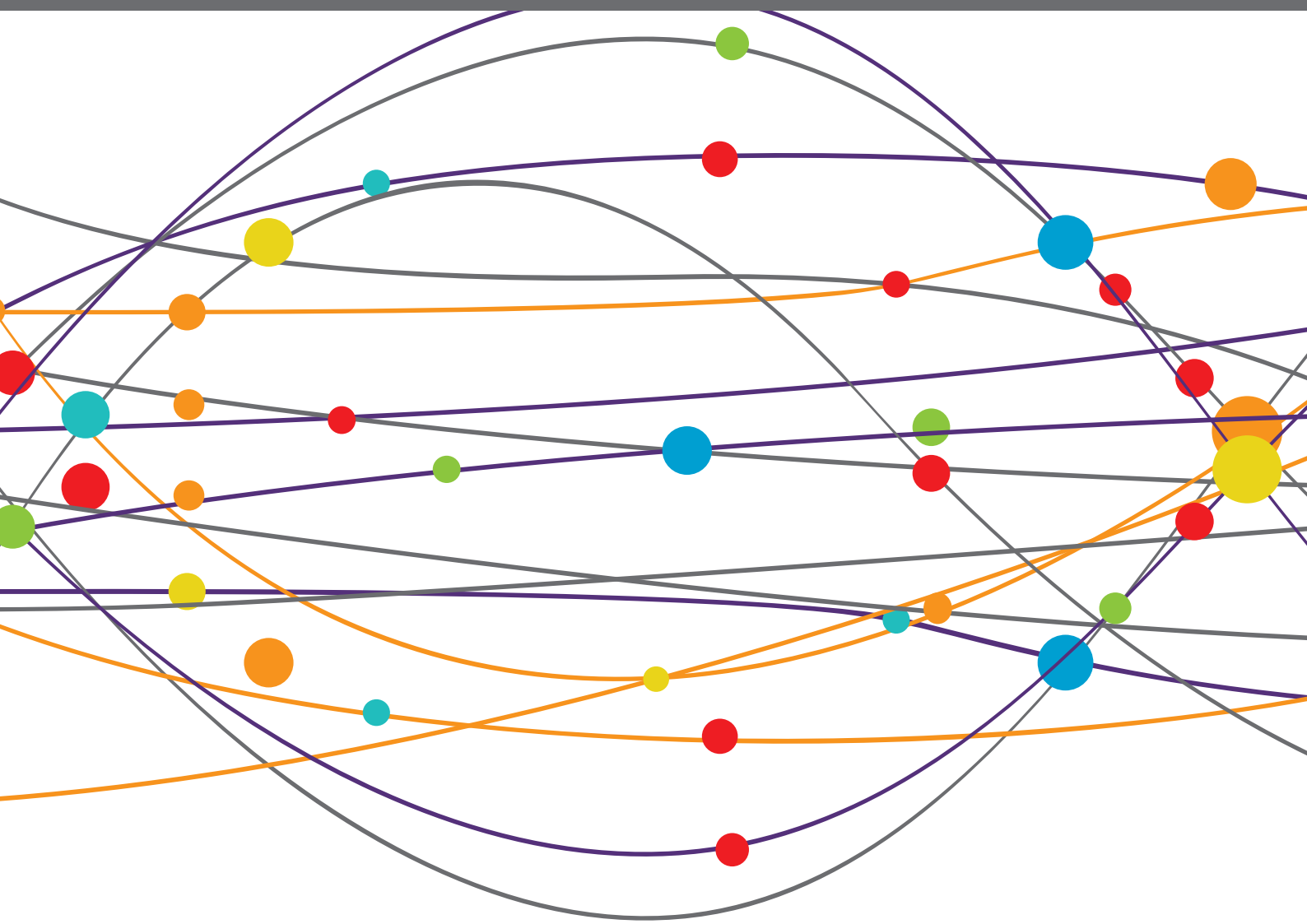
CELL-BASED THERAPIES FOR STROKE: PROMISING SOLUTION OR DEAD END?

EDITED BY: Paulo Henrique Rosado-de-Castro, Andrew N. Clarkson, Johannes Boltze, Koji Abe, Miroslaw Janowski, Pedro M. Pimentel-Coelho and Olivier Detante

PUBLISHED IN: Frontiers in Neurology



frontiers Research Topics

frontiers

Frontiers eBook Copyright Statement

The copyright in the text of individual articles in this eBook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this eBook is the property of Frontiers.

Each article within this eBook, and the eBook itself, are published under the most recent version of the Creative Commons CC-BY licence. The version current at the date of publication of this eBook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or eBook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714
ISBN 978-2-88963-738-6
DOI 10.3389/978-2-88963-738-6

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

CELL-BASED THERAPIES FOR STROKE: PROMISING SOLUTION OR DEAD END?

Topic Editors:
Paulo Henrique Rosado-de-Castro, D'Or Institute for Research and Education (IDOR), Brazil
Andrew N. Clarkson, University of Otago, New Zealand
Johannes Boltze, University of Warwick, United Kingdom
Koji Abe, Okayama University, Japan
Miroslaw Janowski, Johns Hopkins University, United States
Pedro M. Pimentel-Coelho, Federal University of Rio de Janeiro, Brazil
Olivier Detante, Centre Hospitalier Universitaire de Grenoble, France

Citation: Rosado-de-Castro, P. H., Clarkson, A. N., Boltze, J., Abe, K., Janowski, M., Pimentel-Coelho, P. M., Detante, O., eds. (2020). Cell-based Therapies for Stroke: Promising Solution or Dead End?. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88963-738-6

Table of Contents

frontiers in Neurology

EDITORIAL
published: 03 April 2020
doi: 10.3389/fneur.2020.00171

Editorial: Cell-based Therapies for Stroke: Promising Solution or Dead End?

*Johannes Boltze[1], Koji Abe[2], Andrew N. Clarkson[3], Oliver Detante[4,5], Pedro M. Pimentel-Coelho[6,7], Paulo H. Rosado-de-Castro[8,9,10] and Miroslaw Janowski[11,12]**

[1] School of Life Sciences, University of Warwick, Coventry, United Kingdom, [2] Department of Neurology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan, [3] Department of Anatomy, Brain Health Research Centre and Brain Research New Zealand, University of Otago, Dunedin, New Zealand, [4] Stroke Unit, Neurology Department, Grenoble Hospital, Grenoble, France, [5] Grenoble Institute of Neurosciences, Inserm U1216, Université Grenoble Alpes, Grenoble, France, [6] Instituto de Biofísica Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, [7] Instituto Nacional de Ciência e Tecnologia em Medicina Regenerativa, Rio de Janeiro, Brazil, [8] Institute of Biomedical Sciences, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, [9] Department of Radiology, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, [10] D'Or Institute for Research and Education, Rio de Janeiro, Brazil, [11] Department of Diagnostic Radiology and Nuclear Medicine, University of Maryland School of Medicine, Baltimore, MD, United States, [12] NeuroRepair Department, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland

Keywords: stem cells, stroke, mesenchymal stem cells (MSC), brain, treatment, translational medicine, ischemia, clinical trials

Editorial on the Research Topic

Cell-based Therapies for Stroke: Promising Solution or Dead End?

OPEN ACCESS

Edited and reviewed by:
Jean-Claude Baron, University of Cambridge, United Kingdom

****Correspondence:***
Miroslaw Janowski
miroslaw.janowski@som.umaryland.edu

Specialty section:
This article was submitted to Stroke, a section of the journal Frontiers in Neurology

Received: *08 December 2019*
Accepted: *24 February 2020*
Published: *03 April 2020*

Citation:
Boltze J, Abe K, Clarkson AN, Detante O, Pimentel-Coelho PM, Rosado-de-Castro PH and Janowski M (2020) Editorial: Cell-based Therapies for Stroke: Promising Solution or Dead End? Front. Neurol. 11:171. doi: 10.3389/fneur.2020.00171

The introduction of recanalization procedures has revolutionized acute stroke management, although factors such as the narrow time window, strict eligibility criteria, and logistical limitations still exclude the majority of patients from treatment. In addition, residual deficits are present in many patients who undergo therapy, preventing their return to premorbid status. Hence, there is a strong need for novel, and ideally complementary, approaches to stroke management.

In preclinical experiments, cell-based treatments have demonstrated beneficial effects in the subacute and chronic stages following stroke (1–3) and therefore are considered a promising option to supplement current clinical practice. At the same time, great progress has been made in developing clinically feasible delivery and monitoring protocols (4). However, efficacy results initially reported in clinical studies fell short of expectations (5) raising concerns that cell treatment might eventually share the "dead end fate" of many previous experimental stroke therapies. This Research Topic reviews some of the latest and most innovative studies to summarize the state of the art in translational cell treatments for stroke.

NEW MECHANISTIC INSIGHTS FROM PRECLINICAL EXPERIMENTS

Umbilical cord blood (UCB)-derived cells are a widely available and rich source of relatively young cells. However, it is unclear which fraction of this heterogeneous population is responsible for the therapeutic effects reported after stroke. Gornicka-Pawlak et al. investigated $CD34^{-}$ mononuclear cells (MNCs) either freshly prepared or cultured for 3 days vs. a UCB derived neural stem cell line. The study particularly focused on restoring cognitive functions after stroke what is a novel endpoint for the UCB derived neural stem cell line. Freshly prepared cells were found most effective, which is in line with what has been reported for motor and sensory functions using UCB-MNCs

after stroke (6). An enriched environment was provided to the animals, further fostering cognitive recuperation in a clinically meaningful setup. Mu et al. revealed that a combination of adipose stem cells and rehabilitation after experimental stroke is beneficial. This approach follows the newest STem Cells as an Emerging Paradigm in Stroke (STEPS) recommendations and is expected to provide more translationally relevant data (7). Hwang et al. proved that a combination of UCB-MNC and erythropoietin is also beneficial. Green et al. stereotaxically applied neural stem cells in the subacute stage after large cortico-striatal and smaller striatal strokes. Cell graft vitality was better preserved in smaller, striatal lesions, which are associated with a stabilization of functional neuronal networks. However, this effect was only transient, indirectly pointing to other long-term degenerative mechanisms and processes that thus far have not been identified. Encouraging results were reported regarding the efficacy of bone marrow-derived mesenchymal stem cells (MSCs) which have been applied in numerous preclinical trials for almost two decades. Satani et al. performed a systematic review and meta-analysis on 141 preclinical studies, confirming robust efficacy in acute and subacute time windows. It is noteworthy that comparable effects were seen in multiple labs around the world. Based on these robust data, the authors suggest that this approach should advance to carefully planned and implemented clinical trials.

TRANSLATIONAL AND CLINICAL CONSIDERATIONS

Defining the best-suited cell source is crucial to taking the translational process from the preclinical to the clinical stage. Ideally, the respective cells should be applicable for autologous and allogeneic use, and should exert beneficial effects via indirect ("bystander") effects while also exhibiting the potential for replacement of brain cells including astrocytes, oligodendrocytes and, most challenging, neurons thus covering all potential aspects of brain tissue regeneration (8). Recent research by Gancheva et al. revealed that dental pulp stem cells may perfectly fill this role. Another relevant aspect to translation is the safety of cell applications. Potential adverse events, such as secondary microinfarction, were reported when intraarterially administering large diameter cell populations such as MSCs. However, this phenomenon seems to depend on infusion speed and, in particular, cell dose, since lower doses can be safely delivered to the brain (9, 10). Cell engineering is another approach used to mitigate these potential adverse effects, for instance by increasing cell egress from cerebral capillaries (11). Moreover, no strong evidence of such complications has been observed after MSC delivery in clinics (12). The use of MSC-derived extracellular vesicles in place of MSCs also may help circumvent this problem. Bang and Kim, both working at the forefront of clinical translation, summarize the state of the art in this field, focusing on emerging clinical applications and remaining challenges.

Results from clinical cell therapy studies in stroke have been reported for intravenous injections (13, 14) and intracerebral grafts (15). Although overall safety has been confirmed, analysis of efficacy endpoints suggests that magnitude of effect may be smaller in human than animal studies, and a number of logistical challenges also have been identified. Krause et al. reviewed such problems, providing an unbiased overview of bottlenecks in the translational process, and discussing relevant aspects such as cost-to-benefit ratios and the role of industry-driven clinical research. Despite the moderate collective tepid enthusiasm regarding cell-based approaches, encouraging clinical data is available. Haque et al. report metabolic changes observed by magnetic resonance spectroscopy in the brains of patients being treated with autologous bone marrow-derived MNCs. These changes correlated with NIHSS scores and might not only indicate efficacy, but could also be used as surrogate markers for treatment efficacy in future clinical trials.

SUMMARY AND OUTLOOK

Although clinical translation of cell-based therapies is clearly gaining momentum, a number of open questions remain. One is the role of co-morbidities, which are abundantly present in human patients but are rarely modeled preclinically. Laso-Garcia et al. have analyzed this discrepancy and provide a comprehensive summary on effects of the most relevant comorbidities including hypertension, diabetes, and obesity both from clinical and preclinical perspectives. Aspects such as potential cell-drug interactions also await clarification (16). Finally, remarkable developments toward precision stem cell medicine have been achieved, which may facilitate stem cell-based therapies. Stem cell labeling and real-time imaging are capable of improving precision of transplantations (17). Progress in biomarker research (18) and artificial intelligence (19) may soon revolutionize research on outcome assessment, which will be pivotal to the future success of stem cell therapies. In summary, the road on which we travel with cell therapies for stroke is probably not a dead end but the journey remaining is challenging and long. Nevertheless, the overall research progress may finally shed light on the path, with this Research Topic identifying some of the most important past and future milestones along the way.

AUTHOR CONTRIBUTIONS

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

REFERENCES

1. Boltze J, Schmidt UR, Reich DM, Kranz A, Reymann KG, Strassburger M, et al. Determination of the therapeutic time window for human umbilical cord blood mononuclear cell transplantation following experimental stroke in rats. *Cell Transplant.* (2012) 21:1199–211. doi: 10.3727/096368911X589609
2. Shen LH, Li Y, Chen J, Zacharek A, Gao Q, Kapke A, et al. Therapeutic benefit of bone marrow stromal cells administered 1 month after stroke. *J Cereb Blood Flow Metab.* (2007) 27:6–13. doi: 10.1038/sj.jcbfm.9600311

3. Yang B, Strong R, Sharma S, Brenneman M, Mallikarjunarao K, Xi X, et al. Therapeutic time window and dose response of autologous bone marrow mononuclear cells for ischemic stroke. *J Neurosci Res.* (2011) 89:833–9. doi: 10.1002/jnr.22614
4. Guzman R, Janowski M, Walczak P. Intra-arterial delivery of cell therapies for stroke. *Stroke.* (2018) 49:1075–82. doi: 10.1161/STROKEAHA.117.018288
5. Cui LL, Golubczyk D, Tolppanen AM, Boltze J, Jolkkonen J. Cell therapy for ischemic stroke: are differences in preclinical and clinical study design responsible for the translational loss of efficacy? *Ann Neurol.* (2019) 86:5–16. doi: 10.1002/ana.25493
6. Weise G, Lorenz M, Posel C, Maria Riegelsberger U, Storbeck V, Kamprad M, et al. Transplantation of cryopreserved human umbilical cord blood mononuclear cells does not induce sustained recovery after experimental stroke in spontaneously hypertensive rats. *J Cereb Blood Flow Metab.* (2014) 34:e1–9. doi: 10.1038/jcbfm.2013.185
7. Boltze J, Modo MM, Mays RW, Taguchi A, Jolkkonen J, Savitz SI. Stem cells as an emerging paradigm in stroke 4: advancing and accelerating preclinical research. *Stroke.* (2019) 50:3299–306. doi: 10.1161/STROKEAHA.119.025436
8. Janowski M, Wagner DC, Boltze J. Stem cell-based tissue replacement after stroke: factual necessity or notorious fiction? *Stroke.* (2015) 46:2354–63. doi: 10.1161/STROKEAHA.114.007803
9. Janowski M, Lyczek A, Engels C, Xu J, Lukomska B, Bulte JW, et al. Cell size and velocity of injection are major determinants of the safety of intracarotid stem cell transplantation. *J Cereb Blood Flow Metab.* (2013) 33:921–7. doi: 10.1038/jcbfm.2013.32
10. Cui LL, Kerkela E, Bakreen A, Nitzsche F, Andrzejewska A, Nowakowski A, et al. The cerebral embolism evoked by intra-arterial delivery of allogeneic bone marrow mesenchymal stem cells in rats is related to cell dose and infusion velocity. *Stem Cell Res Ther.* (2015) 6:11. doi: 10.1186/scrt544
11. Cui LL, Nitzsche F, Pryazhnikov E, Tibeykina M, Tolppanen L, Rytkonen J, et al. Integrin alpha4 overexpression on rat mesenchymal stem cells enhances transmigration and reduces cerebral embolism after intracarotid injection. *Stroke.* (2017) 48:2895–900. doi: 10.1161/STROKEAHA.117.017809
12. Savitz SI, Yavagal D, Rappard G, Likosky W, Rutledge N, Graffagnino C, et al. A Phase 2 randomized, sham-controlled trial of internal carotid artery infusion of autologous bone marrow-derived ALD-401 cells in patients with recent stable ischemic stroke (RECOVER-Stroke). *Circulation.* (2019) 139:192–205. doi: 10.1161/CIRCULATIONAHA.117.030659
13. Hess DC, Wechsler LR, Clark WM, Savitz SI, Ford GA, Chiu D, et al. Safety and efficacy of multipotent adult progenitor cells in acute ischaemic stroke (MASTERS): a randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet Neurol.* (2017) 16:360–8. doi: 10.1016/S1474-4422(17)30046-7
14. Levy ML, Crawford JR, Dib N, Verkh L, Tankovich N, Cramer SC. Phase I/II study of safety and preliminary efficacy of intravenous allogeneic mesenchymal stem cells in chronic stroke. *Stroke.* (2019) 50:2835–41. doi: 10.1161/STROKEAHA.119.026318
15. Kalladka D, Sinden J, Pollock K, Haig C, McLean J, Smith W, et al. Human neural stem cells in patients with chronic ischaemic stroke (PISCES): a phase 1, first-in-man study. *Lancet.* (2016) 388:787–96. doi: 10.1016/S0140-6736(16)30513-X
16. Ikhsan M, Palumbo A, Rose D, Zille M, Boltze J. Neuronal stem cell and drug interactions: a systematic review and meta-analysis: concise review. *Stem Cells Transl Med.* (2019) 8:1202–11. doi: 10.1002/sctm.19-0020
17. Walczak P, Wojtkiewicz J, Nowakowski A, Habich A, Holak P, Xu J, et al. Real-time MRI for precise and predictable intra-arterial stem cell delivery to the central nervous system. *J Cereb Blood Flow Metab.* (2017) 37:2346–58. doi: 10.1177/0271678X16665853
18. Sloan, Smith-Warner SA, Ziegler RG, Wang M. Statistical methods for biomarker data pooled from multiple nested case-control studies. *Biostatistics.* (2019) doi: 10.1093/biostatistics/kxz051. [Epub ahead of print].
19. Heo J, Yoon JG, Park H, Kim YD, Nam HS, Heo JH. Machine learning-based model for prediction of outcomes in acute stroke. *Stroke.* (2019) 50:1263–5. doi: 10.1161/STROKEAHA.118.024293

Conflict of Interest: 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.

