatic brain injury

INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of mortality and morbidity worldwide (1). The primary trauma is followed by a secondary inflammatory process that may extend for weeks, involving a multitude of inflammatory mediators (2, 3). Cyclooxygenases (COX) are enzymes that form pro-inflammatory prostanooids from arachidonic acid (4). COX-1 is the constitute isoform while COX-2 is induced by growth factors, cytokines, and inflammatory mediators (5). TBI leads to immediate induction of COX-2 in rat brains, persisting for more than 12 days. COX-2 activity reduces prostaglandin, prostacyclin, and thromboxane products in the brain (6, 7). While COX-2 inhibitors improve cognitive functions and motor performance, the exact function of COX-2 in posttraumatic neuroinflammation is unclear (8–10). Human studies report worse outcomes in women than men after TBI, whereas animal studies report better outcomes in females than males (11). The mechanisms for a possible gender dichotomy remain unknown. In an initial study we reported increased COX-2 expression in male rats compared to female after experimental TBI. The increased COX-2 expression correlated with increased apoptotic cell death detected by TUNEL staining at 24 h, while neuronal necrosis was not observed (12). Due to the possibility of COX-2 induction being directly associated with increased apoptosis after TBI a sequential study to investigate blocking of COX-2 specifically was designed, using the established COX-2 inhibitor diclofenac, in an identical model of focal penetrating TBI (13). The penetrating trauma also allowed for direct administration of diclofenac within the perilesional area. Markers of neuronal degeneration and apoptosis at 24 h after injury, corresponding to the peak of post traumatic inflammation in the brain, were analyzed (14, 15).

MATERIALS AND METHODS

The study was conducted in accordance with the Swedish regional Ethics Approval Board for Animal Research (N81/13). A total of 28 male Sprague-Dawley rats (Harlan UK LTD) weighing between 250 and 350 g were divided into the following four groups: experimental trauma + diclofenac treatment (treatment; $n = 8$), experimental trauma and NaCl (controls; $n = 8$), anesthesia and surgery without trauma (sham; $n = 8$), and naïve animals without any manipulation (normal; $n = 4$). The rats were exposed to penetrating focal brain injury as described by Plantman et al. (13) and sacrificed after 24 h. Briefly, the rats were anesthetized by 4% isoflurane and buprenorphine 50 $\mu\text{g}/\text{kg}$. In each rat, a midline incision was made through the skin and periosteum, and a burr hole 2.7 mm in diameter was drilled with its center 3 mm lateral and 3 mm posterior to bregma. Each rat in the experimental trauma and sham groups was placed in a stereotactic frame and positioned with the probe directly above the dura mater. A lead pellet was accelerated by air pressure, hitting a metal cylinder probe with an attached carbon fiber pin, with a tip radius of 1 mm. Depth of penetration into the brain by the pin was limited to 5 mm. Sham operated animals were subjected to identical treatment except for the penetrating

injury. Diclofenac (2-(2,6-dichloranilino) phenylacetic acid), 5 μg in 5 μl 0.9% NaCl, was administered immediately following the trauma in the injury cavity in treatment group, and 5 μl 0.9% NaCl was administered in the injury cavity in the “control” group. In the “treatment” and “control” groups, a 23-gauge needle with a 1 mL plastic syringe (Becton, Dickinson and Company) was inserted 4 mm into the cavity and the injection was performed manually with gentle pressure while the needle was carefully withdrawn from the cavity. The total volume was injected without signs of leakage outside the burr hole in any rat. The timing of the injection, equipment used, injection speed, position of the tip of the syringe and total volumes were identical in all animals receiving injections. The dosage was based on previous reports of rodent brain administration of diclofenac (16, 17). The skin lesion was sutured after the drug administration.

The rats were sacrificed by an overdose of pentobarbital, brains were fresh frozen and cut into 14- μm coronal sections using a Microm HM560 cryostat. Each section was cut starting from the level of the trauma, at the level of corpus callosum being visible, approximately at bregma -2.3 mm, and mounted onto Thermo Scientific Superfrost plus slides and stored at -70°C . The region of interest (ROI) was defined medially by the interhemispheric fissure and the midline, basally by the perilesional area and laterally by the lateral border of the right hemisphere (Figures 1A,B). The central necrotic part of the contusion was omitted from the ROI. The slides were blinded to the assessor in all quantifications.

For evaluation of lesion volume, slides from each rat in treatment and control groups were obtained from bregma $+0.48$, bregma $+0.7$, bregma $+1.0$, bregma $+1.2$, bregma $+1.6$, and bregma $+1.7$ for comparison. The lesion areas were calculated at each level in every rat; the assessor was blinded to the treatment group. The lesion areas at each level were compared between the groups and an aggregated mean difference was obtained as a mean difference of the differences at the six different levels. For COX-2 immunofluorescent detection, the slides were rehydrated in phosphate buffer saline (PBS), fixated for 60 min with 4% formalin, and blocked in the solution of 1% bovine serum albumin (BSA), 0.3% Triton X-100 and NaN_3 (NaAz). The sections were then incubated with the primary antibody (COX-2, dilution 1:100, ab15191 lot: GR146689-1, ABCAM) overnight at 4°C . Next, the slides were incubated with the secondary antibody (Alexa Fluor 488, dilution 1:500, 711-545-152 Jackson ImmunoResearch Laboratories, INC). The nuclear marker used was 4',6-diamidino-2-phenylindole (DAPI). The TACS 2 TdT- Blue Label *in situ* Apoptosis Detection Kit, by Terminal Deoxynucleotidyl transeferase dUTP nick end labeling (TUNEL), was used for the detection of apoptosis in the tissue sections. The slides were put on a heated plate for 30 min and then fixated in 3.7% formaldehyde. The slides were then permeabilized in ethanol for 5 min in -20°C and put in a quenching solution (94 ml methanol and 6 ml 50% hydrogen peroxide) for 5 min before being treated with TdT labeling, incubated for 60 min at 37°C and stopped with a 1XTdT stop buffer. Before detection, the slides were incubated with Streptavidin-HRP (1:500) for 20 min and followed by counter staining with Nuclear Fast Red for 2 min. The slides were then