Copyright © 2020 Boltze, Abe, Clarkson, Detante, Pimentel-Coelho, Rosado-de-Castro and Janowski. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Neurology

ORIGINAL RESEARCH
published: 25 February 2019
doi: 10.3389/fneur.2019.00141

Serial Cerebral Metabolic Changes in Patients With Ischemic Stroke Treated With Autologous Bone Marrow Derived Mononuclear Cells

Muhammad E. Haque[1], Refaat E. Gabr[2], Sarah D. George[1], Seth B. Boren[1], Farhaan S. Vahidy[1], Xu Zhang[3], Octavio D. Arevalo[2], Susan Alderman[1], Ponnada A. Narayana[2], Khader M. Hasan[2], Elliott R. Friedman[2], Clark W. Sitton[2] and Sean I. Savitz[1]*

[1] *Institute for Stroke and Cerebrovascular Diseases, McGovern Medical School, The University of Texas Health Science Center at Houston, Houston, TX, United States,* [2] *Diagnostic and Interventional Imaging, McGovern Medical School, The University of Texas Health Science Center at Houston, Houston, TX, United States,* [3] *Biostatistics, Epidemiology, Research Design Component, Center for Clinical and Translational Sciences, McGovern Medical School, The University of Texas Health Science Center at Houston, Houston, TX, United States*

OPEN ACCESS

Edited by:
Johannes Boltze, Fraunhofer-Institut für Zelltherapie und Immunologie (IZI), Germany

Reviewed by:
Christopher G. Sobey, La Trobe University, Australia
Tracy D. Farr, University of Nottingham, United Kingdom

***Correspondence:**
Muhammad E. Haque
muhammad.e.haque@uth.tmc.edu

Specialty section:
This article was submitted to Stroke, a section of the journal Frontiers in Neurology

Received: *06 December 2018*
Accepted: *04 February 2019*
Published: *25 February 2019*

Citation:
Haque ME, Gabr RE, George SD, Boren SB, Vahidy FS, Zhang X, Arevalo OD, Alderman S, Narayana PA, Hasan KM, Friedman ER, Sitton CW and Savitz SI (2019) Serial Cerebral Metabolic Changes in Patients With Ischemic Stroke Treated With Autologous Bone Marrow Derived Mononuclear Cells. Front. Neurol. 10:141. doi: 10.3389/fneur.2019.00141

Purpose: Cell-based therapy offers new opportunities for the development of novel treatments to promote tissue repair, functional restoration, and cerebral metabolic balance. N-acetylasperate (NAA), Choline (Cho), and Creatine (Cr) are three major metabolites seen on proton magnetic resonance spectroscopy (MRS) that play a vital role in balancing the biochemical processes and are suggested as markers of recovery. In this preliminary study, we serially monitored changes in these metabolites in ischemic stroke patients who were treated with autologous bone marrow-derived mononuclear cells (MNCs) using non-invasive MRS.

Materials and Methods: A sub-group of nine patients (3 male, 6 female) participated in a serial MRS study, as part of a clinical trial on autologous bone marrow cell therapy in acute ischemic stroke. Seven to ten million mononuclear cells were isolated from the patient's bone marrow and administered intravenously within 72 h of onset of injury. MRS data were obtained at 1, 3, and 6 months using a whole-body 3.0T MRI. Single voxel point-resolved spectroscopy (PRESS) was obtained within the lesion and contralesional gray matter. Spectral analysis was done using TARQUIN software and absolute concentration of NAA, Cho, and Cr was determined. National Institute of Health Stroke Scale (NIHSS) was serially recoreded. Two-way analysis of variance was performed and $p < 0.05$ considered statistically significant.

Results: All metabolites showed statistically significant or clear trends toward lower ipsilesional concentrations compared to the contralesional side at all time points. Statistically significant reductions were found in ipsilesional NAA at 1M and 3M, Cho at 6M, and Cr at 1M and 6M ($p < 0.03$), compared to the contralesional side. Temporally, ipsilesional NAA increased between 3M and 6M ($p < 0.01$). On the other hand, ipsilesional Cho showed continued decline till 6M ($p < 0.01$). Ipsilesional Cr was stable over time. Contralesional metabolites were relatively stable over time, with only Cr showing

a reduction 3M ($p < 0.02$). There was a significant ($p < 0.03$) correlation between ipsilesional NAA and NIHSS at 3M follow-up.

Conclusion: Serial changes in metabolites suggest that MRS can be applied to monitor therapeutic changes. Post-treatment increasing trends of NAA concentration and significant correlation with NIHSS support a potential therapeutic effect.

Keywords: autologous mononuclear cells transplantation, cells therapy, ischemic stroke, cerebral metabolites, magnetic resonance spectroscopy

INTRODUCTION

The only therapeutic options for patients with ischemic stroke are approaches that promote recanalization and reperfusion to restore blood flow to the brain. Currently, there are no effective post stroke interventions that can promote repair of damaged tissues and restore brain function. Cell based therapies offer great potential to promote possible repair of damaged tissue, prevent atrophy, and help restore brain function. Extensive animal data have shown the safety and feasibility of the bone marrow-derived mononuclear cells (MNCs) as an attractive therapeutic option for stroke (1–4). Significant physical and cognitive improvement was shown to occurs in animals treated with cell therapies by providing a protective mechanism to attenuate progressive tissue damage (5–8). Clinical trials have shown the feasibility and safety of both autologous and allogenic cell administration in stroke patients (9–13). An important advancement in this field would be to develop post-stroke therapeutic biomarkers.

Number of post-stroke magnetic resonance spectroscopy (MRS) studies have shown elevated lactate (Lac), decreased N-acetylaspartate (NAA) and Creatine (Cr), and variable changes in choline (Cho) concentration in the ipsilesional hemisphere (14–18). Restoration of these cerebral metabolites concentration could facilitate in the evaluation of recovery (19). NAA is synthesized in neurons and is crucial for cerebral lipid synthesis and energy production (20, 21). The cerebral Cr is associated with energy recycling via converting adenosine diphosphate (ADP) to ATP (22). Choline is involved in memory function and muscle control (23). These metabolites have been extensively used as surrogate markers of neuronal health (NAA), membrane integrity (Cho), cellular energy (Cr) and oxidative stress (Lac) (14, 16, 24–28).

The application of MRS to characterize neural progenitor cells (NPCs) *in-vivo*, which can differentiate into neurons, astrocytes, and oligodendrocytes, has been reported (29). Furthermore, two separate studies presented MRS as a tool for monitoring NPCs and human umbilical mesenchymal stem cells differentiation (30, 31). Number of *in-vitro* studies showed the ability of MRS to identify cell types based on their metabolic profile in the cell culture (31). Previously, Brazzini reported an increase in NAA concentration in both left and right basal ganglia in patients with Parkinson treated with bone marrow-derived autologous MNCs transplantation (32). Metabolic improvement was also documented in amyotrophic lateral sclerosis patients treated with stem cells (33).

In this prospective study, we serially monitored NAA, Cho, and Cr concentrations with MRS as possible therapeutic biomarkers following intravenous infusion of autologous MNCs in patients with acute ischemic stroke. Our findings in this preliminary study encourage pursuing a larger study that can be correlated with therapeutic efficacy to predict post-stroke recovery.

MATERIALS AND METHODS

Human Protection

This study was conducted under Federal Investigational New Drug Application BB IND 13775 and was approved by the University of Texas Health Sciences Center at Houston Committee for the Protection of Human Subjects and by the Memorial Hermann Hospital Office of Research.

Patient Enrollment and Serial MRI

A sub-group of nine patients (3 males, 6 females) participated in this serial MRS study, which was an add-on scan to clinical trial testing autologous bone marrow mononuclear cells in patients with ischemic stroke. Written informed consent was obtained after a thorough discussion with the patient family prior to enrollment. The inclusion and exclusion criteria are described elsewhere (10). Post MNCs follow-up imaging was done at one (1M), three (3M), and six (6M) months of onset.

Intervention

Bone Marrow Cells Harvesting

The procedural details about mononuclear cells isolation and intravenous infusion are described elsewhere (10). Briefly, a total of 2 ml/kg bone marrow was harvested aseptically from the posterior iliac bone. All patients were placed in the prone position and a 11-gauge bone marrow needle was placed into the posterior iliac crest, and about 5 to 7 cc was aspirated with a 20-cc syringe and the procedure repeated. IV normal saline was administered if there were changes in the patient's hemodynamics, and all the patients were monitored for oxygen saturation, blood pressure, heart and respiratory rate before, during and after the procedure.

MNCs Isolation and Infusion

Bone marrow was transported in an anticoagulated blood collection bag for isolation and enrichment of the MNCs. The bone marrow was filtered (170-ml blood filter) to remove spicules. The MNCs were enriched from the bone marrow using Ficoll-Paque (GE Healthcare, Milwaukee, WI) and Plus density gradient separation using the density gradient procedure on

the Sepax device (Biosafe SA, Geneva, Switzerland). The MNCs were then washed twice with 5% human serum albumin in normal saline and adjusted to the appropriate concentration for administration (final concentration of 1.25%). A maximum of 10 million cells/kg in normal saline was administered into the antecubital vein over 30 min. Cells Transplantation was done within 72 h from onset of the stroke.

Clinical Assessments

National Institutes of Health Stroke Scale (NIHSS) (34), modified Rankin scale (mRS), and Barthal Index (BI) were recorded at baseline and NIHSS at each follow-up visit.

Imaging Acquisition Protocols

All imaging experiments were performed on a whole body 3.0 T Philips system (Philips Healthcare, Best, The Netherlands) using 8-channel head coil. Structural MRI was obtained using 3D T1-weighted volumes (TE/TR = 3.66 ms/8.2 ms, acquisition matrix = 256 × 256 × 170, FOV = 256 × 256 mm^2) and T2-weighted (TE/TR/ = 80 ms/2.5 s, acquisition matrix = 256 × 256 × 170, and FOV = 256 × 256 mm^2) images. Anatomical localizations of the lesions were performed using 2D fluid-attenuated inversion recovery (FLAIR, TE/TI/TR = 95 ms/2.6 s/11 sec, acquisition matrix = 256 × 256, FOV = 256 × 256 mm^2) images. Single voxel (SV) proton MRS was obtained with point-resolved spectroscopy (PRESS, voxel size 20 × 20 × 20 mm^3 TE/TR = 35/2,000 ms, sampling frequency = 2,000 Hz, data points = 1,024, number of scans = 128) on both the ipsilesional and contralesional regions. Volumes of interest (VOIs) were carefully placed within the infarct and contralateral normal appearing gray matter tissue using FLAIR images. Standard 3-pulse chemical shift selective scheme was used for water suppression with automated shimming. Unsuppressed water spectra were also obtained as an internal reference for metabolite quantification. Saturation bands were placed around the VOI to minimize lipid contamination.

Spectral Analysis and Metabolite Quantification

All the spectral analysis were performed using publicly available Totally Automated Robust Quantitation in NMR (TARQUIN) software (35), available at https://www.nitrc.org/projects/tarquin/. Absolute concentration was calculated in millimole (mM) units by scaling the fitted signal amplitude by the amplitude of the unsuppressed water signal. A detailed description of the measurements are described elsewhere (36, 37). Metabolite peaks at 2.01, 3.03, 3.19 ppm were referenced to NAA, Cr, and Cho, respectively, with respect to unsuppressed water signal at 4.7 ppm. Typically metabolite concentrations are normalized to creatine or choline concentration assuming they were constant; here, we reported absolute concentrations because they were not constant.

Lesion Volume Measurements

A semi-automated seed growing algorithm within Analyze 12.0 (Analyze Direct Inc., KS, USA) was used to delineate lesion volume on T2-weighted images by a single rater. The rater selected two seed points within the hyper and hypointense regions within the lesions and a region-growing algorithm automatically expanded the seed points within the 3D space of the image. Manual editing of the lesion volume was done when necessary.

STATISTICAL ANALYSIS

Cerebral metabolites concentration was analyzed by the mixed model (38) The fixed effects in the model included brain hemisphere (ipsilesional and contralesional), polychotomous time (1, 3, and 6 month), and interaction between hemisphere and polychotomous time. The random effects in the model included patient and interaction between patient and hemisphere. These random effects led to a nested covariance matrix accounting for correlation of measurements due to the same patient and a different level of correlation for measurements due to the same hemisphere of brain. Correlation between cerebral metabolites concentrations and NIHSS scores was depicted in scatter plot, together with Pearson correlation coefficient. Two-sided p-values were reported and p-values less than 0.05 were considered as significant. All statistical analyses were performed using the SAS software (version 9.4, the SAS Institute, Cary, NC).

RESULTS

After cellular intervention, six female and three male patients with average age of 56.5 ± 17.6 years (range 31–78 years) underwent serial metabolite measurements. Patient demographics and lesion laterality, size, location, and severity are summarized in **Table 1**. Patient P01 data were removed from spectroscopic measurement because of noisy spectra at two time points. The lesion size was 13.9–74.4 cc at 1M, and NIHSS was 5–34 at the onset. There was a significant ($p < 0.03$) decrease in lesion volume between 1 and 6 months.

Figure 1 shows a representative MRI of a patient illustrating typical MRS voxel placement in the lesion and contralateral tissue at the three follow-up scans, with their corresponding spectra below it. The contralateral voxel was placed on the normal appearing gray matter regions with some expected partial volume. Spectra were scaled and normalized to the unsuppressed water signal. As shown, the signal amplitude of NAA on the lesion spectra increased at 6 months as compared to 1 month, with minimal change in the contralateral NAA.

Metabolite changes in individual participants and its average values over the study cohort are summarized in **Figure 2**. All three metabolites showed statistically significant or clear trends toward lower ipsilesional concentrations compared to the contralesional side at all time points. Statistically significant reductions were found in ipsilesional NAA at 1M and 3M, Cho at 6M, and Cr at 1M and 6M ($p < 0.03$), compared to the contralesional side.

The temporal NAA concentration within the lesion showed an initial decreasing trend between 1 and 3 months (2.88 ± 2.1 to 2.37 ± 1.9 mM, $p = 0.24$) followed by an increase between 3 and 6 (2.37 ± 1.9 to 3.72 ± 2.6 mM, $p < 0.01$) months.

TABLE 1 | Patient demographics, clinical score, and lesion volume.

PID	Laterality	Lesion location	NIHSS				Lesion volume (cm^3)		
			Baseline	01M	03M	06M	01M	03M	06M
P01	L	Insula, frontal lobe	14	3	3	1	74.4	67.6	68.6
P02	R	Insula, IFG	9	6	3	2	50.2	26.4	25.1
P03	L	Insula, IFG	10	1	1	1	23.6	14.1	25.13
P04	R	Putamen, GP, CR	15	7	5	2	33.1	15.1	13.8
P05	R	Putamen, GP, CR	34	7	5	4	22.2	8.0	7.12
P06	R	Putamen, insula, FG	17	7	9	11	27.5	26.4	28.4
P07	R	Putamen	8	6	5	5	17.9	7.54	6.54
P08	R	TL, OL	16	9	8	7	13.9	12.9	11.5
P09	L	ITG, STG, FG, insula	8	5	5	6	6.08	6.8	6.03

IFG, Inferior Frontal Gyrus; GP, Globus Pallidus; CR, Corona Radiata; FG, Frontal Gyrus; TL, Temporal Lobe; OL, Occipital Lobe; ITG, Inferior Temporal Gyrus; STG, Superior Temporal Gyrus; NIHSS, National Institute of Health Stroke Scale.

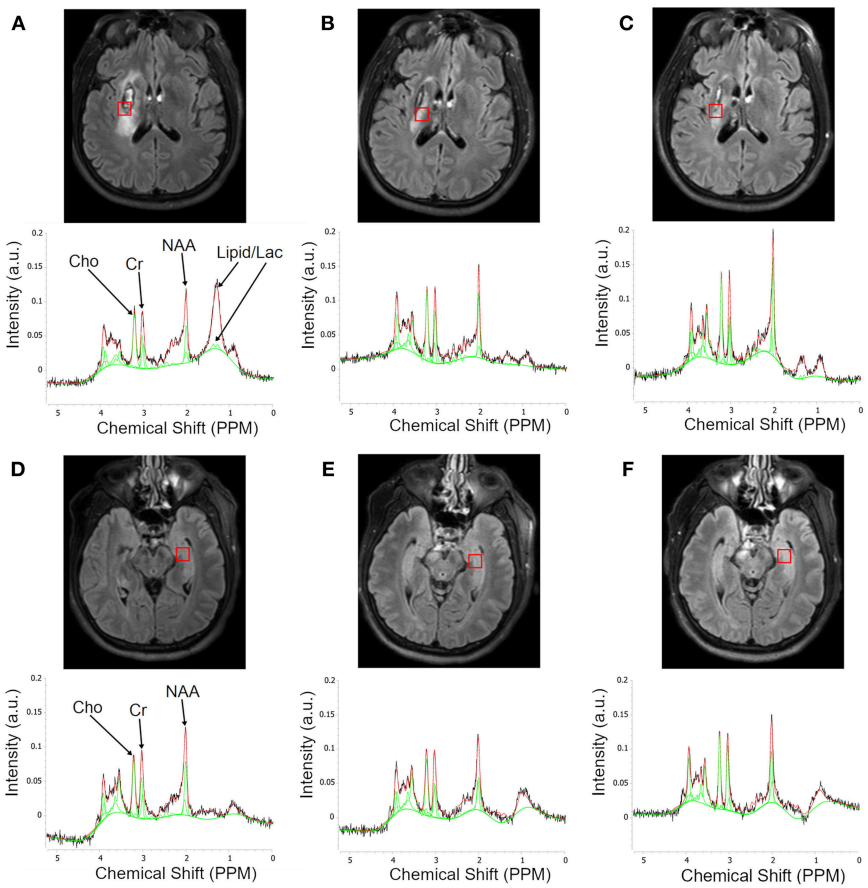


FIGURE 1 | Serial Flair MR images illustrating typical MRS voxel placement in the lesion and contralateral tissues and corresponding MR spectra. The first row of images **(A–C)** is a single slice showing voxel placement in the lesion at 1, 3, and 6 months, respectively. The second row shows the corresponding spectra within the lesion. Each plot shows the raw spectrum (black), library spectra of matching metabolites (red), and fitted function (green). Third row **(D–F)** shows voxel placement in the contralesional gray matter with corresponding spectra in the fourth row. Note the questionable peak at 1.33 ppm at 1 month that disappears at the later time point.

The contralesional NAA also showed a strong trend toward lower concentration between 1 and 3 months (5.95 ± 1.0 to 5.09 ± 0.93 mM, $p < 0.053$), but stabilized between 3 and 6 months (5.09 ± 0.93 to 5.17 ± 0.70 mM, $p =$ NS). Post graft Cho concentration in the lesion showed a continuing trend for reduction, which reached statistical significance at 6 months (1.62 ± 0.51 to 1.09 ±

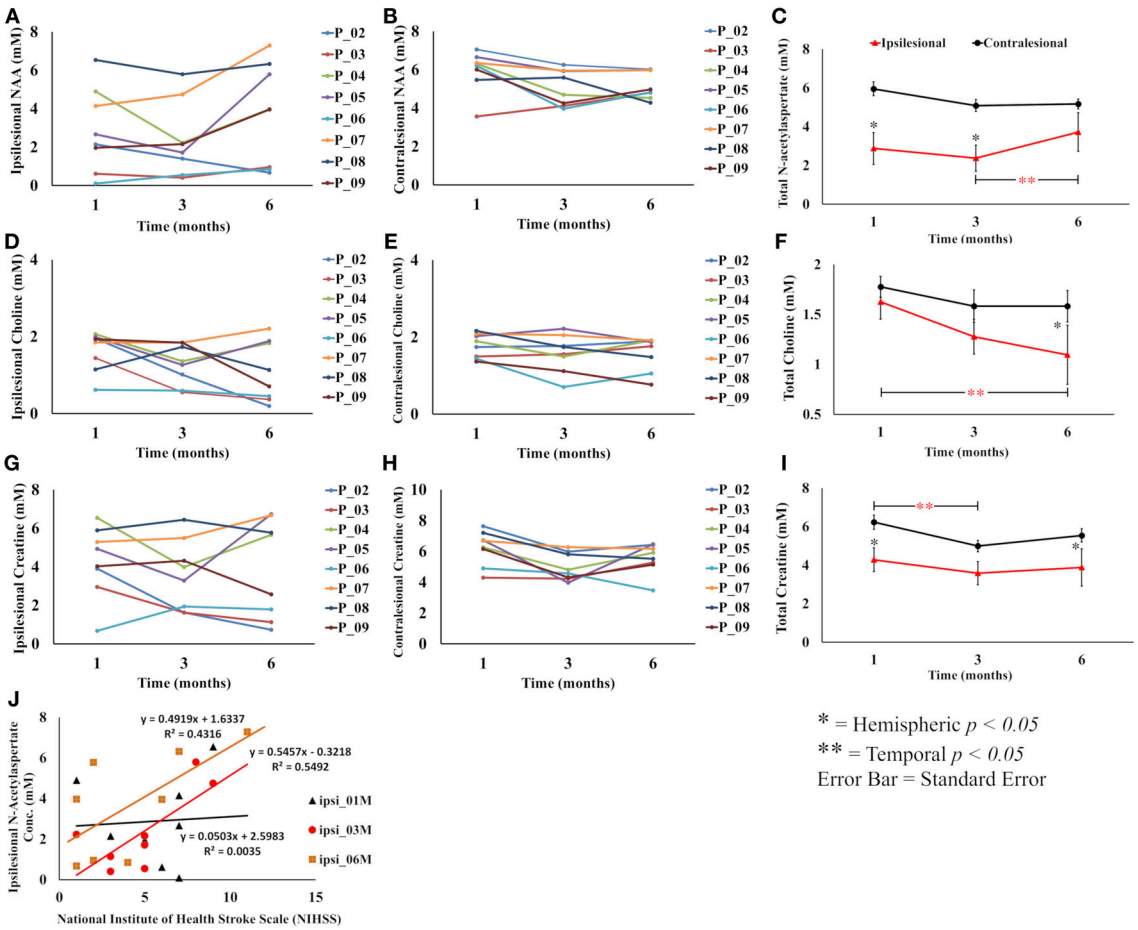


FIGURE 2 | Overall summary of post treatment serial change in metabolite concentration in the lesion and contralesion voxels. **(A,B)** Show serial ipsilesional and contralesional NAA concentrations in individual participants, respectively. **(C)** Shows overall trend of significant hemispheric decrease of NAA concentration between 1 and 3 months. Temporally, ipsilesional NAA increased between 3 and 6 months. **(D,E)** Show serial ipsilesional and contralesional choline concentrations in individual participants, respectively. **(F)** Shows an overall significant continual decline of ipsilesional choline concentration between 1 and 6 months, whereas in the contralesion it decreased between 1 and 3 months and then stabilized between 3 and 6 months. **(G,H)** Show serial ipsilesional and contralesional creatine concentrations in individual participants, respectively. **(I)** Illustrates the overall hemispheric and temporal decrease of creatine concentration between 1 and 3 months that stabilized between 3 and 6 months. A correlation between ipsilesional NAA concentration and NIHSS at 1, 3, and 6 months is shown in **(J)**. A significant ($p < 0.03$) correlation was observed at 3 months.

0.78 mM, $p < 0.01$), while contralesional measurements revealed a decreasing trend between 1 and 3 months (1.77 ± 0.31 to 1.58 ± 0.49 mM, p = NS) that stabilized through 6 months (1.58 ± 0.44 mM). Ipsilesional Cr was stable over the study period, but contralesional Cr reduced between 1 and 3 month (6.23 ± 1.12 to 4.99 ± 0.89 mM, $p < 0.02$).

Ipsilesional NAA concentration and NIHSS score were significantly correlated ($p < 0.05$) at 3 month follow-up and almost reached statistical significance at 6 months ($p = 0.07$), but no correlation was found at 1 month as shown in **Figure 2J**. There was no statistically significant correlation between Cho, Cr, and NIHSS in either ipsi or contralesional hemisphere. There was no significant correlation between lesion volume change and metabolite concentrations.

DISCUSSION

A handful of clinical trials have shown safety and feasibility of cell administration as an exploratory new therapeutic option in patients with recent ischemic stroke; however, to the best of our knowledge, this is the first *prospective* study in which patient's cerebral metabolites were serially measured over 6 months after the cellular intervention.

Our finding is in-line with an animal study reporting a significant increase in NAA/Cr and NAA/Cho ratios within the lesion in treated animals with gadolinium labeled mesenchymal stem cells compared to the sham group (39). In another animal study, Qian reported an initial decrease followed by an increase in ipsilesional NAA concentrations without any treatment after inducing transient ischemic stroke (TIA). However, unlike the

permanent stroke model, both the lesions and symptoms resolve within a few hours of onset in most patients with TIA (40).

The correlation between the NAA and NIHSS is in support of the previous study (19) suggesting NAA as a marker of therapeutic efficacy. However, the small sample size is the major limitation of our study. Interestingly the decreasing trends of all three metabolites concentrations in the contralesional measurement suggest global depletion of available energy which is supported by a decrease in Cr concentration which is considered a marker of neuronal energy.

Prior studies investigated brain metabolites as a marker of post-stroke recovery especially NAA because it is exclusively found within neurons. A reduction in NAA in the ipsilesional hemisphere has been associated with continued neuronal death and expansion of the infarct (41–43). A continued decline in NAA concentration within the lesion or other ipsilesional tissues at 1, 3, and 6 months has been documented, with few exceptions (44–46). Contrary to the literature, our preliminary results show a trend of regaining NAA concentrations within the lesion between 3 and 6 months following MNC infusion. This is in agreement with a previous study in patients with Parkinson disease who were also treated with MNCs, which reported an increase in NAA in the basal ganglia. Here we found increased NAA within the lesion.

We also noted that most of the patients had one or two broad peaks between 0.9 and 1.5 ppm, resonance regions of lipid and lactate, in the ipsilesional voxel at 1 month (**Figure 1**) suggesting possible hypoxic environment or cells proliferation which resolved at later follow-up visits. Interestingly, there was no peak between 0.9 and 1.5 ppm in the contralesional voxel. Further investigation of this finding requires a non-treated matching control patients as a control group.

A higher choline concentration around the penumbra region in acute phase has been documented (47). Here we observed a decreasing trend of choline concentration in both ipsilesional and contralesional measurement between 1 and 3 months. While the choline signal stabilized in the contralesional side, it continued to decrease in the lesion suggestion progressive membrane disintegration. The opposite trend between NAA (increasing) and Cho (decreasing) between 3 and 6 months in the lesion could be due to a higher number of immature neurons without membrane, which is typically developed at the final stages of neuronal maturation. However, we will investigate this hypothesis in future animal study combining MRS and histology.

STUDY LIMITATION

The limitations of this exploratory pilot study included the small sample size, the absence of non-treated patient group, the limited coverage of single voxel spectroscopy, and known partial volume effects. Future studies will address these limitations by increasing sample size, adding non-treated patients group and obtaining multi-voxel MRS.

CONCLUSION

Our study supports using MRS as an innovative means to evaluate treatment efficacies by quantifying lesion metabolites. In this preliminary study we observed signals of a slow treatment effect, regain in NAA concentrations in a damaged tissue area, and patients with increased NAA recovering better on clinical deficits scores.

AUTHOR CONTRIBUTIONS

MH designed the study, carried MRI quantitative analysis, and drafted the manuscript. RG contributed to data analysis, assisted in optimizing MRI acquisition. SG and SB created plots and figures. SA and FV recruited, consented, and obtained patient's serial neurological assessment. XZ performed statistical analysis. KH and PN provided qualitative and quantitative quality assurance of images and data analysis. OA, EF, and CS provided radiology reports and assisted in lesion locations. SS supervised the study and provided necessary resources. All authors contributed to and approved the final manuscript.

FUNDING

This work was funded by the National Institutes of Health (NIH/NINDS 1R21HD060978-01A1).

ACKNOWLEDGMENTS

We wish to thank Mr. Vipulkumar Patel and Dr. Kasam Mallikarjunarao for helping in data acquisition and management.

REFERENCES

1. Kamiya N, Ueda M, Igarashi H, Nishiyama Y, Suda S, Inaba T, et al. Intra-arterial transplantation of bone marrow mononuclear cells immediately after reperfusion decreases brain injury after focal ischemia in rats. *Life Sci.* (2008) 83:433–7. doi: 10.1016/j.lfs.2008.07.018
2. Shichinohe H, Kuroda S, Maruichi K, Osanai T, Sugiyama T, Chiba Y, et al. Bone marrow stromal cells and bone marrow-derived mononuclear cells: which are suitable as cell source of transplantation for mice infarct brain? *Neuropathology* (2010) 30:113–22. doi: 10.1111/j.1440-1789.2009.01050.x
3. Vasconcelos-Dos-Santos A, Rosado-De-Castro PH, Lopes De Souza SA, Da Costa Silva J, Ramos AB, Rodriguez De Freitas G, et al. Intravenous and intra-arterial administration of bone marrow mononuclear cells after focal cerebral ischemia: Is there a difference in biodistribution and efficacy? *Stem Cell Res.* (2012) 9:1–8. doi: 10.1016/j.scr.2012.02.002
4. Yang B, Li W, Satani N, Nghiem DM, Xi X, Aronowski J, et al. Protective effects of autologous bone marrow mononuclear cells after administering t-PA in an embolic stroke model. *Transl Stroke Res.* (2018) 9:135–45. doi: 10.1007/s12975-017-0563-1
5. Bjorklund A, Lindvall O. Cell replacement therapies for central nervous system disorders. *Nat Neurosci.* (2000) 3:537–44. doi: 10.1038/75705
6. Honma T, Honmou O, Iihoshi S, Harada K, Houkin K, Hamada H, et al. Intravenous infusion of immortalized human mesenchymal stem cells protects against injury in a cerebral ischemia model in adult rat. *Exp Neurol.* (2006) 199:56–66. doi: 10.1016/j.expneurol.2005.05.004