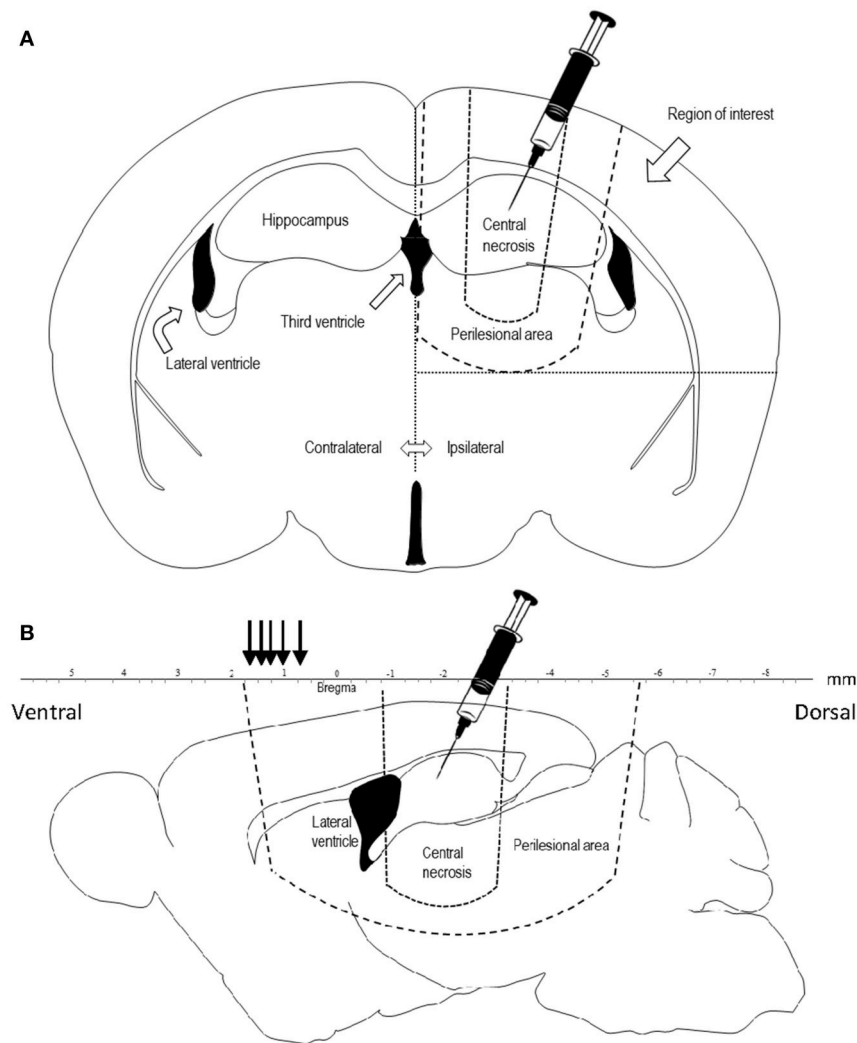


FIGURE 1 | (A) Coronal projection of a rat brain, showing the area of penetrating injury and the region of interest for quantification of COX-2, TUNEL, and Fluoro Jade. Diclofenac was injected in the trauma cavity directly after injury. **(B)** Sagittal projection 1.44 mm laterally of the midline of a rat brain showing the central necrosis and perilesional area. Lesion area was measured at comparable sections at bregma +0.48, +0.7, +1.0, +1.2, +1.6, +1.7 mm (black arrows) representing the frontal part of the perilesional area.

dehydrated in a series of dH₂O, 70% ethanol, 90% ethanol, 100% ethanol and xylene, and finally mounted in Entellan. To detect degenerating neurons, the slides were fixated in 4% formalin and immersed in 0.06% potassium permanganate (KMnO₄) for 10 min before being incubated for 30 min in Fluoro Jade B. The slides were dried at 50°C, immersed in xylene for 15 min and mounted with Entellan.

Sections were first examined at 1x magnification through a Nikon E600 microscope using dark field illumination. The level of each examined coronal section was identified using the 3rd edition of Paxinos and Watson's rat brain atlas (18). The lateral ventricles, anterior commissure and the shape of the corpus callosum were used as landmarks. For COX-2 protein expression detection, eight sections per animal were digitally photographed in 40x using dark field illumination in

a Nikon Eclipse E600 microscope. Eight whole brain sections were photographed per animal. In the ipsilateral side one picture was taken from the perilesional area and one from the ventrolateral cortex, with corresponding areas being photographed in the contralateral side. Thus, 32 pictures (four pictures per brain section and eight sections per animal) were analyzed for each animal. The integrated intensity of the COX-2 protein expression was analyzed in ImageJ. The following macro-code was used as the batch process for automated counting of the integrated intensity with a set threshold: run("8-bit");run("Invert");setAutoThreshold("Yen");//run("Threshold..."); run("Measure");close();saveAs("Results" ...). Quantification of Fluoro Jade and TUNEL positive cells was done manually in 40x magnification. Fluoro Jade positive cells were counted in the ipsilateral and contralateral side of eight sections

per animal. TUNEL positive cells were counted similarly in four sections per animal. The lesion area was analyzed by Fiji/ImageJ software from 1x dark field photographs.

Statistical Analyses

Statistical analyses were carried out in GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla, USA). All error bars represent the standard error of the mean (SEM). COX-2, TUNEL and Fluoro Jade was analyzed by the non-parametric Kruskal Wallis ANOVA followed by Benjamini Hochberg *post-hoc* test. Lesion area was analyzed by two-way unpaired *t*-test. $p < 0.05$ was considered significant. Significance levels: * $p < 0.05$, *** $p < 0.005$.

RESULTS

COX-2 protein expression was increased in the ipsilateral side following trauma, in both the “controls” and “diclofenac treatment” groups compared to the “sham” and “normal” ($p < 0.05$) groups. No differences were observed between the “controls” and the “diclofenac” group. Weak COX-2 expression was seen in the contralateral side in all groups except for “normal” (Figures 2A–F). TUNEL positive cells were increased in the injured hemispheres of the “diclofenac” and “control” groups compared to the “sham” and “normal” groups. TUNEL positive cells were not detected in the contralateral hemispheres. The number of TUNEL positive cells were lower in the diclofenac treatment group compared to the “control” group with a mean change of 54% (“diclofenac” 39.8 cells/view—“control” 85.6 cells/view) ($p < 0.05$) (Figures 2G–L). Fluoro Jade positive cells were increased in both the “diclofenac” and “control” groups compared to “sham” and “normal.” Positive cells were not detected in the contralateral sides. Furthermore, no differences were detected between the different groups (Figures 2M–R). The lesion area was measured in the groups exposed to penetrating trauma. The lesion areas measured at six different levels were significantly smaller at four levels and smaller with statistical significance at four. The mean differences were 55% (“diclofenac” 9.2 mm^2 —“control” 16.9 mm^2) ($p < 0.005$) (Figures 3A–S).