7. Gao Q, Li Y, Shen L, Zhang J, Zheng X, Qu R, et al. Bone marrow stromal cells reduce ischemia-induced astrocytic activation *in vitro*. *Neuroscience* (2008) 152:646–55. doi: 10.1016/j.neuroscience.2007.10.069
8. Sammali E, Alia C, Vegliante G, Colombo V, Giordano N, Pischiutta F, et al. Intravenous infusion of human bone marrow mesenchymal stromal cells promotes functional recovery and neuroplasticity after ischemic stroke in mice. *Sci Rep*. (2017) 7:6962. doi: 10.1038/s41598-017-07274-w
9. Kondziolka D, Steinberg GK, Wechsler L, Meltzer CC, Elder E, Gebel J, et al. Neurotransplantation for patients with subcortical motor stroke: a phase 2 randomized trial. *J Neurosurg.* (2005) 103:38–45. doi: 10.3171/jns.2005.103.1.0038
10. Savitz SI, Misra V, Kasam M, Juneja H, Cox CS Jr, Alderman S, et al. Intravenous autologous bone marrow mononuclear cells for ischemic stroke. *Ann Neurol.* (2011) 70:59–69. doi: 10.1002/ana.22458
11. Bhasin A, Srivastava MV, Mohanty S, Bhatia R, Kumaran SS, Bose S. Stem cell therapy: a clinical trial of stroke. *Clin Neurol Neurosurg.* (2013) 115:1003–8. doi: 10.1016/j.clineuro.2012.10.015
12. Steinberg GK, Kondziolka D, Wechsler LR, Lunsford LD, Coburn ML, Billigen JB, et al. Clinical outcomes of transplanted modified bone marrow-derived mesenchymal stem cells in stroke: a phase 1/2a study. *Stroke* (2016) 47:1817–24. doi: 10.1161/STROKEAHA.116.012995
13. Hess DC, Wechsler LR, Clark WM, Savitz SI, Ford GA, Chiu D, et al. Safety and efficacy of multipotent adult progenitor cells in acute ischaemic stroke (MASTERS): a randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet Neurol.* (2017) 16:360–8. doi: 10.1016/S1474-4422(17)30046-7
14. Felber SR, Luef GJ, Birbamer GG, Aichner FT, Gerstenbrand F. Magnetic resonance studies in stroke. *Lancet* (1992) 339:879–80. doi: 10.1016/0140-6736(92)90328-Z
15. Ford CC, Griffey RH, Matwiyoff NA, Rosenberg GA. Multivoxel 1H-MRS of stroke. *Neurology* (1992) 42:1408–12. doi: 10.1212/WNL.42.7.1408
16. Kang DW, Roh JK, Lee YS, Song IC, Yoon BW, Chang KH. Neuronal metabolic changes in the cortical region after subcortical infarction: a proton MR spectroscopy study. *J Neurol Neurosurg Psychiatr.* (2000) 69:222–7. doi: 10.1136/jnnp.69.2.222
17. Kobayashi M, Takayama H, Suga S, Mihara B. Longitudinal changes of metabolites in frontal lobes after hemorrhagic stroke of basal ganglia: a proton magnetic resonance spectroscopy study. *Stroke* (2001) 32:2237–45. doi: 10.1161/hs1001.096621
18. Munoz Maniega S, Cvoro V, Chappell FM, Armitage PA, Marshall I, Bastin ME, et al. Changes in NAA and lactate following ischemic stroke: a serial MR spectroscopic imaging study. *Neurology* (2008) 71:1993–9. doi: 10.1212/01.wnl.0000336970.85817.4a
19. Bivard A, Yassi N, Krishnamurthy V, Lin L, Levi C, Spratt NJ, et al. A comprehensive analysis of metabolic changes in the salvaged penumbra. *Neuroradiology* (2016) 58:409–15. doi: 10.1007/s00234-015-1638-x
20. Madhavarao CN, Namboodiri AM. NAA synthesis and functional roles. *Adv Exp Med Biol.* (2006) 576:49–66. Discussion: 361–3. doi: 10.1007/0-387-30172-0_4
21. Rigotti DJ, Inglese M, Babb JS, Rovaris M, Benedetti B, Filippi M, et al. Serial whole-brain N-acetylaspartate concentration in healthy young adults. *AJNR Am J Neuroradiol.* (2007) 28:1650–1. doi: 10.3174/ajnr.A0712
22. Rackayova V, Cudalbu C, Pouwels PJW, Braissant O. Creatine in the central nervous system: from magnetic resonance spectroscopy to creatine deficiencies. *Anal Biochem.* (2017) 529:144–57. doi: 10.1016/j.ab.2016.11.007
23. Prado MA, Reis RA, Prado VF, De Mello MC, Gomez MV, De Mello FG. Regulation of acetylcholine synthesis and storage. *Neurochem Int.* (2002) 41:291–9. doi: 10.1016/S0197-0186(02)00044-X
24. Fenstermacher MJ, Narayana PA. Serial proton magnetic resonance spectroscopy of ischemic brain injury in humans. *Invest Radiol.* (1990) 25:1034–9. doi: 10.1097/00004424-199009000-00016
25. Felber SR, Aichner FT, Sauter R, Gerstenbrand F. Combined magnetic resonance imaging and proton magnetic resonance spectroscopy of patients with acute stroke. *Stroke* (1992) 23:1106–10. doi: 10.1161/01.STR.23.8.1106
26. Graham GD, Blamire AM, Howseman AM, Rothman DL, Fayad PB, Brass LM, et al. Proton magnetic resonance spectroscopy of cerebral lactate and other metabolites in stroke patients. *Stroke* (1992) 23:333–40. doi: 10.1161/01.STR.23.3.333
27. Kamada K, Houkin K, Iwasaki Y, Abe H, Kashiwaba T. Metabolic and neurological patterns in chronic cerebral infarction: a single-voxel 1H-MR spectroscopy study. *Neuroradiology* (1997) 39:560–5. doi: 10.1007/s002340050467
28. Saunders DE. MR spectroscopy in stroke. *Br Med Bull.* (2000) 56:334–45. doi: 10.1258/0007142001903256
29. Manganas LN, Zhang X, Li Y, Hazel RD, Smith SD, Wagshul ME, et al. Magnetic resonance spectroscopy identifies neural progenitor cells in the live human brain. *Science* (2007) 318:980–5. doi: 10.1126/science.1147851
30. Xu ZF, Pan AZ, Yong F, Shen CY, Chen YW, Wu RH. Human umbilical mesenchymal stem cell and its adipogenic differentiation: profiling by nuclear magnetic resonance spectroscopy. *World J Stem Cells* (2012) 4:21–7. doi: 10.4252/wjsc.v4.i4.21
31. Chung YL, El Akabawy G, So PW, Solanky BS, Leach MO, Modo M. Profiling metabolite changes in the neuronal differentiation of human striatal neural stem cells using 1H-magnetic resonance spectroscopy. *Neuroreport* (2013) 24:1035–40. doi: 10.1097/WNR.0000000000000056
32. Brazzini A, Cantella R, De La Cruz A, Yupanqui J, Leon C, Jorquiera T, et al. Intraarterial autologous implantation of adult stem cells for patients with Parkinson disease. *J Vasc Interv Radiol.* (2010) 21:443–51. doi: 10.1016/j.jvir.2010.01.008
33. Garcia Santos JM, Inuggi A, Gomez Espuch J, Vazquez C, Iniesta F, Blanquer M, et al. Spinal cord infusion of stem cells in amyotrophic lateral sclerosis: magnetic resonance spectroscopy shows metabolite improvement in the precentral gyrus. *Cytotherapy* (2016) 18:785–96. doi: 10.1016/j.jcyt.2016.03.296
34. Brott T, Adams HP Jr, Olinger CP, Marler JR, Barsan WG, Biller J, et al. Measurements of acute cerebral infarction: a clinical examination scale. *Stroke* (1989) 20:864–70. doi: 10.1161/01.STR.20.7.864
35. Wilson M, Reynolds G, Kauppinen RA, Arvanitis TN, Peet AC. A constrained least-squares approach to the automated quantitation of *in vivo* (1)H magnetic resonance spectroscopy data. *Magn Reson Med.* (2011) 65:1–12. doi: 10.1002/mrm.22579
36. Ernst T, Lee JH, Ross BD. Direct 31P imaging in human limb and brain. *J Comput Assist Tomogr.* (1993) 17:673–80. doi: 10.1097/00004728-199309000-00001
37. Kreis R, Ernst T, Ross BD. Development of the human brain: *in vivo* quantification of metabolite and water content with proton magnetic resonance spectroscopy. *Magn Reson Med.* (1993) 30:424–37. doi: 10.1002/mrm.1910300405
38. Littell RC, Henry PR, Ammerman CB. Statistical analysis of repeated measures data using SAS procedures. *J Anim Sci.* (1998) 76:1216–31. doi: 10.2527/1998.7641216x
39. Shyu WC, Chen CP, Lin SZ, Lee YJ, Li H. Efficient tracking of non-iron-labeled mesenchymal stem cells with serial MRI in chronic stroke rats. *Stroke* (2007) 38:367–74. doi: 10.1161/01.STR.0000254463.24655.14
40. Qian J, Qian B, Lei H. Reversible loss of N-acetylaspartate after 15-min transient middle cerebral artery occlusion in rat: a longitudinal study with *in vivo* proton magnetic resonance spectroscopy. *Neurochem Res.* (2013) 38:208–17. doi: 10.1007/s11064-012-0910-2
41. Petroff OA, Graham GD, Blamire AM, Al-Rayess M, Rothman DL, Fayad PB, et al. Spectroscopic imaging of stroke in humans: histopathology correlates of spectral changes. *Neurology* (1992) 42:1349–54. doi: 10.1212/WNL.42.7.1349
42. Demougeot C, Marie C, Giroud M, Beley A. N-acetylaspartate: a literature review of animal research on brain ischaemia. *J Neurochem.* (2004) 90:776–83. doi: 10.1111/j.1471-4159.2004.02583.x
43. Glodzik-Sobanska L, Slowik A, Kieltyka A, Kozub J, Sobiecka B, Urbanik A, et al. Reduced prefrontal N-acetylaspartate in stroke patients with apathy. *J Neurol Sci.* (2005) 238:19–24. doi: 10.1016/j.jns.2005.06.004
44. Houkin K, Kamada K, Kamiyama H, Iwasaki Y, Abe H, Kashiwaba T. Longitudinal changes in proton magnetic resonance spectroscopy in cerebral infarction. *Stroke* (1993) 24:1316–21. doi: 10.1161/01.STR.24.9.1316
45. Wardlaw JM, Marshall I, Wild J, Dennis MS, Cannon J, Lewis SC. Studies of acute ischemic stroke with proton magnetic resonance spectroscopy: relation between time from onset, neurological

deficit, metabolite abnormalities in the infarct, blood flow, and clinical outcome. *Stroke* (1998) 29:1618–24. doi: 10.1161/01.STR.29.8.1618

46. Cirstea CM, Brooks WM, Craciunas SC, Popescu EA, Choi IY, Lee P, et al. Primary motor cortex in stroke: a functional MRI-guided proton MR spectroscopic study. *Stroke* (2011) 42:1004–9. doi: 10.1161/STROKEAHA.110.601047
47. Munoz Maniega S, Cvoro V, Armitage PA, Marshall I, Bastin ME, Wardlaw JM. Choline and creatine are not reliable denominators for calculating metabolite ratios in acute ischemic stroke. *Stroke* (2008) 39:2467–9. doi: 10.1161/STROKEAHA.107.507020

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.

Copyright © 2019 Haque, Gabr, George, Boren, Vahidy, Zhang, Arevalo, Alderman, Narayana, Hasan, Friedman, Sitton and Savitz. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Neurology

REVIEW
published: 12 March 2019
doi: 10.3389/fneur.2019.00211

Mesenchymal Stem Cell-Derived Extracellular Vesicle Therapy for Stroke: Challenges and Progress

Oh Young Bang[1,2*] *and Eun Hee Kim*[2,3,4]

[1] Department of Neurology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea, [2] Translational and Stem Cell Research Laboratory on Stroke, Samsung Medical Center, Seoul, South Korea, [3] Medical Research Institute, Sungkyunkwan University School of Medicine, Seoul, South Korea, [4] Stem cell and Regenerative Medicine Institute, Samsung Biomedical Research Institute, Seoul, South Korea

Stroke is the leading cause of physical disability among adults. Stem cells such as mesenchymal stem cells (MSCs) secrete a variety of bioactive substances, including trophic factors and extracellular vesicles (EVs), into the injured brain, which may be associated with enhanced neurogenesis, angiogenesis, and neuroprotection. EVs are circular membrane fragments (30 nm−1 μm) that are shed from the cell surface and harbor proteins, microRNAs, etc. Since 2013 when it was first reported that intravenous application of MSC-derived EVs in a stroke rat model improved neurological outcomes and increased angiogenesis and neurogenesis, many preclinical studies have shown that stem cell-derived EVs can be used in stroke therapy, as an alternative approach to stem cell infusion. Although scientific research regarding MSC-derived EV therapeutics is still at an early stage, research is rapidly increasing and is demonstrating a promising approach for patients with severe stroke. MSC therapies have already been tested in preclinical studies and clinical trials, and EV-mediated therapy has unique advantages over cell therapies in stroke patients, in terms of biodistribution (overcoming the first pass effect and crossing the blood-brain-barrier), cell-free paradigm (avoidance of cell-related problems such as tumor formation and infarcts caused by vascular occlusion), whilst offering an off-the-shelf approach for acute ischemic stroke. Recently, advances have been made in the understanding of the function and biogenesis of EVs and EVs therapeutics for various diseases. This review presents the most recent advances in MSC-derived EV therapy for stroke, focusing on the application of this strategy for stroke patients.

Keywords: stroke, ischemic stroke, extracellular vesicles, stem cells, mesenchymal stem cells, microRNA

OPEN ACCESS

Edited by:
Pedro M. Pimentel-Coelho, Federal University of Rio de Janeiro, Brazil

Reviewed by:
Claire Rome, Université Grenoble Alpes, France
Jialing Liu, University of California, San Francisco, United States

***Correspondence:**
Oh Young Bang
ohyoung.bang@samsung.com

Specialty section:
This article was submitted to Stroke, a section of the journal Frontiers in Neurology

Received: *16 December 2018*
Accepted: *18 February 2019*
Published: *12 March 2019*

Citation:
Bang OY and Kim EH (2019) Mesenchymal Stem Cell-Derived Extracellular Vesicle Therapy for Stroke: Challenges and Progress. Front. Neurol. 10:211. doi: 10.3389/fneur.2019.00211

INTRODUCTION

Stroke is the leading cause of physical disability among adults. One-fourth to a half of stroke survivors are left with significant disabilities. Stem cell therapy is considered a potential regenerative strategy for patients with neurologic deficits. Adult stem cells, such as mesenchymal stem cells (MSCs) may be a good option for stroke therapy, as they secrete a variety of bioactive substances, including trophic factors and extracellular vesicles (EVs, 30 nm−1 μm sized circular membrane fragments shed from the cell surface) into the injured brain, which is associated with enhanced

neurogenesis, angiogenesis, and synaptogenesis (1–5). In addition, MSCs are thought to play multiple roles, such as attenuating inflammation (6), reducing scar thickness (7), enhancing autophagy (8), and possibly replacing damaged cells (9), in various brain diseases. Over the past 15 years, several randomized stem cell therapy trials have been conducted in patients with ischemic stroke (10–17), which showed mixed results. Possible reasons for conflicting results include, heterogeneous study populations (therefore requiring the selection of optimal candidate patients), delayed treatment (thus requiring an off-the shelf approach as soon as possible following a stroke), the limited restorative potential of stem cell therapy (especially in elderly patients with chronic illness), and a lack of objective measurements for the assessment of efficacy in stem cell therapy (5, 18).

It is widely accepted that MSCs exert their action via paracrine effects via secretomes or EVs, rather than through transdifferentiation to replace damaged neurons. Approximately 80% of cells disappeared in the infarcted brain within several days after transplantation with MSCs (19), yet the effects of stem cells persisted for several weeks following treatment. Our biodistribution study showed that MSCs exhibit a dynamic release of EVs in the ischemic brain condition, and that systemic administration of MSC-derived EVs led to a dose-dependent increase of MSC EVs in the infarcted hemisphere (bypassing the lung and liver) and functional improvement, suggesting that MSC EV therapy has a similar functional outcome, yet an improved safety profile compared to MSC administration (20).

This review presents the most recent advances in MSC-derived EV therapy for stroke, focusing on the clinical application of this strategy for stroke patients.

BIOLOGY AND FUNCTION OF EXTRACELLULAR VESICLES

EV Biogenesis

EVs are a broad term that usually refers to heterogeneous vesicles that are released from cells. EVs containing cellular proteins, DNAs and RNAs of cells are classified into exosomes (30–200 nm), microvesicles (200–1000 nm) and apoptosomes (1–10 μm) depending on their size (21). Among them, exosomes and microvesicles released from living cells, are involved in many processes, such as proliferation, differentiation and angiogenesis, and are known to act as a means of intercellular communication (22–24).

Exosomes and microvesicles originate from the plasma membrane, and are formed through distinct mechanisms (**Figure 1**) (23). The generation of microvesicles begins with the recruitment of cytoplasmic proteins and nucleic acids by the endosomal sorting complex required for transport (ESCRT)-dependent and independent pathways {mediated by ADP ribosylation factor 6 [ARF6] and phospholipase D2 [PLD2]. Lipid flipping then occurs, and membrane budding takes place.

Microvesicles are a more heterogeneous population and more sensitive to external stimulation than exosomes. For example, an increase in the extracellular concentration of ATP induces activation of the P2X7 receptor and consequential release of microvesicles (21). The production of exosomes begins with the membrane folding inward, the creation of empty intraluminal vesicles (ILVs), and the maturation of ILVs into multivesicular bodies (MVBs). They are released into the extracellular space through fusion of MVBs and the plasma membrane by small GTPases, such as RAB27A, RAB11, and RAB35, or by ESCRT (25).

Knowledge regarding EV biogenesis is essential for understanding EV characteristics and for the development of EV therapeutics. For example, activation of P2X7R by the pathogen-associated molecular pattern (PAMP) or damage-associated molecular pattern (DAMP) can induce membrane blebbing, or fusion of MVBs. PLD2, which regulates lipids by degrading phosphatidylcholine into choline and phosphatidic acid, and ARF6, which regulates membrane trafficking and actin cytoskeleton remodeling, may play an important role in endocytosis and exocytosis (23). Studies have shown that overexpression of ARF6 increases the number of exosomes released from the cells, whereas inhibition of ARF6 and PLD2 reduces the release of exosomes (26, 27).

Mechanisms of Action of Stem Cell-Derived EVs

Stem cell-derived EVs could play a critical role in the exchange of information between stem cells and damaged cells and alter the behavior of the target cells. Ischemia induces an increase in the circulating or regional levels of EVs, and it has been identified that EVs have their own function. Stroke triggers the mobilization of bone marrow (BM) MSC-derived EVs in patients with severe stroke (28), and EVs released by ischemic stimulation have restorative capacity (20). In addition, EVs from ischemic tissue facilitated vasculogenesis in the ischemic limb model (29). EVs from ischemic muscles induce BM mononuclear cell differentiation into cells with an endothelial phenotype (29).

Formation of new neuronal cells and blood vessels are the fundamental processes for the recovery after ischemic brain injury. During acute phase of stoke, both ischemic/reperfusion injury and inflammatory response are pivotal to the pathophysiology of ischemic stroke. In addition, stroke patients are often elderly and have chronic diseases, which may attenuate regenerative potential after stroke. As shown in **Table 1**, regenerative potential could be enhanced by treatment of MSCs or MSC-derived EVs (67). In addition, treatment of MSC-derived EVs in animal models of brain diseases resulted in central and peripheral immunotolerance (63, 68). EVs harbor bioactive molecules and EVs secreted from stem cells carry more complex cargos than other cellular sources (69). Stem cell-derived EVs contain many molecules that may have therapeutic effects in stroke (70), such as microRNAs, proteins, and mitochondria (**Table 1**). MicroRNAs, are a class of short, single-stranded, non-coding RNAs that can be horizontally shuttled by EVs, and EVs-encapsulated proteins have been implicated in the regulation of protective and restorative processes (71). Beside microRNAs, MSC EVs may shuttle other genetic components, such as mRNAs (72). In addition, damage