DISCUSSION

This study showed that specific COX-2 inhibition by diclofenac was associated with decreased apoptosis in the perilesional area after focal penetrating TBI. The focal penetrating TBI model is highly reproducible and has a known gender specific COX-2 response (12). In this specific context, the model mimics an open brain contusion, which is a particular form of head trauma (19). This kind of injury allowed direct intralesional administration of diclofenac. The mode of administration is unusual but provides direct access to the intended target area and reduces the risk of a heterogeneous uptake in the brain from intravenously or intraperitoneally injected substances. Intralesional administration in the brain is not standard clinical practice in head injuries, which may comprise a limitation of the clinical applicability. Direct administration into surgical sites is, however, practiced with gliadel wafers for brain tumor

therapy and antibiotics for management of ventriculitis; hence intra-axial administration for clinical focal brain injuries should be considered as a novel clinical option. There are potential risks associated with intralesional administration of a vasoactive substance, and coagulation derangements including a possible higher risk of hemorrhage. Safety needs to be addressed in clinical studies since our study only intended to analyse the impact of COX-2 inhibition on cell death; the large effect size that may foster hopes of neuroprotection was not expected. We targeted the immediate inflammation known to occur 24 h after TBI (14) and found important differences probably caused by diclofenac treatment. Yet, the present study cannot determine the precise effects of diclofenac; a complete time series is necessary to evaluate final cell survival and comprehensive kinetics.

COX-2 was upregulated in the perilesional area after TBI, similarly to our experience (12) and previous reports (8, 10, 20–22). Most expression was found in the ipsilateral hemisphere, with little expression also in the contralateral side. Diclofenac did not decrease protein expression of COX-2. Earlier studies report conflicting results of COX inhibition. The COX-2 inhibitor rofecoxib did not alter the COX-2 expression (21), while the COX-2 inhibitor DFU decreased COX-2 when the drug was administered 10 min before trauma, but not when administered 2–6 h post trauma (10). It is possible that systemic inflammation in the body is sufficient for general COX-2 induction, and that the acute blood brain barrier disruption by the focal injury allowed for COX-2 to enter the brain.

TUNEL staining, indicative of apoptosis was increased in the perilesional area in all trauma groups compared to the “sham” group. Diclofenac decreased TUNEL staining compared to “control.” The number of neurons positive for Fluoro Jade were increased after TBI, although they were not affected by diclofenac. Fluoro Jade labels degeneration, both necrosis and apoptosis, while TUNEL staining is specific in labeling apoptosis (23). Fluoro Jade is specific in labeling neurons, while TUNEL labels all cell types undergoing apoptosis. It is therefore possible that the inflammatory amelioration by diclofenac occurs primarily in cells other than neurons. It is also possible that high levels of neuronal necrosis resulting from the penetrating injury conceals possible anti-apoptotic effects in the neurons specifically. DFU decreased activated caspase-3 levels, further suggesting specific antiapoptotic effects (10), while no effects on TUNEL or Fluoro Jade staining were detected after rofecoxib treatment following rodent fluid percussion TBI (21). Potentially, COX-2 inhibition is more effective in specific TBI subgroups, which should be explored in future studies. The amount of TUNEL positive cells in the “control” group (male rats) corresponded to the amount found in male rat brains when comparing male and female responses in an identical setting in the preceding study (12). TUNEL positive cells in the “diclofenac” group also corresponded to the amount in untreated female rats exposed to identical trauma. It is possible that lower levels of COX-2 in female brains is protective, and that male subgroups should be targeted in future studies of neuroprotective properties of diclofenac. TUNEL positive cells in the “control” (NaCl vehicle) group corresponded to the amount in male rats