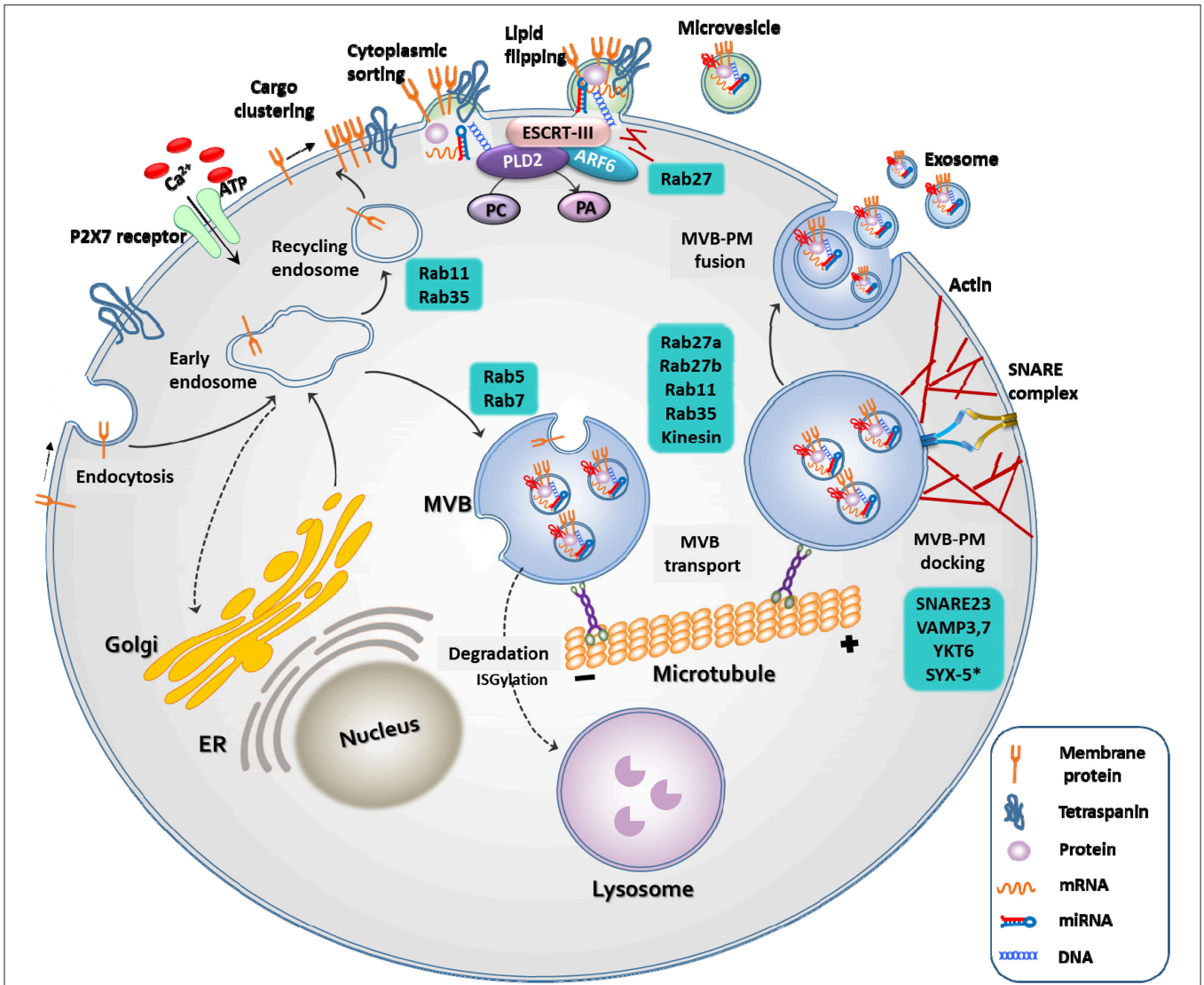


FIGURE 1 | Biogenesis of extracellular vesicles. EVs are released through two different pathways. When extracellular adenosine triphosphate (ATP) increases in response to external stimuli, the P2X7 receptor opens and calcium ions enter the cell. Membrane-associated proteins, tetraspanins, and cytoplasmic cargos are clustered in discrete membrane of the plasma membrane for microvesicles. The cargo of MVs are composed of cytoplasmic proteins, mRNAs, miRNAs, and DNAs. Similar to exosomes, RAS-related protein (RAB), actin, the endosomal sorting complex required for transport (ESCRT), ADP ribosylation factor 6 (ARF6) and phospholipase D2 (PLD2), and soluble N-ethylmaleimide-sensitive protein receptor (SNARE) proteins play important roles in MV release. However, MVs differ from exosomes in that they bud directly through flipping of lipid from the plasma membrane. The cargo of multivesicular bodies (MVBs) are either derived from endocytosis of the plasma membrane or from the trans-Golgi network. The reverse flow in the direction of the Golgi or recirculation to the plasma membrane is controlled by various Rab GTPases. Once MVB has matured, it is transported to the plasma membrane along the microtubule, and not by lysosomes. As a final step in exosome release, MVBs are docked and fused with the plasma membrane. Rab, actin, and SNARE proteins play important roles in these exosome release steps.

to the mitochondria caused by tissue injury, aggravates the severity of injury. Restoration of mitochondria dysfunction, through stem cell-derived mitochondria transplantation via EVs could potentially be an effective therapeutic strategy (66, 73).

ADVANTAGES OF EXTRACELLUALR VESICLES OVER STEM CELLS IN STROKE

Allogeneic stem cells have many advantages over autologous stem cells. Allogeneic MSCs are scalable from a manufacturing perspective, with standardized procedures. The use of allogeneic MSCs reduces the time required to obtain a sufficient number of cells (the "off the shelf" approach). Through the application of allogeneic stem cell therapy in the acute phase of stroke, both neurorestorative and neuroprotective actions can be expected (74). In recent clinical trials of intravenous application of allogeneic stem cells (MultiStem® in patients with acute stroke, stem cells were applied within 24–48 h, following the onset of symptoms (16). In addition, MSCs from younger healthy donors may differ in terms of their proliferation and neurorestorative

TABLE 1 | Mode of action of stem cell-derived EV in animal models of stroke or other ischemic disease.

Mode of action	Intravesicular contents
Angio-/neuro-genesis	miR-17-92 cluster targeting phosphatase and tensin homolog (30) miR-124a (31) miR-126 targeting portocadherin 7 (32) miR-133b targeting RABEPK (33) and RhoA (34) miR-134 targeting caspase-8 (35) miR-181b-5p targeting TRPM7 (36) miR-184 targeting Numbl, miR-210 targeting ephrin-A3 (20, 37, 38) miR-210 targeting Efna3 (39) miR-294 (40) Angiopoietin-1 mRNA to restore vascular permeability (41) CXCR4 via Akt signaling pathway (42) CXCR4, VEGF, VEGFR2, HGF, c-Met, Akt (20) VEGF, HIF-1α (43) PDGF (44) ICAM-1, bFGF, CHI3L1, CD147, CD105 (37) Transcription factors (STAT3) and signaling pathways (NF-κB) (45, 46) No specified (47–49)
Neuroprotection	miR-19a targeting PTEN (50) miR-21 via MAPK signaling pathway (51) miR-22 targeting Mecp2 (52) miR-125b targeting p53 (53) miR-145 targeting AQP4 (54) miR-199a via sirt1 pathway (55) miR-214 targeting CaMKII (56) miR-494 via Akt pathway (57) miR-711 targeting PPARγ (58) Neuron-specific enolase (59) Plasminogen activator inhibitor-1 targeting STAT3 and Akt (60)
Immunomodulation	miR-181a via BCL2, XIAP (61) CD73 promote adenosine accumulation (62) Anti-inflammatory cytokines (63) Not specified (64)
Rejuvenation	miR-17, 34a via Akt signaling (65) Mitochondria (66)
Thrombus resolution and recanalization	miR-126 targeting portocadherin 7 (32)

miR, microRNA; RABEPK, Rab9 effector protein with kelch motifs; RhoA, Ras homolog gene family member A; TRPM7, transient recent potential melastatin 7; NF-κB, nuclear factor-κB; PDGF, platelet-derived growth factor.

capacity, from those obtained from elderly stroke patients with chronic illness (75).

However, conflicting results exist. Following serum contact, allogeneic MSCs can be injured by complement, and the viability of allogeneic MSCs after infusion is greatly reduced, compared with autologous MSCs (76). High mortality following intravenous transplantation of MSCs in animal stroke models, and reports of pulmonary embolism following intravenous injection of allogeneic adipose-derived MSCs have been accounted (77). MSC-related procoagulation status could be a possible explanation for such lethal pulmonary thromboembolism (78). Lastly, cell diameters of MSCs are large, ranging from 15 to 30 μm, which leads to passive arrest of MSCs in small diameter vessels, causing vascular occlusion and reduction in cerebral blood flow, when administered through intra-arterial routes, and also trapping in systemic vessels such as the lungs, when administered systemically (the first pass effect) (79–81).

The cell-free paradigm, using allogeneic MSC-derived EVs could avoid such cell-related problems of allogeneic stem cell therapy. EVs have low toxicity, high stability in the circulation, advantages in scalable production and storage, and high transport efficiency to donor cells (passing the blood-brain barrier [BBB] and avoiding the first pass effect).

APPLICATIONS OF STEM CELL-EXTRACELLULAR VESICLES FOR TREATING STROKE

Preclinical Evidence of the Effects of EVs Derived From Various Stem Cells in Stroke

Xin et al. reported for the first time, that intravenous application of MSC-derived EVs in a stroke rat model improved neurological outcomes and increased angiogenesis and neurogenesis (47). Other investigators have also demonstrated that stem cell-derived EVs can be used for stroke therapy, as an alternative approach to stem cell infusion methods (**Table 2**) (67, 87, 88). In addition, several advances in EV-based strategy were introduced, including: (a) the use of stem cells other than MSCs, such as EVs from embryonic stem cells (ESCs), neural stem cells (NSCs), or induced pluripotent stem cells (iPSC)-derived MSC/NSCs (64, 84, 86), (b) application of EVs via the intra-nasal approach (84), (c) EV production other than conventional two-dimensional (2D) culture methods to increase the production of EVs and regulate the contents of EVs, e.g., 3D dynamic culture (37) and stimulation with ischemic brain extracts (20, 59), and (d) various EV isolation methods other than ultracentrifugation (82, 83, 85). Very recently, the effects of EVs on stroke has been tested in a large animal model of stroke (86).

Recent Advances for EV Therapeutics

Various approaches are currently being employed to drive the MSC secretome toward a more anti-inflammatory and regenerative phenotype (88). Because secretomes include a wide array of growth factors, cytokines, and EVs, such approaches could also improve the efficacy of EV-based therapy.

Firstly, conventional 2D cell culture systems often disregard the mechanical stimuli that significantly influence the intricate *in vivo* cellular microenvironment. Characteristics of EVs as well as phenotypes of stem cells could be affected by mechanical forces (89). For example, shear stress enhances the immune regulatory function of MSCs (90). In addition, compared to conventional 2D cultured MSCs, MSCs cultured in spheroid showed higher efficacy and safety profiles, and decreased the expression of integrins, resulting in increased secretion of EVs (91, 92). Cha et al. successfully amplified EV sections and therapeutic EV contents (microRNAs and cytokines) from MSCs using a dynamic 3D culture method,

TABLE 2 | Various applications of stem cell-derived EV in stroke.

References	Animals	Stem cells /mode of application	EV production /culture media	EV isolation /dose per animal	Major finding
Xin et al. (47)	Rat	Rat BM MSCs /intravenous	2D culture /Exosome-free serum	UC /100 μg total exosome protein	Angiogenesis Neurogenesis Neurological recovery
Doeppner et al. (82)	Mice	Human BM MSCs /intravenous	2D culture /MSC basal media	PEG /EVs released by 2×10^6 cells	Neuroprotection Angiogenesis Neurogenesis Immunomodulation Neurological recovery
Chen et al. (83)	Rat	Mini-pig adipose MSCs /intravenous	2D culture /10% fetal bovine serum	KISO^{TM} system /100 μg total exosome protein	Reduction of infarct volume Neurological recovery
Lee et al. (59)	Rat	Human adipose MSCs /intravenous	2D culture /Serum free media with brain extract	UC /0.2 mg /kg	Angiogenesis Neurogenesis Immunomodulation
Kalani et al. (84)	Mice	Mice ESCs /intra-nasal	2D culture on fibroblast monolayer /Exosome-free serum	UC /NA	Restoration of neurovascular unit Immunomodulation
Otero-Ortega et al. (85)	Rat	Rat adipose MSCs /intravenous	2D culture /Exosome-free serum	Exosome extraction kit (miRCURY) /100 μg total exosome protein	Neuroplasticity White matter recovery Neurological recovery
Xin et al. (30)	Rat	Rat BM MSCs /intravenous	2D culture /Exosome-free serum	UC /100 μg total exosome protein	Neuroplasticity Neurological recovery
Xin et al. (33)	Rat	microRNA-133b overexpressing Rat BM MSCs /intra-arterial	2D culture /Exosome-free serum	UC /3×10^{11} EVs, comparable to 100 μg total exosome protein	Neuroplasticity Neurological recovery
Moon et al. (20)	Rat	Rat BM MSCs /intravenous	2D culture /Serum free media with brain extract	UC /30 μg total exosome protein	Angiogenesis Neurogenesis Neuroplasticity Neurological recovery
Cha et al. (37)	*In vitro*	Human BM MSCs	3D dynamic culture /Serum free media	UC /NA	Angiogenesis Neurogenesis Neurological recovery
Webb et al. (86)	Pig	Human NSCs /intravenous	2D culture /NSC basal culture media	UC /2×10^{10} EVs /kg	Improve neural tissue preservation Neurological recovery
Webb et al. (64)	Mice	iPSC-derived NSC or MSCs /intravenous	NA	NA	Neuroprotection Immunomodulation Neurological recovery

MSC, mesenchymal stem cells; BM, bone marrow; UC, ultracentrifugation; PEG, polyethylene glycol precipitation method; ESCs, embryonic stem cells; iPSC, induced pluripotent stem cell; NSCs, neural stem cells; NA, not available.

instead of using the conventional culture method (37). In a traumatic brain injury model, EVs derived from MSCs cultured in 3D scaffolds provided better outcomes than EVs from MSCs cultured in 2D conditions, probably by promoting neurogenesis and angiogenesis (93). Either native (decellularizing tissues) or synthetic 3D extracellular matrix-based scaffolds can be utilized to provide a 3D environment for cell attachment and growth (23).

Second, although MSC-derived EVs show promise in their application for regenerative therapies, their use is often limited by very low-yield conventional cell culture systems. Both microcarriers and hollow-fiber bioreactors are currently used for large-scale cell expansion of MSCs in the 3D environment (23) (89). These methods may be particularly useful in MSC EV production, because (a) large volumes of media would be required to get a sizable number of EVs for clinical use, (b) viability of MSCs could be maintained by continuous medium perfusion and avoiding metabolic by-product accumulation in a bioreactor, without the use of serum, which contains a large number of xenogeneic EVs, and (c) continuous processing, by controlling culture medium flow in and out of a bioreactor, as is often required because of the high advantages of reproducibility and safety of the resulting EV products.

Third, preconditioning of sublethal stimuli can trigger an adaptive response to further injury or damage. A wide variety of molecules and culture methods can be used to prime MSCs and modify their EVs. For example, Moon et al. showed that cultivation of MSCs with either serum obtained from stroke patients, or treatment of ischemic brain extracts on culture media, could activate restorative properties of MSCs and the release of EVs, suggesting that signals from an ischemic brain can affect the efficacy of MSCs and MSC-derived EVs and activate the secretion of EVs from MSCs (20, 94). Similar findings were also

reported by another research group (59). It is widely accepted that hypoxic conditions (i.e., 0.1–2% O_2, conditions similar to BM) were beneficial to MSCs and might stimulate MSCs to exhibit adaptive responses. MSC culture in hypoxic conditions with/without serum deprivation amplified EV sections, increased therapeutic EV contents (e.g., microRNAs), and improved the EV efficacy in tissue-injury models (48, 49, 56, 95). Inflammatory stimulation of MSCs renders release of EVs that have enhanced anti-inflammatory properties (96).

Fourth, as mentioned before, there have been advances in our current knowledge on the regulation of EV biogenesis (**Figure 1**). The modification of certain molecular pathways in EV biogenesis could lead to increased yield of EV production (23). For example, activation of EV biogenesis during membrane blebbing (P2X7 receptor, phospholipase D2) or multivesicular body fusion with the plasma membrane (Rab GTPase, SNARES) could increase EV secretion, leading to an increased yield (23, 25, 97–100). In addition, genetic modification to overexpress certain therapeutic proteins or RNAs within EVs (**Table 2**) could lead to an increased efficacy of EVs. For example, EVs harvested from microRNA-133b-overexpressing MSCs improved neuronal plasticity and functional recovery following stroke (33). Furthermore, bioengineering techniques can be applied to produce semi-synthetic artificial EVs to increase the expression of functional/traceable molecules on EV surfaces/membranes or cargo, and fully synthetic artificial EVs can be engineered to increase the yield of EV production (101). For example, "exosome-like nanovesicles," which have morphological and biochemical characteristics similar to EVs, can be made from cells through cell membrane fragmentation (102).

Lastly, the source of EVs could be an important determinant in the efficacy of stem cell-derived EVs in stroke. MSCs have limited restorative potential in elderly patients. Similarly, MSC EVs may have significant age-dependent differences in their cargo contents (103). The transfer of EVs from young MSCs rejuvenated aged stem cells (65). Fetal MSCs from amniotic fluid, cord blood, or Wharton's Jelly-derived stem cells are reported to have intermediate cellular phenotypes between ESCs/iPSC and MSC, in terms of expression patterns of both marker/transcription factors of pluripotency and mesenchymal commitment, as well as their broadly multipotent nature (104). Although the use of ESC/iPSC-derived EV therapy may be safer than the use of ESC/iPSC cell therapy, in terms of tumorigenicity, limited data is available within the field of stroke and in human trials (64, 84). Therefore, fetal MSCs could be good sources of EVs in clinical application.

CLINIAL APPLICATIONS OF EXTRACELLULAR VESICLE-BASED THERAPY

The effects of EV therapeutics have increasingly been reported in various animal disease/injury models (87). However, only a few clinical studies on the effects of EV therapy have been reported in humans. Kordelas et al. reported a case study, whereby refractory graft-versus-host disease was treated with allogeneic MSC EVs (105). In this report, allogeneic MSCs were cultured in MSC conditioned media and EVs were isolated by the polyethylene glycol (PEG) precipitation method. EVs obtained from 4×10^7 MSCs were administered repetitively four times. Clinical symptoms were improved, and no adverse effects were observed. Katagiri et al. applied allogeneic MSC EVs via local injection for alveolar bone regeneration in eight patients who were diagnosed as needing bone augmentation prior to dental implant placement, which revealed this method was safe and may have great osteogenic potential (106). Lastly, Zhang et al. applied MSC EVs via intravitreal injection in five patients with refractory macular holes (107). All three clinical studies are small case series, and although this data suggests that MSC EVs are safe and may improve patient outcomes, randomization trials are needed to investigate the efficacy and safety of MSC EV therapy. No studies have examined the effects of stem cell-derived EVs in stroke patients. Several phase I/II clinical trials are ongoing to evaluate the application of EVs in cancer patients (108–110).

Considering MSC EVs are the therapeutically active component of MSCs, are non-self-replicating and small sized, the regulatory items required to produce EV fractions for clinical treatment strategies could be less complicated than for MSC therapies. However, compared to MSC therapy, clinical evaluation of EV therapeutics is still at an early stage. Several issues must be considered and need to be solved before the clinical application of EVs, including specific guidelines targeting EV-based therapeutics, characterization, isolation, and storage of EVs, quality control requirements, and *in vivo* analyses of EV. These issues were discussed precisely elsewhere (87, 111, 112), yet the following issues deserve mention in the application of EV for stroke patients.