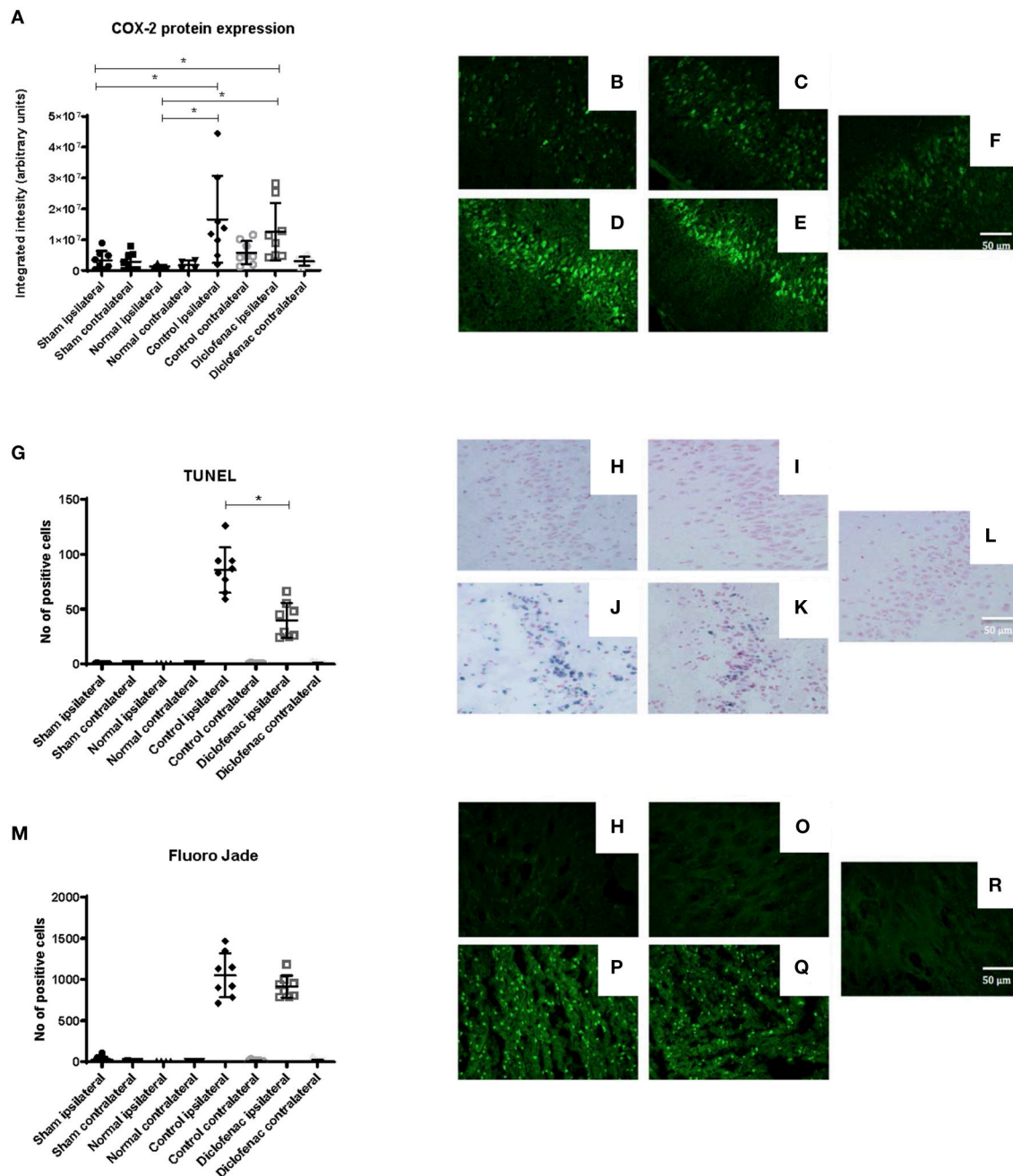


FIGURE 2 | (A) COX-2 protein expression was upregulated 24 h following injury. Diclofenac did not affect protein expression. Photomicrographs of COX-2 fluorescence **(B)** "normal" ipsilateral **(C)** "sham" ipsilateral **(D)** "control" trauma ipsilateral **(E)** "diclofenac" treated trauma ipsilateral. **(F)** Contralateral side **(G)** TUNEL staining showing lower apoptosis levels by diclofenac treatment. Photomicrographs of TUNEL **(H)** "normal" ipsilateral **(I)** "sham" ipsilateral **(J)** "control" trauma ipsilateral **(K)** "diclofenac" treated trauma ipsilateral. **(L)** Contralateral side **(M)** diclofenac treatment did not affect Fluoro Jade detected neuronal degeneration. Photomicrographs of Fluoro Jade **(N)** "normal" ipsilateral **(O)** "sham" ipsilateral **(P)** "control" trauma ipsilateral **(Q)** "diclofenac" treated trauma ipsilateral. **(R)** Contralateral side. Data expressed as mean $\pm$ SEM. * $p < 0.05$.

receiving no injection in the preceding study (12), why possible cell death resulting from the injection only could be excluded.

Diclofenac appeared to decrease the macroscopic lesion area after 24 h. We could only quantify and compare the lesion areas in a limited number of brain sections which were available

after the immunohistochemical analyses; the extensive effect on lesion size was unexpected and serendipitous. We did not have material to make a three-dimensional analysis of the lesion volume, which comprised a limitation. The size effect was, however, extensive from the analyses we made and the