First, the optimal time and mode of application of EVs should be studied in stroke patients. Most recovery occurs in the first few months following a stroke, with only minor additional measureable improvements occurring thereafter. The levels of chemokines, trophic factors, and related miRNAs increase markedly in the infarcted brain during the acute phase of stroke but decrease over time. Such changes in the brain microenvironment may greatly affect the biodistribution of EVs, as well as the degree of recovery and neurogenesis/angiogenesis after EV therapeutics in stroke patients.

Second, since EVs have many therapeutic components and multiple modes of action, markers for potency and quality control should be chosen carefully and should be measured during the freezing/thawing procedures and storage period. EV therapeutics for stroke patients may differ depending on the time (acute vs. chronic phase) of application. For example, EV cargo components targeting neuroprotection and immunomodulation are needed in patients with acute ischemic stroke, while EV components targeting neurogenesis and angiogenesis are required for neurorestoration in both acute and chronic stroke patients. Differential markers for the potency of EVs (*in vitro* bioassays) may be needed for patients with acute and chronic ischemic stroke. In addition, customized stem cell-EV properties for stroke treatment are needed. Given the heterogeneity of EVs in terms of cargo proteins and RNAs, further studies are needed to increase the

therapeutic components of EVs for stroke patients in clinically feasible ways (33, 37, 56, 96, 113).

Lastly, the BBB is formed by the brain capillary endothelium and excludes ∼100% of large-molecule neurotherapeutics from the brain and more than 98% of all small-molecule drugs (114). As a result, compared with local application of EVs for topical diseases or other systemic illnesses, stroke patients often require large amounts of stem cells and stem cell-derived EVs. Therefore, selection of culture media and isolation methods are particularly important in EV therapeutics for stroke. Many different cell culture media have been used in the production of EVs, including serum-supplemented media, serum-free media, and EV-free/reduced serum-supplemented media. Because a prior elimination of EVs from fetal bovine serum is crucial, and commercial exosome/EV-depleted serum is expensive and may be imperfect, various methods to deplete EVs are being investigated, such as through the ultrafiltration method (115). In addition, various techniques have currently been used for EV isolation that include (but are not limited to) ultracentrifugation, PEG precipitation, size exclusion chromatography, and tangential-flow filtration. However, each method has advantages and disadvantages, and there is no reliable method for isolation techniques for EVs (112). Recently, GMP-compatible methods for clinical scale production, purification, and isolation of EVs have been introduced (116). Another important issue in improving the therapeutic effects of EV-based therapy in stroke is BBB manipulation, which may enhance endogenous repair mechanisms following stroke, by allowing entry of paracrine factors (e.g., trophic factors and EVs) more easily to the brain (117).

CONCLUSION AND FUTURE PERSPECTIVES

Cell therapy using EVs derived from stem cells could represent a new, clinically feasible, and cell-free paradigm that would avoid cell-related problems. Development of scientific research has just begun in this stem cell-derived EV strategy when compared to that of stem cell therapy. However, MSC-derived EV is rapidly expanding and could be a promising approach for patients with severe stroke, as MSC therapies have already been tested in preclinical and clinical trials and EV-mediated therapy has unique advantages over MSC therapies in stroke patients, in terms of biodistribution (cross the BBB and avoid the first pass effect) and off-the-shelf approaches for acute ischemic stroke.

There have been significant advances in the application of stem cell-derived EVs for human diseases and our understanding of the function and biogenesis of EVs. The efficacy of stem cell-derived EV therapeutics will be improved with advances in our understanding of the biology of stem cells and their EVs, together with advances in techniques to modulate stem cell-derived EV characteristics, including biotechnology and bioengineering. Future studies should focus on our need for more well-designed preclinical studies of EV therapeutics in animal models of stroke. Further studies should particularly focus on biodistribution studies, optimal time/dose/mode of application, and functional outcome measures with neuroimaging data. In addition, the optimal cargo of EVs for EV therapies for stroke patients is unsettled. Moreover, quality management of EVs and establishing standard operating procedures for EV therapeutics are needed, as randomized trials of EV for stroke patients are warranted.

AUTHOR CONTRIBUTIONS

OB and EK: study concept and design, acquisition of data, analysis and interpretation of data, drafting/revising the manuscript for content.

FUNDING

This research was supported by a grant from the Korean Healthcare Technology R&D Project, Ministry of Health and Welfare (HI17C1256), and the Bio and Medical Technology Development Program of the National Research Foundation (NRF) funded by the Korean government (MSIT) (2018M3A9H1023675).

REFERENCES

1. Chen X, Li Y, Wang L, Katakowski M, Zhang L, Chen J, et al. Ischemic rat brain extracts induce human marrow stromal cell growth factor production. *Neuropathology.* (2002) 22:275–9. doi: 10.1046/j.1440-1789.2002.00450.x
2. Liu Z, Li Y, Zhang RL, Cui Y, Chopp M. Bone marrow stromal cells promote skilled motor recovery and enhance contralesional axonal connections after ischemic stroke in adult mice. *Stroke.* (2011) 42:740–4. doi: 10.1161/STROKEAHA.110.607226
3. Song M, Mohamad O, Gu X, Wei L, Yu SP. Restoration of intracortical and thalamocortical circuits after transplantation of bone marrow mesenchymal stem cells into the ischemic brain of mice. *Cell Transplant.* (2013) 22:2001–15. doi: 10.3727/096368912X657909
4. Lai RC, Chen TS, Lim SK. Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. *Regen Med.* (2011) 6:481–92. doi: 10.2217/rme.11.35
5. Bang OY, Kim EH, Cha JM, Moon GJ. Adult stem cell therapy for stroke: challenges and progress. *J Stroke.* (2016) 18:256–66. doi: 10.5853/jos.2016.01263
6. Acosta SA, Tajiri N, Hoover J, Kaneko Y, Borlongan CV. Intravenous bone marrow stem cell grafts preferentially migrate to spleen and abrogate chronic inflammation in stroke. *Stroke.* (2015) 46:2616–27. doi: 10.1161/STROKEAHA.115.009854
7. Shen LH, Li Y, Chen J, Zacharek A, Gao Q, Kapke A, et al. Therapeutic benefit of bone marrow stromal cells administered 1 month after stroke. *J Cereb Blood Flow Metab.* (2007) 27:6–13. doi: 10.1038/sj.jcbfm.9600311
8. Shin JY, Park HJ, Kim HN, Oh SH, Bae JS, Ha HJ, et al. Mesenchymal stem cells enhance autophagy and increase beta-amyloid clearance in Alzheimer disease models. *Autophagy.* (2014) 10:32–44. doi: 10.4161/auto.26508
9. Yamauchi T, Kuroda Y, Morita T, Shichinohe H, Houkin K, Dezawa M, et al. Therapeutic effects of human multilineage-differentiating stress enduring (MUSE) cell transplantation into infarct brain of mice. *PLoS ONE.* (2015) 10:e0116009. doi: 10.1371/journal.pone.0116009

10. Kondziolka D, Steinberg GK, Wechsler L, Meltzer CC, Elder E, Gebel J, et al. Neurotransplantation for patients with subcortical motor stroke: a phase 2 randomized trial. *J Neurosurg.* (2005) 103:38–45. doi: 10.3171/jns.2005.103.1.0038
11. Lee JS, Hong JM, Moon GJ, Lee PH, Ahn YH, Bang OY, et al. A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke. *Stem Cells.* (2010) 28:1099–106. doi: 10.1002/stem.430
12. Kim SJ, Moon GJ, Chang WH, Kim YH, Bang OY, STARTING-2 (STem cell Application Researches and Trials In NeuroloGy-2) collaborators. Intravenous transplantation of mesenchymal stem cells preconditioned with early phase stroke serum: current evidence and study protocol for a randomized trial. *Trials.* (2013) 14:317. doi: 10.1186/1745-6215-14-317
13. Prasad K, Sharma A, Garg A, Mohanty S, Bhatnagar S, Johri S, et al. Intravenous autologous bone marrow mononuclear stem cell therapy for ischemic stroke: a multicentric, randomized trial. *Stroke.* (2014) 45:3618–24. doi: 10.1161/STROKEAHA.114.007028
14. Chen DC, Lin SZ, Fan JR, Lin CH, Lee W, Lin CC, et al. Intracerebral implantation of autologous peripheral blood stem cells in stroke patients: a randomized phase II study. *Cell Transplant.* (2014) 23:1599–612. doi: 10.3727/096368914X678562
15. Ghali AA, Yousef MK, Ragab OA, ElZamarany EA. Intra-arterial infusion of autologous bone marrow mononuclear stem cells in subacute ischemic stroke patients. *Front Neurol.* (2016) 7:228. doi: 10.3389/fneur.2016.00228
16. Hess DC, Wechsler LR, Clark WM, Savitz SI, Ford GA, Chiu D, et al. Safety and efficacy of multipotent adult progenitor cells in acute ischaemic stroke (MASTERS): a randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet Neurol.* (2017) 16:360–8. doi: 10.1016/S1474-4422(17)30046-7
17. Fang J, Guo Y, Tan S, Li Z, Xie H, Chen P, et al. Autologous endothelial progenitor cells transplantation for acute ischemic stroke: a 4-year follow-up study. *Stem Cells Transl Med.* (2018) 8:14–21. doi: 10.1002/sctm.18-0012
18. Bang OY. Stem cell therapy for stroke: lessons learned from recent successful randomized trials of interventional therapy for stroke. *Precis Future Med.* (2018) 2:109–16. doi: 10.23838/pfm.2018.00058
19. Rosenblum S, Wang N, Smith TN, Pendharkar AV, Chua JY, Birk H, et al. Timing of intra-arterial neural stem cell transplantation after hypoxia-ischemia influences cell engraftment, survival, and differentiation. *Stroke.* (2012) 43:1624–31. doi: 10.1161/STROKEAHA.111.637884
20. Moon GJ, Sung JH, Kim DH, Kim EH, Cho YH, Son JP, et al. Application of mesenchymal stem cell-derived extracellular vesicles for stroke: biodistribution and microRNA study. *Transl Stroke Res.* (2018) 1–13. doi: 10.1007/s12975-018-0668-1. [Epub ahead of print].
21. Basso M, Bonetto V. Extracellular vesicles and a novel form of communication in the brain. *Front Neurosci.* (2016) 10:127. doi: 10.3389/fnins.2016.00127
22. Burrello J, Monticone S, Gai C, Gomez Y, Kholia S, Camussi G. Stem cell-derived extracellular vesicles and immune-modulation. *Front Cell Dev Biol.* (2016) 4:83. doi: 10.3389/fcell.2016.00083
23. Phan J, Kumar P, Hao D, Gao K, Farmer D, Wang A. Engineering mesenchymal stem cells to improve their exosome efficacy and yield for cell-free therapy. *J Extracell Vesicles.* (2018) 7:1522236. doi: 10.1080/20013078.2018.1522236
24. Todorova D, Simoncini S, Lacroix R, Sabatier F, Dignat-George F. Extracellular vesicles in angiogenesis. *Circ Res.* (2017) 120:1658–73. doi: 10.1161/CIRCRESAHA.117.309681
25. Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol.* (2014) 30:255–89. doi: 10.1146/annurev-cellbio-101512-122326
26. Ghossoub R, Lembo F, Rubio A, Gaillard CB, Bouchet J, Vitale N, et al. Syntenin-ALIX exosome biogenesis and budding into multivesicular bodies are controlled by ARF6 and PLD2. *Nat Commun.* (2014) 5:3477. doi: 10.1038/ncomms4477
27. Muralidharan-Chari V, Clancy J, Plou C, Romao M, Chavrier P, Raposo G, et al. ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles. *Curr Biol.* (2009) 19:1875–85. doi: 10.1016/j.cub.2009.09.059
28. Kim SJ, Moon GJ, Cho YH, Kang HY, Hyung NK, Kim D, et al. Circulating mesenchymal stem cells microparticles in patients with cerebrovascular disease. *PLoS ONE.* (2012) 7:e37036. doi: 10.1371/journal.pone.0037036
29. Leroyer AS, Ebrahimian TG, Cochain C, Recalde A, Blanc-Brude O, Mees B, et al. Microparticles from ischemic muscle promotes postnatal vasculogenesis. *Circulation.* (2009) 119:2808–17. doi: 10.1161/CIRCULATIONAHA.108.816710
30. Xin H, Katakowski M, Wang F, Qian J, Lin XS, Ali MM, et al. MicroRNA-17–92 cluster in exosomes enhance neuroplasticity and functional recovery after stroke in rats. *Stroke.* (2017) 48:747–53. doi: 10.1161/STROKEAHA.116.015204
31. Yang J, Zhang X, Chen X, Wang L, Yang G. Exosome mediated delivery of miR-124 promotes neurogenesis after ischemia. *Mol Ther Nucleic Acids.* (2017) 7:278–87. doi: 10.1016/j.omtn.2017.04.010
32. Sun J, Zhang Z, Ma T, Yang Z, Zhang J, Liu X, et al. Endothelial progenitor cell-derived exosomes, loaded with miR-126, promoted deep vein thrombosis resolution and recanalization. *Stem Cell Res Ther.* (2018) 9:223. doi: 10.1186/s13287-018-0952-8
33. Xin H, Wang F, Li Y, Lu QE, Cheung WL, Zhang Y, et al. Secondary release of exosomes from astrocytes contributes to the increase in neural plasticity and improvement of functional recovery after stroke in rats treated with exosomes harvested from microRNA 133b-overexpressing multipotent mesenchymal stromal cells. *Cell Transplant.* (2017) 26:243–57. doi: 10.3727/096368916X693031
34. Holtje M, Djalali S, Hofmann F, Munster-Wandowski A, Hendrix S, Boato F, et al. A 29-amino acid fragment of clostridium botulinum C3 protein enhances neuronal outgrowth, connectivity, and reinnervation. *FASEB J.* (2009) 23:1115–26. doi: 10.1096/fj.08-116855
35. Xiao Y, Geng F, Wang G, Li X, Zhu J, Zhu W. Bone marrow-derived mesenchymal stem cells-derived exosomes prevent oligodendrocyte apoptosis through exosomal miR-134 by targeting caspase-8. *J Cell Biochem.* (2018) doi: 10.1002/jcb.27519. [Epub ahead of print].
36. Yang Y, Cai Y, Zhang Y, Liu J, Xu Z. Exosomes secreted by adipose-derived stem cells contribute to angiogenesis of brain microvascular endothelial cells following oxygen-glucose deprivation *in vitro* through microRNA-181b/TRPM7 axis. *J Mol Neurosci.* (2018) 65:74–83. doi: 10.1007/s12031-018-1071-9
37. Cha JM, Shin EK, Sung JH, Moon GJ, Kim EH, Cho YH, et al. Efficient scalable production of therapeutic microvesicles derived from human mesenchymal stem cells. *Sci Rep.* (2018) 8:1171. doi: 10.1038/s41598-018-19211-6
38. Liu C, Teng ZQ, Santistevan NJ, Szulwach KE, Guo W, Jin P, et al. Epigenetic regulation of miR-184 by MBD1 governs neural stem cell proliferation and differentiation. *Cell Stem Cell.* (2010) 6:433–44. doi: 10.1016/j.stem.2010.02.017
39. Wang N, Chen C, Yang D, Liao Q, Luo H, Wang X, et al. Mesenchymal stem cells-derived extracellular vesicles, via miR-210, improve infarcted cardiac function by promotion of angiogenesis. *Biochim Biophys Acta Mol Basis Dis.* (2017) 1863:2085–92. doi: 10.1016/j.bbadis.2017.02.023
40. Khan M, Nickoloff E, Abramova T, Johnson J, Verma SK, Krishnamurthy P, et al. Embryonic stem cell-derived exosomes promote endogenous repair mechanisms and enhance cardiac function following myocardial infarction. *Circ Res.* (2015) 117:52–64. doi: 10.1161/CIRCRESAHA.117.305990
41. Hu S, Park J, Liu A, Lee J, Zhang X, Hao Q, et al. Mesenchymal stem cell microvesicles restore protein permeability across primary cultures of injured human lung microvascular endothelial cells. *Stem Cells Transl Med.* (2018) 7:615–24. doi: 10.1002/sctm.17-0278
42. Kang K, Ma R, Cai W, Huang W, Paul C, Liang J, et al. Exosomes secreted from CXCR4 overexpressing mesenchymal stem cells promote cardioprotection via Akt signaling pathway following myocardial infarction. *Stem Cells Int.* (2015) 2015:659890. doi: 10.1155/2015/659890
43. Zou X, Gu D, Xing X, Cheng Z, Gong D, Zhang G, et al. Human mesenchymal stromal cell-derived extracellular vesicles alleviate renal ischemic reperfusion injury and enhance angiogenesis in rats. *Am J Transl Res.* (2016) 8:4289–99.

44. Ma J, Zhao Y, Sun L, Sun X, Zhao X, Sun X, et al. Exosomes derived from Akt-modified human umbilical cord mesenchymal stem cells improve cardiac regeneration and promote angiogenesis via activating platelet-derived growth factor D. *Stem Cells Transl Med.* (2017) 6:51–9. doi: 10.5966/sctm.2016-0038
45. Anderson JD, Johansson HJ, Graham CS, Vesterlund M, Pham MT, Bramlett CS, et al. Comprehensive proteomic analysis of mesenchymal stem cell exosomes reveals modulation of angiogenesis via nuclear factor-kappaB signaling. *Stem Cells.* (2016) 34:601–13. doi: 10.1002/stem.2298
46. Shabbir A, Cox A, Rodriguez-Menocal L, Salgado M, Van Badiavas E. Mesenchymal stem cell exosomes induce proliferation and migration of normal and chronic wound fibroblasts, and enhance angiogenesis *in vitro*. *Stem Cells Dev.* (2015) 24:1635–47. doi: 10.1089/scd.2014.0316
47. Xin H, Li Y, Cui Y, Yang JJ, Zhang ZG, Chopp M. Systemic administration of exosomes released from mesenchymal stromal cells promote functional recovery and neurovascular plasticity after stroke in rats. *J Cereb Blood Flow Metab.* (2013) 33:1711–5. doi: 10.1038/jcbfm.2013.152
48. Zhang HC, Liu XB, Huang S, Bi XY, Wang HX, Xie LX, et al. Microvesicles derived from human umbilical cord mesenchymal stem cells stimulated by hypoxia promote angiogenesis both *in vitro* and *in vivo*. *Stem Cells Dev.* (2012) 21:3289–97. doi: 10.1089/scd.2012.0095
49. Bian S, Zhang L, Duan L, Wang X, Min Y, Yu H. Extracellular vesicles derived from human bone marrow mesenchymal stem cells promote angiogenesis in a rat myocardial infarction model. *J Mol Med (Berl).* (2014) 92:387–97. doi: 10.1007/s00109-013-1110-5
50. Yu B, Kim HW, Gong M, Wang J, Millard RW, Wang Y, et al. Exosomes secreted from GATA-4 overexpressing mesenchymal stem cells serve as a reservoir of anti-apoptotic microRNAs for cardioprotection. *Int J Cardiol.* (2015) 182:349–60. doi: 10.1016/j.ijcard.2014.12.043
51. Yao X, Wang Y, Zhang D. microRNA-21 confers neuroprotection against cerebral ischemia-reperfusion injury and alleviates blood-brain barrier disruption in rats via the MAPK signaling pathway. *J Mol Neurosci.* (2018) 65:43–53. doi: 10.1007/s12031-018-1067-5
52. Feng Y, Huang W, Wani M, Yu X, Ashraf M. Ischemic preconditioning potentiates the protective effect of stem cells through secretion of exosomes by targeting Mecp2 via miR-22. *PLoS ONE.* (2014) 9:e88685. doi: 10.1371/journal.pone.0088685
53. Xie YL, Zhang B, Jing L. MiR-125b blocks bax/cytochrome C/caspase-3 apoptotic signaling pathway in rat models of cerebral ischemia-reperfusion injury by targeting p53. *Neurol Res.* (2018) 40:828–37. doi: 10.1080/01616412.2018.1488654
54. Zheng L, Cheng W, Wang X, Yang Z, Zhou X, Pan C. Overexpression of microRNA-145 ameliorates astrocyte injury by targeting aquaporin 4 in cerebral ischemic stroke. *Biomed Res Int.* (2017) 2017:9530951. doi: 10.1155/2017/9530951
55. Xu WH, Yao XY, Yu HJ, Huang JW, Cui LY. Downregulation of miR-199a may play a role in 3-nitropropionic acid induced ischemic tolerance in rat brain. *Brain Res.* (2012) 1429:116–23. doi: 10.1016/j.brainres.2011.10.007
56. Wang Y, Zhao R, Liu D, Deng W, Xu G, Liu W, et al. Exosomes derived from miR-214-enriched bone marrow-derived mesenchymal stem cells regulate oxidative damage in cardiac stem cells by targeting CaMKII. *Oxid Med Cell Longev.* (2018) 2018:4971261. doi: 10.1155/2018/4971261
57. Wang X, Zhang X, Ren XP, Chen J, Liu H, Yang J, et al. MicroRNA-494 targeting both proapoptotic and antiapoptotic proteins protects against ischemia/reperfusion-induced cardiac injury. *Circulation.* (2010) 122:1308–18. doi: 10.1161/CIRCULATIONAHA.110.964684
58. Zhao N, Mi L, Zhang X, Xu M, Yu H, Liu Z, et al. Enhanced MiR-711 transcription by PPARgamma induces endoplasmic reticulum stress-mediated apoptosis targeting calnexin in rat cardiomyocytes after myocardial infarction. *J Mol Cell Cardiol.* (2018) 118:36–45. doi: 10.1016/j.yjmcc.2018.03.006
59. Lee JY, Kim E, Choi SM, Kim DW, Kim KP, Lee I, et al. Microvesicles from brain-extract-treated mesenchymal stem cells improve neurological functions in a rat model of ischemic stroke. *Sci Rep.* (2016) 6:33038. doi: 10.1038/srep33038
60. Scheibe F, Klein O, Klose J, Priller J. Mesenchymal stromal cells rescue cortical neurons from apoptotic cell death in an *in vitro* model of cerebral ischemia. *Cell Mol Neurobiol.* (2012) 32:567–76. doi: 10.1007/s10571-012-9798-2
61. Xu LJ, Ouyang YB, Xiong X, Stary CM, Giffard RG. Post-stroke treatment with miR-181 antagomir reduces injury and improves long-term behavioral recovery in mice after focal cerebral ischemia. *Exp Neurol.* (2015) 264:1–7. doi: 10.1016/j.expneurol.2014.11.007
62. Amarnath S, Foley JE, Farthing DE, Gress RE, Laurence A, Eckhaus MA, et al. Bone marrow-derived mesenchymal stromal cells harness purinergenic signaling to tolerize human Th1 cells *in vivo*. *Stem Cells.* (2015) 33:1200–12. doi: 10.1002/stem.1934
63. Mokarizadeh A, Delirezh N, Morshedi A, Mosayebi G, Farshid AA, Mardani K. Microvesicles derived from mesenchymal stem cells: potent organelles for induction of tolerogenic signaling. *Immunol Lett.* (2012) 147:47–54. doi: 10.1016/j.imlet.2012.06.001
64. Webb RL, Kaiser EE, Scoville SL, Thompson TA, Fatima S, Pandya C, et al. Human neural stem cell extracellular vesicles improve tissue and functional recovery in the murine thromboembolic stroke model. *Transl Stroke Res.* (2018) 9:530–9. doi: 10.1007/s12975-017-0599-2
65. Kulkarni R, Bajaj M, Ghode S, Jalnapurkar S, Limaye L, Kale VP. Intercellular transfer of microvesicles from young mesenchymal stromal cells rejuvenates aged murine hematopoietic stem cells. *Stem Cells.* (2018) 36:420–33. doi: 10.1002/stem.2756
66. Wang J, Li H, Yao Y, Zhao T, Chen YY, Shen YL, et al. Stem cell-derived mitochondria transplantation: a novel strategy and the challenges for the treatment of tissue injury. *Stem Cell Res Ther.* (2018) 9:106. doi: 10.1186/s13287-018-0832-2
67. Doeppner TR, Bahr M, Giebel B, Hermann DM. Immunological and non-immunological effects of stem cell-derived extracellular vesicles on the ischaemic brain. *Ther Adv Neurol Disord.* (2018) 11:1756286418789326. doi: 10.1177/1756286418789326
68. Laso-Garcia F, Ramos-Cejudo J, Carrillo-Salinas FJ, Otero-Ortega L, Feliu A, Gomez-de Frutos M, et al. Therapeutic potential of extracellular vesicles derived from human mesenchymal stem cells in a model of progressive multiple sclerosis. *PLoS ONE.* (2018) 13:e0202590. doi: 10.1371/journal.pone.0202590
69. Lai RC, Tan SS, Teh BJ, Sze SK, Arslan F, de Kleijn DP, et al. Proteolytic potential of the MSC exosome proteome: implications for an exosome-mediated delivery of therapeutic proteasome. *Int J Proteomics.* (2012) 2012:971907. doi: 10.1155/2012/971907
70. Katsuda T, Ochiya T. Molecular signatures of mesenchymal stem cell-derived extracellular vesicle-mediated tissue repair. *Stem Cell Res Ther.* (2015) 6:212. doi: 10.1186/s13287-015-0214-y
71. Yuan Y, Du W, Liu J, Ma W, Zhang L, Du Z, et al. Stem cell-derived exosome in cardiovascular diseases: macro roles of micro particles. *Front Pharmacol.* (2018) 9:547. doi: 10.3389/fphar.2018.00547
72. Ragni E, Banfi F, Barilani M, Cherubini A, Parazzi V, Larghi P, et al. Extracellular vesicle-shuttled mRNA in mesenchymal stem cell communication. *Stem Cells.* (2017) 35:1093–105. doi: 10.1002/stem.2557
73. Hayakawa K, Chan SJ, Mandeville ET, Park JH, Bruzzese M, Montaner J, et al. Protective effects of endothelial progenitor cell-derived extracellular mitochondria in brain endothelium. *Stem Cells.* (2018) 36:1404–10. doi: 10.1002/stem.2856
74. Savitz SI. Cell therapies: careful translation from animals to patients. *Stroke.* (2013) 44:S107–9. doi: 10.1161/STROKEAHA.112.679605
75. Efimenko AY, Kochegura TN, Akopyan ZA, Parfyonova YV. Autologous stem cell therapy: how aging and chronic diseases affect stem and progenitor cells. *Biores Open Access.* (2015) 4:26–38. doi: 10.1089/biores.2014.0042
76. Li Y, Lin F. Mesenchymal stem cells are injured by complement after their contact with serum. *Blood.* (2012) 120:3436–43. doi: 10.1182/blood-2012-03-420612
77. Jung JW, Kwon M, Choi JC, Shin JW, Park IW, Choi BW, et al. Familial occurrence of pulmonary embolism after intravenous, adipose tissue-derived stem cell therapy. *Yonsei Med J.* (2013) 54:1293–6. doi: 10.3349/ymj.2013.54.5.1293
78. Tatsumi K, Ohashi K, Matsubara Y, Kohori A, Ohno T, Kakidachi H, et al. Tissue factor triggers procoagulation in transplanted mesenchymal stem cells leading to thromboembolism. *Biochem Biophys Res Commun.* (2013) 431:203–9. doi: 10.1016/j.bbrc.2012.12.134

79. Krueger TEG, Thorek DLJ, Denmeade SR, Isaacs JT, Brennen WN. Concise review: mesenchymal stem cell-based drug delivery: the good, the bad, the ugly, and the promise. *Stem Cells Transl Med.* (2018) 7:651–63. doi: 10.1002/sctm.18-0024
80. Pendharkar AV, Chua JY, Andres RH, Wang N, Gaeta X, Wang H, et al. Biodistribution of neural stem cells after intravascular therapy for hypoxic-ischemia. *Stroke.* (2010) 41:2064–70. doi: 10.1161/STROKEAHA.109.575993
81. Nguyen PK, Neofytou E, Rhee JW, Wu JC. Potential strategies to address the major clinical barriers facing stem cell regenerative therapy for cardiovascular disease: a review. *JAMA Cardiol.* (2016) 1:953–62. doi: 10.1001/jamacardio.2016.2750
82. Doeppner TR, Herz J, Gorgens A, Schlechter J, Ludwig AK, Radtke S, et al. Extracellular vesicles improve post-stroke neuroregeneration and prevent postischemic immunosuppression. *Stem Cells Transl Med.* (2015) 4:1131–43. doi: 10.5966/sctm.2015-0078
83. Chen KH, Chen CH, Wallace CG, Yuen CM, Kao GS, Chen YL, et al. Intravenous administration of xenogenic adipose-derived mesenchymal stem cells (ADMSC) and ADMSC-derived exosomes markedly reduced brain infarct volume and preserved neurological function in rat after acute ischemic stroke. *Oncotarget.* (2016) 7:74537–56. doi: 10.18632/oncotarget.12902
84. Kalani A, Chaturvedi P, Kamat PK, Maldonado C, Bauer P, Joshua IG, et al. Curcumin-loaded embryonic stem cell exosomes restored neurovascular unit following ischemia-reperfusion injury. *Int J Biochem Cell Biol.* (2016) 79:360–9. doi: 10.1016/j.biocel.2016.09.002
85. Otero-Ortega L, Laso-Garcia F, Gomez-de Frutos MD, Rodriguez-Frutos B, Pascual-Guerra J, Fuentes B, et al. White matter repair after extracellular vesicles administration in an experimental animal model of subcortical stroke. *Sci Rep.* (2017) 7:44433. doi: 10.1038/srep44433
86. Webb RL, Kaiser EE, Jurgielewicz BJ, Spellicy S, Scoville SL, Thompson TA, et al. Human neural stem cell extracellular vesicles improve recovery in a porcine model of ischemic stroke. *Stroke.* (2018) 49:1248–56. doi: 10.1161/STROKEAHA.117.020353
87. Fuster-Matanzo A, Gessler F, Leonardi T, Iraci N, Pluchino S. Acellular approaches for regenerative medicine: on the verge of clinical trials with extracellular membrane vesicles? *Stem Cell Res Ther.* (2015) 6:227. doi: 10.1186/s13287-015-0232-9
88. Cunningham CJ, Redondo-Castro E, Allan SM. The therapeutic potential of the mesenchymal stem cell secretome in ischaemic stroke. *J Cereb Blood Flow Metab.* (2018) 38:1276–92. doi: 10.1177/0271678X18776802
89. Cha JM, Lee MY, Hong J. Bioreactor systems are essentially required for stem cell bioprocessing. *Precis Future Med.* (2018) doi: 10.23838/pfm.2018.00128. [Epub ahead of print].
90. Diaz MF, Vaidya AB, Evans SM, Lee HJ, Aertker BM, Alexander AJ, et al. Biomechanical forces promote immune regulatory function of bone marrow mesenchymal stromal cells. *Stem Cells.* (2017) 35:1259–72. doi: 10.1002/stem.2587
91. Xie L, Mao M, Zhou L, Jiang B. Spheroid mesenchymal stem cells and mesenchymal stem cell-derived microvesicles: two potential therapeutic strategies. *Stem Cells Dev.* (2016) 25:203–13. doi: 10.1089/scd.2015.0278
92. Mo M, Zhou Y, Li S, Wu Y. Three-dimensional culture reduces cell size by increasing vesicle excretion. *Stem Cells.* (2018) 36:286–92. doi: 10.1002/stem.2729
93. Zhang Y, Chopp M, Zhang ZG, Katakowski M, Xin H, Qu C, et al. Systemic administration of cell-free exosomes generated by human bone marrow derived mesenchymal stem cells cultured under 2D and 3D conditions improves functional recovery in rats after traumatic brain injury. *Neurochem Int.* (2017) 111:69–81. doi: 10.1016/j.neuint.2016.08.003
94. Moon GJ, Cho YH, Kim DH, Sung JH, Son JP, Kim S, et al. Serum-mediated activation of bone marrow-derived mesenchymal stem cells in ischemic stroke patients: a novel preconditioning method. *Cell Transplant.* (2018) 27:485–500. doi: 10.1177/0963689718755404
95. Park H, Park H, Mun D, Kang J, Kim H, Kim M, et al. Extracellular vesicles derived from hypoxic human mesenchymal stem cells attenuate gsk3beta expression via miRNA-26a in an ischemia-reperfusion injury model. *Yonsei Med J.* (2018) 59:736–45. doi: 10.3349/ymj.2018.59.6.736
96. Harting MT, Srivastava AK, Zhaorigetu S, Bair H, Prabhakara KS, Toledano Furman NE, et al. Inflammation-stimulated mesenchymal stromal cell-derived extracellular vesicles attenuate inflammation. *Stem Cells.* (2018) 36:79–90. doi: 10.1002/stem.2730
97. Qu Y, Dubyak GR. P2X7 receptors regulate multiple types of membrane trafficking responses and non-classical secretion pathways. *Purinergic Signal.* (2009) 5:163–73. doi: 10.1007/s11302-009-9132-8
98. Urbanelli L, Magini A, Buratta S, Brozzi A, Sagini K, Polchi A, et al. Signaling pathways in exosomes biogenesis, secretion and fate. *Genes (Basel).* (2013) 4:152–70. doi: 10.3390/genes4020152
99. Rao SK, Huynh C, Proux-Gillardeaux V, Galli T, Andrews NW. Identification of SNAREs involved in synaptotagmin VII-regulated lysosomal exocytosis. *J Biol Chem.* (2004) 279:20471–9. doi: 10.1074/jbc.M400798200
100. Hsu C, Morohashi Y, Yoshimura S, Manrique-Hoyos N, Jung S, Lauterbach MA, et al. Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-C. *J Cell Biol.* (2010) 189:223–32. doi: 10.1083/jcb.200911018
101. Garcia-Manrique P, Matos M, Gutierrez G, Pazos C, Blanco-Lopez MC. Therapeutic biomaterials based on extracellular vesicles: classification of bio-engineering and mimetic preparation routes. *J Extracell Vesicles.* (2018) 7:1422676. doi: 10.1080/20013078.2017.1422676
102. Yoon J, Jo W, Jeong D, Kim J, Jeong H, Park J. Generation of nanovesicles with sliced cellular membrane fragments for exogenous material delivery. *Biomaterials.* (2015) 59:12–20. doi: 10.1016/j.biomaterials.2015.04.028
103. Fafian-Labora J, Lesende-Rodriguez I, Fernandez-Pernas P, Sangiao-Alvarellos S, Monserrat L, Arntz OJ, et al. Effect of age on pro-inflammatory miRNAs contained in mesenchymal stem cell-derived extracellular vesicles. *Sci Rep.* (2017) 7:43923. doi: 10.1038/srep43923
104. Loukogeorgakis SP, De Coppi P. Concise review: amniotic fluid stem cells: the known, the unknown, and potential regenerative medicine applications. *Stem Cells.* (2017) 35:1663–73. doi: 10.1002/stem.2553
105. Kordelas L, Rebmann V, Ludwig AK, Radtke S, Ruesing J, Doeppner TR, et al. MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia.* (2014) 28:970–3. doi: 10.1038/leu.2014.41
106. Katagiri W, Osugi M, Kawai T, Hibi H. First-in-human study and clinical case reports of the alveolar bone regeneration with the secretome from human mesenchymal stem cells. *Head Face Med.* (2016) 12:5. doi: 10.1186/s13005-016-0101-5
107. Zhang X, Liu J, Yu B, Ma F, Ren X, Li X. Effects of mesenchymal stem cells and their exosomes on the healing of large and refractory macular holes. *Graefes Arch Clin Exp Ophthalmol.* (2018) 256:2041–52. doi: 10.1007/s00417-018-4097-3
108. Escudier B, Dorval T, Chaput N, Andre F, Caby MP, Novault S, et al. Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial. *J Transl Med.* (2005) 3:10. doi: 10.1186/1479-5876-3-10
109. Dai S, Wei D, Wu Z, Zhou X, Wei X, Huang H, et al. Phase I clinical trial of autologous ascites-derived exosomes combined with GM-CSF for colorectal cancer. *Mol Ther.* (2008) 16:782–90. doi: 10.1038/mt.2008.1
110. Morse MA, Garst J, Osada T, Khan S, Hobeika A, Clay TM, et al. A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. *J Transl Med.* (2005) 3:9. doi: 10.1186/1479-5876-3-9
111. Lener T, Gimona M, Aigner L, Borger V, Buzas E, Camussi G, et al. Applying extracellular vesicles based therapeutics in clinical trials - an ISEV position paper. *J Extracell Vesicles.* (2015) 4:30087. doi: 10.3402/jev.v4.30087
112. Reiner AT, Witwer KW, van Balkom BWM, de Beer J, Brodie C, Corteling RL, et al. Concise review: developing best-practice models for the therapeutic use of extracellular vesicles. *Stem Cells Transl Med.* (2017) 6:1730–9. doi: 10.1002/sctm.17-0055
113. Domenis R, Cifu A, Quaglia S, Pistis C, Moretti M, Vicario A, et al. Pro inflammatory stimuli enhance the immunosuppressive functions of adipose mesenchymal stem cells-derived exosomes. *Sci Rep.* (2018) 8:13325. doi: 10.1038/s41598-018-31707-9
114. Pardridge WM. Blood-brain barrier drug targeting: the future of brain drug development. *Mol Interv.* (2003) 3:90–105. doi: 10.1124/mi.3.2.90
115. Kornilov R, Puhka M, Mannerstrom B, Hiidenmaa H, Peltoniemi H, Siljander P, et al. Efficient ultrafiltration-based protocol to deplete

extracellular vesicles from fetal bovine serum. *J Extracell Vesicles.* (2018) 7:1422674. doi: 10.1080/20013078.2017.1422674

116. Watson DC, Yung BC, Bergamaschi C, Chowdhury B, Bear J, Stellas D, et al. Scalable, cGMP-compatible purification of extracellular vesicles carrying bioactive human heterodimeric IL-15/lactadherin complexes. *J Extracell Vesicles.* (2018) 7:1442088. doi: 10.1080/20013078.2018.1442088
117. Borlongan CV, Hadman M, Sanberg CD, Sanberg PR. Central nervous system entry of peripherally injected umbilical cord blood cells is not required for neuroprotection in stroke. *Stroke.* (2004) 35:2385–9. doi: 10.1161/01.STR.0000141680.49960.d7

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.