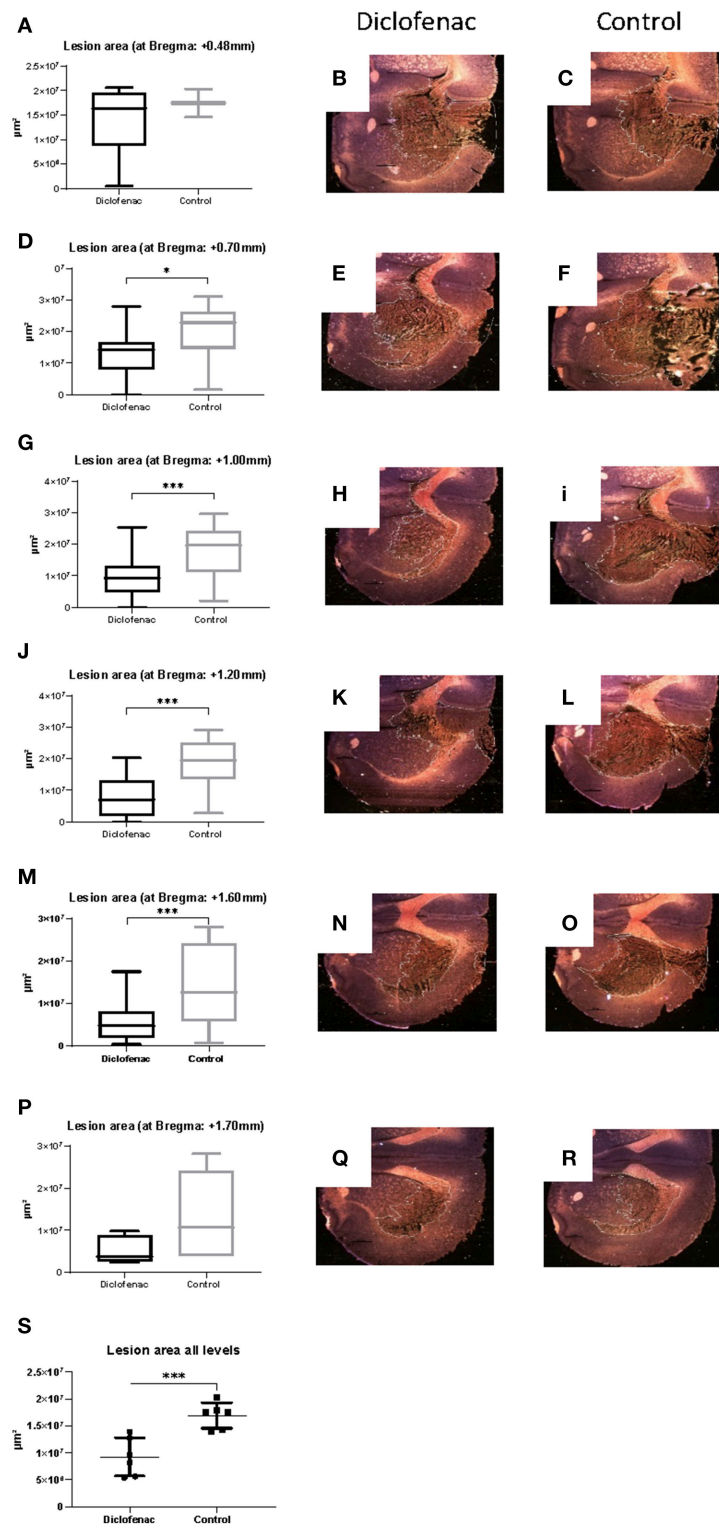


FIGURE 3 | (A–S) Diclofenac treatment decreased lesion area in the frontal perilesional area. Lesion area was measured at comparable sections at bregma +0.48, +0.7, +1.0, +1.2, +1.6, +1.7 mm (black arrows) representing the frontal part of the perilesional area. Data expressed as mean $\pm$ SEM. * p < 0.05, *** p < 0.005.

intralesional treatment needs to be evaluated with prospective quantification of lesion volumes. Similar results of COX-2 inhibition reducing lesion size together with increased glia cell proliferation in the perilesional area have been reported (9). It is therefore possible that the anti-apoptotic and tissue sparing effects were not a direct effect by COX-2 inhibition but mediated by other cells. Microglial activation occurs within 24 h after experimental TBI (24). Sites of activation often coincide with neuronal degeneration and axonal abnormality, hence anti-inflammatory treatments targeting microglia are suggested as potential therapeutic strategies (25). It is also feasible that astrocytes mediate apoptosis. Astrocytes maintain the homeostasis of ions, transmitters, water, and blood flow, critical for neuronal function. In response to TBI, astrocytes become active in response to axonal injury, vascular disruption, ischemia and inflammation (26), and are emerging as both potent pro-inflammatory and anti-inflammatory cells (27). Astrocytes are associated with T-cell apoptosis in autoimmune inflammation (28). Future studies should therefore aim at investigating specific apoptotic pathways and the effect of diclofenac on neurons as well as glia cells. These studies may also target protein markers of axonal functionality, such as phosphorylated neurofilaments to determine if diclofenac in addition to reducing cell death favors the restoration of axonal functionality decreased by injury.

The findings suggest that the gender-related difference in apoptotic cells after TBI (12) is associated with COX-2 regulation.

Most anti-inflammatory drugs have failed to produce lasting effects (29). It is possible that COX-2 inhibition is beneficial in focal TBI, making diclofenac a potential candidate for further clinical applications. In addition, local administration of the drug may provide a novel treatment that can be developed clinically.

CONCLUSION

COX-2 inhibition by diclofenac is associated with decreased apoptosis after focal penetrating TBI and may be beneficial in preventing brain tissue damage.

ETHICS STATEMENT

Swedish regional ethics approval board for animal research (N81/13).

AUTHOR CONTRIBUTIONS

MG, MR, JD, and TM planned the study. MG, JD, and KD performed the experiments. KD, EL, MA, and AH performed preparation and analysis. All authors contributed to the text.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Gut Microbiota as a Therapeutic Target to Ameliorate the Biochemical, Neuroanatomical, and Behavioral Effects of Traumatic Brain Injuries

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Current efficacious treatments for traumatic brain injury (TBI) are lacking. Establishment of a protective gut microbiota population offers a compelling therapeutic avenue, as brain injury induces disruptions in the composition of the gut microbiota, i.e., gut dysbiosis, which has been shown to contribute to TBI-related neuropathology and impaired behavioral outcomes. The gut microbiome is involved in the modulation of a multitude of cellular and molecular processes fundamental to the progression of TBI-induced pathologies including neuroinflammation, blood brain barrier permeability, immune system response, microglial activation, and mitochondrial dysfunction, as well as intestinal motility and permeability. Additionally, gut dysbiosis further aggravates behavioral impairments in animal models of TBI and spinal cord injury, as well as negatively affects health outcomes in murine stroke models. Recent studies indicate that microbiota transplants and probiotics ameliorate neuroanatomical damage and functional impairments in animal models of stroke and spinal cord injury. In addition, probiotics have been shown to reduce the rate of infection and time spent in intensive care of hospitalized patients suffering from brain trauma. Perturbations in the composition of the gut microbiota and its metabolite profile may also serve as potential diagnostic and theragnostic biomarkers for injury severity and progression. This review aims to address the etiological role of the gut microbiome in the biochemical, neuroanatomical, and behavioral/cognitive consequences of TBI, as well as explore the potential of gut microbiome manipulation in the form of probiotics as an effective therapeutic to ameliorate TBI-induced pathology and symptoms.

Keywords: traumatic brain injury, therapy, gut microbiome, microbiota-gut-brain axis, gut dysbiosis

BRIEF OVERVIEW OF TRAUMATIC BRAIN INJURY

Traumatic brain injury (TBI) is a major cause of death and disability in the United States and represents one of the most prevalent injury types sustained by the worldwide population (1). Reports spanning the last two decades underscore the human and financial burden of TBI in the United States, with an annual incidence of ~1.4 million cases (2), prevalence of ~3.17 million with a long-term TBI-induced disability (3), and an annual economic burden of billions of dollars (4). Importantly, these disabilities are a result of not only the mechanical damage

sustained due to the initial injury (primary), but also the subsequent cellular and molecular damage that exacerbates in the following hours, days, weeks, and years post-injury (secondary) (5, 6). The etiology of secondary injury is multifaceted and may constitute altered cerebral blood flow, excitotoxicity, inflammation, microglial activation, metabolic anomalies, mitochondrial dysfunction, and oxidative stress resulting in transient or lifelong behavioral and cognitive deficits (5–9). TBI severity is categorized based on the Glasgow Coma Scale (GCS), in which patients are scored on the basis of clinical symptoms, and the resulting overall score classifies their injury as mild (score: 13–15), moderate (score: 9–12), or severe (score: <9) (10, 11). Overall, TBI complexity occurs on a spectrum ranging from mild to severe, diffuse to focal, and single to repeated exposures in brain vs. multi-organs, which leads to injury-specific heterogeneous pathobiological responses that cannot be regarded as a single condition (12).

Despite decades of rigorous preclinical research in which much insight into the heterogeneous nature of brain injury has been gained, efficacious therapeutics for TBI-induced neuropathologies and behavioral/cognitive impairments are lacking (13–15). Given the prevalence of TBI-related disabilities, it is imperative to consider novel treatment strategies. Restoration of the gut microbiome by gut eubiotic therapeutics is one such compelling avenue, which is capable of modulating the bi-directional relationship between TBI-induced disruptions of the gut microbiome and the influence of this gut dysbiosis on the pathophysiology of TBI-induced secondary injury progression (16, 17).

MICROBIOTA-GUT-BRAIN AXIS (MGBA)

Gut microbiota refer to the bacteria, archaea, viruses, and eukaryotic microbes that reside primarily within the colon, but also within the stomach and small intestine (18). This commensal bacterial community accounts for 0.2–1 kg of an adult's bodyweight (18, 19), outnumbering mammalian cells by as much as 10:1, though more recent estimates indicate a ratio of ~1:1 (18), and contains ~100 fold more unique genes than the human genome (20). Bacteroidetes and Firmicutes phyla compose the majority of the gut microbiota, with Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia being present in fewer numbers. However, gut microbiota composition differs among individuals as diet, age, gender, environment, and genetics all influence bacterial strains/populations (21–23). The activity and composition of this microbial population is involved in a surprising number of biological processes, including homeostasis of the central nervous system (CNS) (24–26). This relationship is referred to as the microbiota-gut-brain axis (MGBA) (27), with communication between the gut microbiota and the CNS occurring through a neuro-endocrino-immunological network (28).

Perhaps the most direct route of communication within the MGBA is among the gut microbiota, enteric nervous system (ENS), and vagus nerve. Neuroactive compounds produced

by gut bacteria influence the activity of sensory neurons of the ENS, which in turn modulates the afferent activity of the vagus nerve (29). These compounds consist of bacterial metabolites, neurotransmitters, neurotrophic factors, cytokines, and endotoxins (30–32). Nervous system signaling originating from the gastrointestinal tract is then integrated by the nucleus of the solitary tract (33) and relayed to other brain nuclei (34). Gut microbiota also play a fundamental role in the development and functioning of the host immune system (35). Homeostasis of host immune system function is predicated upon proper gastrointestinal neuromuscular control, maintenance of intestinal wall integrity, and intact ENS/vagus nerve signaling (36, 37), aspects of gastrointestinal health that are, in part, regulated by the gut microbiome. Perturbations in the composition of the gut microbiota are known to lead to a weakening of the intestinal-host barrier (38), allowing gastrointestinal content to be released into the blood stream and other parts of the body, a condition referred to as “leaky gut” (39), which can lead to neuroinflammation. For example, peripheral administration of the bacterial endotoxin lipopolysaccharide induces cytokine expression within the hypothalamus-pituitary-axis, resulting in regional neurotoxicity and systemic inflammation (40, 41). Notably, the cross-talk among the gut bacteria, ENS, and vagus nerve cohesively regulates the host immune and inflammatory responses to modulate CNS function (42, 43). Finally, cognitive and behavioral changes (e.g., stress) have repeatedly been shown to alter the composition of the gut microbiota, demonstrating both feed-forward and feedback mechanisms within the MGBA (44).

Gut microbiome composition has been linked to a variety of illness and disease states (45, 46), with research dating back over seven decades establishing a relationship between the metabolic products of gut bacteria and hepatic encephalopathy (47, 48). More recent research has linked the gut microbiota to inflammatory diseases (49) and several CNS-related disorders, including autism (50, 51), depression (28, 52), and anxiety (53, 54), as well as Alzheimer's disease (55) and Parkinson's disorder (55, 56). However, it is difficult to prove causation and directionality when discussing gut microbiome changes observed in human neuropsychiatric and neurodegenerative conditions (57). For these reasons, rodents are commonly used when investigating the MGBA as they (1) possess similar, but not identical, core intestinal bacterial populations to humans (58, 59) and (2) can be maintained “germ free” (devoid of gut microbiota) or gnotobiotic (gut microbiota of known composition).

Eubiotic therapeutics that alter the gut microbiome through diet, microbiota transplants, antibiotics, and pre-/probiotics influence both systemic and CNS-related processes. Microbiota transplants have been shown to influence obesity levels in rodents (60) and humans (61), as well as effectively treat recurrent *Clostridium difficile* infection (62). Meanwhile, probiotics have shown promise in the treatment of patients with ulcerative colitis (63) and antibiotics are now commonly used to eliminate the bacterial populations involved in hepatic encephalopathy (64). Probiotics have also been shown to reduce anxiety- and depressive-like symptoms in animals, with limited evidence indicating similar results in humans (53). Furthermore, gut

microbiome alterations have been shown to ameliorate autism-like behaviors in mice (65), with probiotics having been suggested as a therapeutic strategy for individuals with post-traumatic stress disorder (66). Among other research findings [as reviewed by (67)], this has led some researchers to suggest “psychobiotics” as a new therapeutic approach for neurological and neuropsychiatric illnesses (68, 69).

ROLE OF THE MGBA IN CNS INJURIES

Pertinent for TBI research is the bi-directional relationship that exists between brain injury and the gut microbiome (**Figure 1**). Research in brain and spinal cord injury (SCI) animal models has demonstrated that CNS injury disrupts the motility and permeability of the intestinal wall (70, 71) and perturbs the composition of the gut microbiome (17, 72), leading to a host-maladaptive state referred to as gut dysbiosis (73). Conversely, gut dysbiosis influences the pathophysiology of traumatic CNS injury (74, 75). For example, following SCI, significant changes in the composition of the gut microbiota were observed, namely a decrease in Bacteroidetes and increase in Firmicutes, with post-injury changes in the gut microbiome persisting out to 1 month and predicting the degree of locomotor impairment (76). A similar relationship was observed in a controlled cortical impact (CCI) rodent model of moderate TBI, with bacterial changes occurring as early as 2 h following injury, persisting out to 7 days post-injury, and correlating with lesion volume. However, the opposite alteration in gut microbiota was observed with a decrease in Firmicutes and increase in bacterial families within the Bacteroidetes and Proteobacteria phyla (77). Furthermore, a recent study by Treangen et al. (78) reported gut dysbiosis with significant decreases in *Lactobacillus gasseri*, *Ruminococcus flavefaciens*, and *Eubacterium ventriosum* and significant increases in *Eubacterium sulci* and *Marvinbryantia formatexigens* at 24 h post-CCI in mice. *L. gasseri* displayed the most drastic change with a 4-fold log decrease in abundance as compared to baseline, though it should be noted that a less pronounced decrease was also observed following sham procedures. As *L. gasseri* is a member of the phylum Firmicutes, this work complements the findings of Nicholson et al., and provides for a potential eubiotic target as *L. gasseri* inhabits the human gut microbiome (79). Investigations into TBI-induced gut dysbiosis in humans is limited, though a recent study in severely injured patients with polytrauma reported a decrease in Bacteroidales, Fusobacteriales, and Verrucomicrobiales, as well as an increase in Clostridiales and Enterococcus within 72 h of injury (80).

Gut dysbiosis also affects the integrity and permeability of the blood brain barrier (BBB) (81). Coupled with TBI-induced physical disruptions to the BBB (82), intestinal contents and the associated upregulation of the pro-inflammatory immune response more easily permeate the CNS, resulting in increased microglial activity, neuroinflammation, and neuropathology (83, 84). Microglial maturation and function within the CNS have been shown to be influenced by the gut microbiome in BBB-intact animals (85, 86), a relationship expected to be enhanced

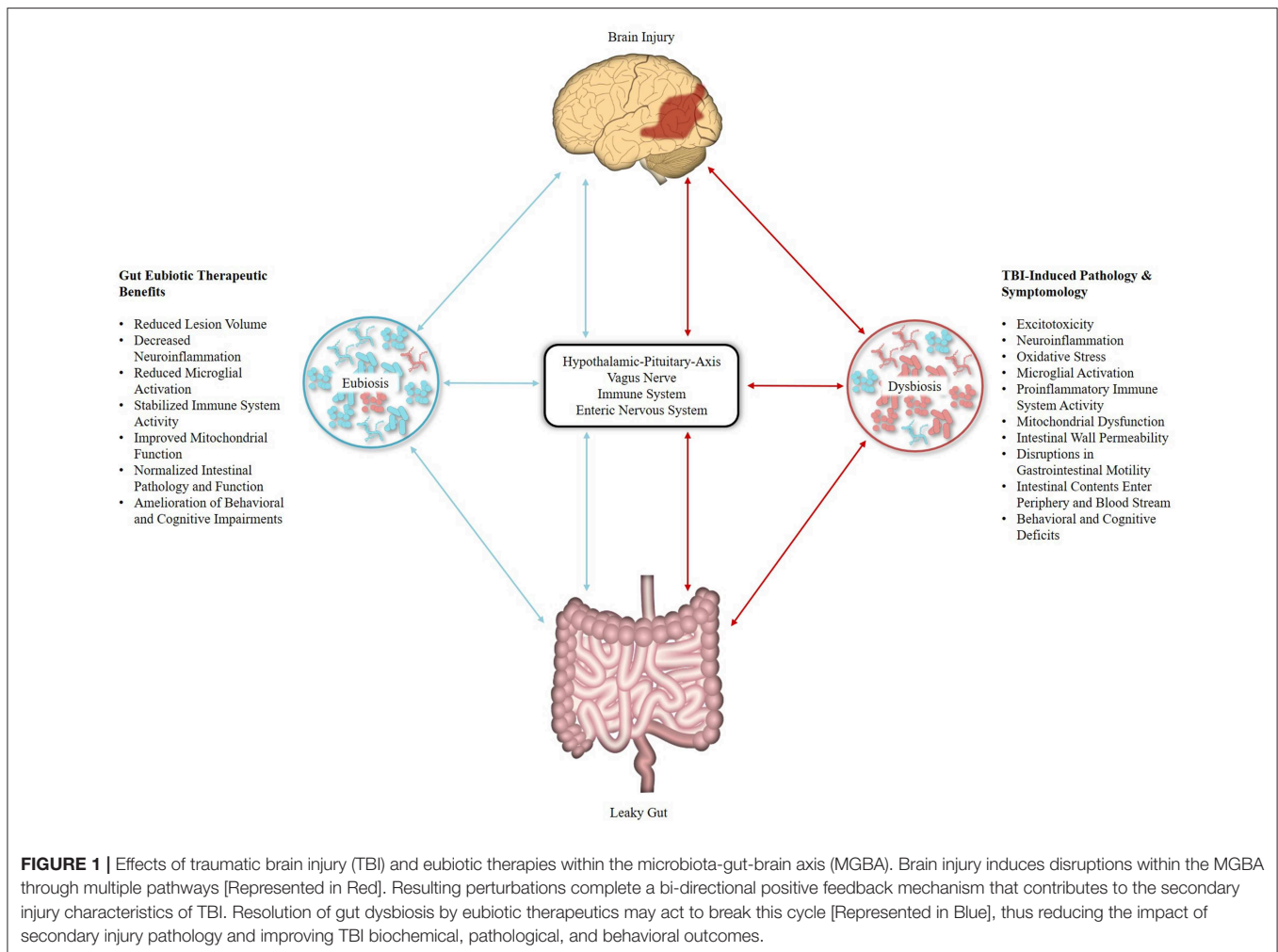
by increased BBB permeability. Therefore, it is likely that TBI-induced gut dysbiosis is a contributing factor in increased microglial activation following CNS injury (86). Post-injury mitochondrial dysfunction in terms of energy production (i.e., ATP synthesis) observed in TBI (87, 88) may also be impacted by gut dysbiosis, as studies have revealed a link between gut bacterial metabolites and mitochondrial function (26, 89).

Importantly, experimenter-induced alterations in the composition of the gut microbiota community regulate immune system activity, neuropathology, and behavior following CNS injury. In a gnotobiotic mouse model of ischemic stroke, an expansion of Proteobacteria accompanied by a contraction in Firmicutes and Bacteroidetes altered immune system homeostasis by increasing peripheral neuroprotective anti-inflammatory T_{reg} cells and decreasing pro-inflammatory $\gamma\delta$ T cells, resulting in a reduction in ischemic brain injury (90). However, the large-scale depletion of cultivatable gut microbiota by a broad-spectrum antibiotic in a mouse model of focal cerebral ischemia prior to injury resulted in decreased rates of survival and an increase in the development of severe acute colitis (74). Furthermore, if gut dysbiosis was experimentally induced by a broad-spectrum antibiotic prior to SCI, both neurological impairment and spinal cord pathology were exacerbated, likely due to changes in immune system activity (76). These studies demonstrate the complex relationships within the MGBA, revealing that the bacterial populations present at the time of injury influence the degree of neuropathology and functional impairment following TBI. Such knowledge establishes the basis for both the monitoring and manipulation of the gut microbiota as a means to diagnose and ameliorate the pathophysiology and symptomatology of brain injuries.

GUT MICROBIOTA AS A POTENTIAL DIAGNOSTIC AND THERAPEUTIC TARGET FOR TBI

Monitoring the extent of gut dysbiosis may provide a diagnostic tool for the identification of TBI severity, providing information for treatment guidance. Fecal metabolomes have already been used as biomarkers for several ailments including Crohn's disease and colorectal cancer (91, 92), and a recent study by Houlden et al. (72) demonstrated a positive correlation between the degree of gut dysbiosis and the severity of a closed-head-impact rodent model. Importantly, the profile of gut microbiota changes observed following TBI differed from those following ischemic brain injury by 72 h post-injury, indicating that different forms of brain injury uniquely impact the gut microbiome (72).

Beyond monitoring, manipulation of the gut microbiome via eubiotic therapies (e.g., microbiota transplants and pre/probiotics) presents an exciting treatment target for TBI (**Figure 1**). Several of the ailments associated with TBI-induced pathology that affect the microbiota are improved by the intake of probiotics, such as intestinal motility and permeability, health of the intestinal cellular lining, intestinal inflammation, and systemic immune response (93–95).



Furthermore, perturbations in bacterial composition initially appear 24–72 h following trauma (72, 80); a time period corresponding to the pathophysiology of TBI-induced secondary injury, representing an ideal treatment window. As substantial alterations in the gut microbiome can occur 24–48 h following dramatic changes in diet (96, 97), eubiotic therapies could fundamentally shift the gut microbiome to a beneficial state in time to mitigate aspects of TBI-associated secondary injury. Preclinical studies support this concept as microbiota transplants have been shown to reduce brain lesion size and improve health outcomes in mouse models of ischemic stroke (98) and restore microglial function (85). Probiotic derived bacterial metabolites may also serve to modulate mitochondrial homeostasis (99) as gut microbiota generate short-chain fatty acid products such as butyrate, propionate, and acetate (100). Together with dietary ketones, these gut microbiome products serve as alternative energy sources for the injured brain and may improve bioenergetics function following TBI and SCI (101, 102). Additionally, gut microbiota-generated butyrate serves as a histone deacetylation (HDAC) inhibitor, offering additional benefits as HDACs play an important

role in neuroprotection following CNS injuries (103) and enhance cognitive function in neuropsychiatric disorders (104). Furthermore, the butyric acid-producing probiotic *Clostridium butyricum* improved neurological deficits, reduced brain edema, attenuated neurodegeneration, and ameliorated BBB impairment (105), as well as improved spatial memory in mouse models of weight-drop impact head injury and cerebral ischemia, respectively (83). Probiotic supplements rich in lactobacilli and bifidobacteria have also been shown to improve spatial memory in a cognitively impaired mouse model (106) and one explanation for these observed improvements is evidenced by VSL#3 (a commercial, medical-grade probiotic rich in lactic acid bacteria) rescuing hippocampal neurogenesis via Ly6C^{hi} monocytes in mice with antibiotic-induced gut dysbiosis (107). Treatment with VSL#3 also decreases circulating levels of TNF α , lessens cerebral monocyte infiltration, and reduces microglial activation (108). In mice that received SCI, VSL#3 provided the day of injury and extending for 35 days post-injury reduced neuropathology, improved locomotor recovery, and triggered a protective immune response through an increase in the number of T_{reg} cells (109).

Importantly, human preclinical trials in brain injury patients with GCSs of 5–12 (i.e., moderate to severe TBI) indicate that manipulation of the gut microbiome through lactobacilli-rich probiotic supplementation within the first 48 h of admission with continued treatment for between 5 and 21 days can reduce nosocomial infection rate (110), decrease gastrointestinal dysfunction (111), lessen the incidence of ventilator-associated pneumonia (111), and shorten the time spent in intensive care (112). These observed benefits are commonly attributed to probiotic-induced reductions in systemic and central inflammation (113, 114). No studies exist examining the behavioral/cognitive outcomes of probiotic supplementation on TBI patients; however, probiotics have been shown to improve behavior and cognition in individuals with Alzheimer's disease (115) and depression (116), as well as healthy individuals (117). Probiotic supplementation for patients with penetrating TBI may be additionally useful as the long-term use of antibiotics is recommended for the reduction of infection, morbidity, and mortality rates (118, 119). As discussed, antibiotic-induced disruptions of the gut microbiome can lead to worsened TBI-related outcomes, potentially guiding medical practices toward adjunctive probiotic treatments to mitigate or minimize complex downstream pathobiological responses following TBI.

CONCLUSION

Provided the bi-directional relationship between the gut microbiome and TBI-associated pathology, resolution of gut dysbiosis represents a compelling therapeutic target. Probiotics consisting of lactobacilli, bifidobacteria,

and other butyrate-producing gut bacteria appear most beneficial, providing a eubiotic therapy that enhances MGBA function through their anti-inflammatory and positive mitochondrial energetic properties. However, recent work revealed that antibiotic-induced microbiome perturbations and probiotic colonization display strong inter-species and inter-individual differences that may not have been apparent in previous investigations (120, 121). Additionally, differing courses/compositions of eubiotic treatments may need to be considered based on the type and severity of CNS injury, as these parameters produce dissimilar gut dysbiosis profiles (72). Therefore, resolution of gut dysbiosis as a therapeutic option requires investigations that yield information on the specific changes that occur to the gut microbiota following different types and severities of TBI, as well as optimal doses, treatment window, duration of treatment, and efficacy of experimentally-induced gut microbiome alterations across age and gender. Data that are sorely lacking (17, 93). Ultimately, this information could be used to develop a powerful diagnostic tool or eubiotic therapy to alleviate trauma brought on by brain injury.

AUTHOR CONTRIBUTIONS

MR wrote the manuscript. JP contributed to manuscript revision. DS read and approved the submitted version.

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