Copyright © 2019 Bang and Kim. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Neurology

ORIGINAL RESEARCH
published: 26 March 2019
doi: 10.3389/fneur.2019.00235

Combined Adipose Tissue-Derived Mesenchymal Stem Cell Therapy and Rehabilitation in Experimental Stroke

Jingwei Mu[1,2†], *Abdulhameed Bakreen*[2†], *Miia Juntunen*[3,4†], *Paula Korhonen*[5], *Ella Oinonen*[2], *Lili Cui*[2], *Mikko Myllyniemi*[2], *Shanshan Zhao*[2], *Susanna Miettinen*[3,4] and *Jukka Jolkkonen*[2,5,6*]

[1] Department of Neurology, The People's Hospital of China Medical University, Shenyang, China, [2] Department of Neurology, University of Eastern Finland, Kuopio, Finland, [3] Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland, [4] Research, Development and Innovation Centre, Tampere University Hospital, Tampere, Finland, [5] A. I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, Kuopio, Finland, [6] Neurocenter, Kuopio University Hospital, Kuopio, Finland

OPEN ACCESS

Edited by:
Paulo Henrique Rosado-de-Castro, Instituto D'Or de Pesquisa e Ensino (IDOR), Brazil

Reviewed by:
Aurel Popa-Wagner, University Hospital Essen, Germany
Fivos Panetsos, Complutense University of Madrid, Spain

***Correspondence:**
Jukka Jolkkonen
jukka.jolkkonen@uef.fi

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Stroke, a section of the journal Frontiers in Neurology

Received: 21 December 2018
Accepted: 22 February 2019
Published: 26 March 2019

Citation:
Mu J, Bakreen A, Juntunen M, Korhonen P, Oinonen E, Cui L, Myllyniemi M, Zhao S, Miettinen S and Jolkkonen J (2019) Combined Adipose Tissue-Derived Mesenchymal Stem Cell Therapy and Rehabilitation in Experimental Stroke. Front. Neurol. 10:235. doi: 10.3389/fneur.2019.00235

Background/Objective: Stroke is a leading global cause of adult disability. As the population ages as well as suffers co-morbidities, it is expected that the stroke burden will increase further. There are no established safe and effective restorative treatments to facilitate a good functional outcome in stroke patients. Cell-based therapies, which have a wide therapeutic window, might benefit a large percentage of patients, especially if combined with different restorative strategies. In this study, we tested whether the therapeutic effect of human adipose tissue-derived mesenchymal stem cells (ADMSCs) could be further enhanced by rehabilitation in an experimental model of stroke.

Methods: Focal cerebral ischemia was induced in adult male Sprague Dawley rats by permanently occluding the distal middle cerebral artery (MCAO). After the intravenous infusion of vehicle ($n = 46$) or ADMSCs (2×10^6) either at 2 ($n = 37$) or 7 ($n = 7$) days after the operation, half of the animals were housed in an enriched environment mimicking rehabilitation. Subsequently, their behavioral recovery was assessed by a neurological score, and performance in the cylinder and sticky label tests during a 42-day behavioral follow-up. At the end of the follow-up, rats were perfused for histology to assess the extent of angiogenesis (RECA-1), gliosis (GFAP), and glial scar formation.

Results: No adverse effects were observed during the follow-up. Combined ADMSC therapy and rehabilitation improved forelimb use in the cylinder test in comparison to MCAO controls on post-operative days 21 and 42 ($P < 0.01$). In the sticky label test, ADMSCs and rehabilitation alone or together, significantly decreased the removal time as compared to MCAO controls on post-operative days 21 and 42. An early initiation of combined therapy seemed to be more effective. Infarct size, measured by MRI on post-operative days 1 and 43, did not differ between the experimental groups. Stereological counting revealed an ischemia-induced increase both in the density of blood vessels and the numbers of glial cells in the perilesional cortex, but there were no differences among MCAO groups. Glial scar volume was also similar in MCAO groups.

Conclusion: Early delivery of ADMSCs and combined rehabilitation enhanced behavioral recovery in an experimental stroke model. The mechanisms underlying these treatment effects remain unknown.

Keywords: stroke, cell therapy, rehabilitation, combination therapy, functional outcome, mechanisms, translational research

INTRODUCTION

Stroke is one of the leading global causes of death and long-term disability, with about 5 million survivors becoming permanently disabled annually (1–3). Despite advances in acute stroke care (4), the narrow therapeutic time windows for early thrombolysis and thrombectomy make them available to only about 10% of stroke patients (5, 6). Safe and effective treatments beyond the acute phase are urgently needed.

Cell therapy represents a potential breakthrough in the treatment of stroke. In particular, mesenchymal stem cells (MSCs) are of major interest due to their advantages over other cell types, including their abundance and good availability (7), their relatively low immunogenicity (8) and tumorigenicity (9), and the lack of ethical concerns (10, 11). The non-invasive intravenous (IV) route has been most commonly used for delivery of MSCs in both preclinical and clinical studies (Cui et al. in press). More importantly, preclinical studies have revealed evidence for facilitation of behavioral recovery in animal models of stroke, e.g., improvements in sensorimotor functions (12–14).

Although still unclear, the putative mechanisms include secretion of neurotrophic factors that promote neuroprotection against inflammation (15), oxidative stress (14), and apoptosis (16). Neurorestorative mechanisms such as angiogenesis (17), neurogenesis (18), synaptogenesis (12), oligodendrogenesis (18), repair of white matter fiber tracts (19), and remodeling of neural circuits (20, 21) have also been proposed. In particular, local angiogenesis is required to provide sufficient oxygen and nutrients during cerebral reconstruction and remodeling of damaged tissue, thus this phenomenon plays an important role in the recovery of neural function after stroke (22). Indeed, it has been reported that a higher density of blood vessels resulted in reduced morbidity and prolonged survival of stroke patients (23, 24).

Recently, adipose tissue-derived mesenchymal stem cells (ADMSCs) have demonstrated their therapeutic potential in stroke models by improving the gross neurological condition (17, 18, 20, 25, 26), sensorimotor function (14, 15, 18, 19, 25, 27), as well as exerting beneficial effects on spatial learning and memory (7). In fact, the promising preclinical data laid the foundation for the first safety trial of ADMSCs in stroke patients (28). Unfortunately, the limited therapeutic efficacy in early patient studies have indicated that further preclinical studies are necessary in order to optimize the current cell treatment protocols.

After the acute phase, rehabilitation therapy is the only approved treatment for stroke survivors presenting with neurological deficits (29). In experimental settings, various rehabilitative approaches such as physical training (30, 31), skilled training (32–34), and special rehabilitative training devices (35, 36) have all been employed. In addition, housing the experimental animals in an enriched environment (EE) has also been used to provide multiple sensory, motor, social, and visual stimuli (37). Although very non-specific, housing in EE is one of the most promising approaches for improving an animal's sensorimotor functions after an experimental stroke (38, 39). EE has also been shown to improve spatial learning and memory in ischemia-reperfusion models (40).

The combination of different restorative approaches represents an intriguing approach to maximize treatment effects (41). Furthermore, cell-based therapies offer the possibility of combining different neurorestorative strategies to achieve an additive or even a synergistic therapeutic effect. However, only a few studies have been published (12, 13, 37, 42–45), and thus, more research is required in this regard to examine not only the stand-alone effects of each therapy, but also their potential combined effect (46, 47). Here, we hypothesized that the combination of an enriched environment with the IV infusion of ADMSCs after permanent middle cerebral artery occlusion (MCAO) would result in an improved behavioral recovery, perhaps even a maximal therapeutic effect. In order to explore the therapeutic window, we infused ADMSCs at either 2 or 7 d post-MCAO. Angiogenesis was evaluated as a possible repair mechanism related to treatment effect. In addition, glial cell staining was used to assess the extent of gliosis since the presence of a glial scar is considered to impede neuronal plasticity and prevent the functional recovery.

MATERIALS AND METHODS

Animals

Ninety-seven adult male Sprague Dawley rats (Envigo, operation weight 274–340 g) were maintained in a controlled environment (temperature 20 ± 1°C; humidity 50–60%; light period 07:00–19:00) with free access to food and fresh water throughout the experiment. Animal care procedures were conducted according to the guidelines set by the European Community Council Directives 86/609/EEC; and this work was approved by the Animal Ethics Committee (Hämeenlinna, Finland).

Preparation and Characterization of Human ADMSCs

The adipose tissue stem cell line RESSTORE01 (Master Cell Bank/Stock n°1—Donor RESSSTORE01, Batch n°: 591133643763) was cultured in the growth medium Alpha MEM (Gibco, Life technologies) supplemented with 5% human platelet lysate (Stemulate, Cook Medical, USA) and 1% Penicillin-Streptomycin (Lonza, Belgium). The medium was changed

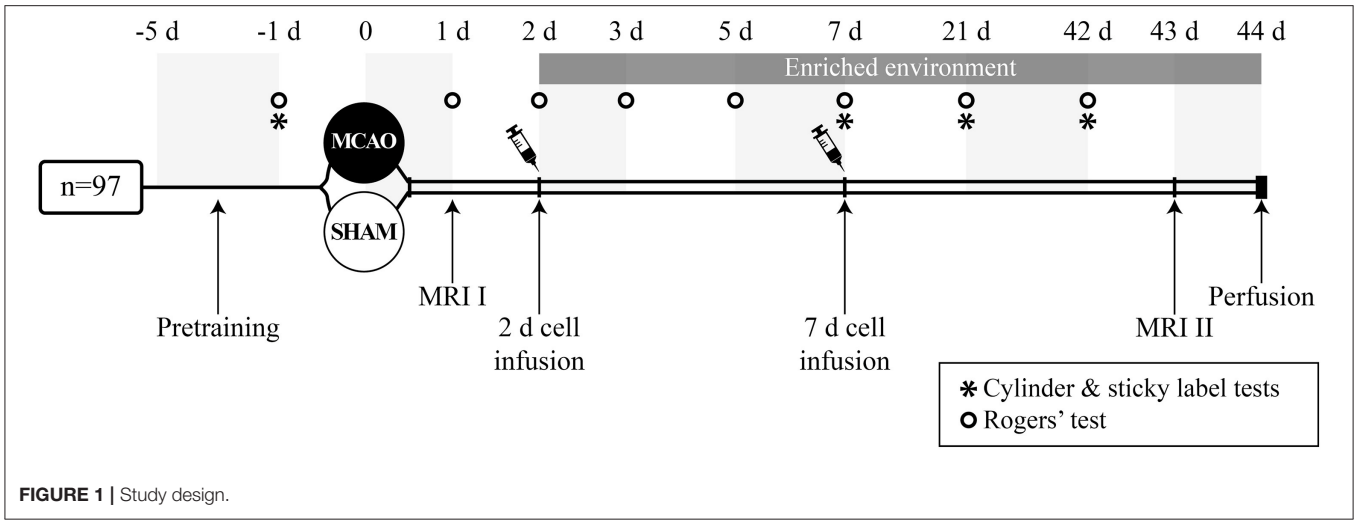


FIGURE 1 | Study design.

twice each week and cells were passaged when they reached 100% confluence. The cells were detached with TrypLE Select (Life Technologies™, Thermo Fisher Scientific) for 10 min at 37°C and then centrifuged at 1,000 rpm for 5 min. The RESSTORE01 cell phenotype was analyzed with flow cytometry (FACSAria Fusion Cell Sorter, BD Biosciences) at passage PX +1. Monoclonal antibodies against CD19-phycoerythrincyanine (PE-Cy7), CD45RO-allophycocyanin (APC), CD73-PE, CD90-APC (BD Biosciences), CD11a-APC, CD105-PE (R&D Systems Inc., Minneapolis, MN, USA), CD34-APC and HLA-DR-PE (Immunotools GmbH, Friesoythe, Germany) were used. The cells expressed (>95%) surface markers CD73, CD90, and CD105 and lacked the expression (<2%) of CD11a, CD19, CD34, CD45, and HLA-DR. Cells at passages PX +2/3 were used for the study.

Permanent Middle Cerebral Artery Occlusion

Anesthesia was induced with 5% isoflurane in 30 O_2/70% N_2O and maintained during the operation with 2% isoflurane. The temperature of the rats was kept constant (37 ± 0.5°C) with a heating blanket and rectal probe (Harvard Homeothermic Blanket Control Unit; PanLab, Barcelona, Spain). In the occlusion of the right middle cerebral artery (MCA), the temporal muscle was removed to expose the temporal bone and a 2–3 mm diameter hole was drilled on top of the artery while cooling the bone with ice-cold 0.9% NaCl. The dura was carefully removed after which the artery was occluded with an electrocoagulator (Aesculap, Center Valley, PA, USA). Immediately after the MCA occlusion, both common carotid arteries (CCA) were occluded with micro-aneurysm clips for 60 min. After 1 h, the clamps were slowly released, the temporal muscle was replaced, and the incision/wound was sutured. The sham-operated rats went through all of the same procedures except for the occlusion of MCA and CCAs. To assist rehydration, 5 ml of 0.9% NaCl was given intraperitoneally. Buprenorfine (Temgesic, 0.03 mg/kg) was injected subcutaneously immediately after surgery as an analgesic.

Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) was performed 24 h after the operation and on post-operative day 43 (**Figure 1**) using a Bruker 9.4 T horizontal scanner. The rats were anesthetized with 5% isoflurane in 30 O_2/70% N_2. After induction, the anesthesia was maintained throughout the imaging with 1.5% isoflurane inhaled through a nose mask. In the determination of the infarct volume, T2 weighted multi-slice images were acquired using a RARE sequence with the following parameters: time-to-repetition TR = 2.5 s, effective time-to-echo effTE = 40 ms, RARE factor 8, matrix size of 256 × 256, field-of-view of 30 × 30 mm, 15 slices with a slice thickness of 1 mm. T_2^* weighted images were obtained using a standard gradient echo imaging sequence from the same slices with identical resolution and TR = 700 ms, TE = 15 ms, flip angle ∼50°. The cortical infarct volume was measured using in-house written Matlab software. Animals with infarct size <20 mm^3 (n = 5) or >150 mm^3 (n = 2) were excluded from the data analysis. These exclusion criteria had been decided before the experiment.

Cell Treatment and Housing in Enriched Environment

The animals were sequentially assigned to experimental groups based on initial screening on MRI to ensure that the infarct size did not differ between experimental groups before treatment (**Table 1**). Two days after the occlusion procedure, isoflurane anesthetized rats were slowly infused with 2 million cells/1 ml 0.9% NaCl into the tail vein. Vehicle groups were treated with 1 ml 0.9% NaCl. Additional animals were treated 7 days after MCAO (**Figure 1**). Body weight was recorded during the follow-up as part of the safety assessment. After the infusion of the cells, half of the rats were moved to an enriched environment that consisted of two large metal cages (61 × 46 × 46 cm) that were connected by a tunnel. The cages contained ladders, tunnels, shelves and a running wheel to provide sensorimotor stimuli. Novel objects (e.g., toys, wooden balls) were changed

TABLE 1 | Experimental groups.

	Groups	Treatment		Housing		Timing	
		Vehicle	Cell	Standard	EE	2 d	7 d
SHAM	SHAM+V+S ($n = 8$)	x		x		x	
	SHAM+C+S ($n = 8$)		x	x		x	
	SHAM+V+EE ($n = 8$)	x			x	x	
	SHAM+C+EE ($n = 8$)		x		x	x	
MCAO	MCAO+V+S ($n = 12$)	x		x		x	
	MCAO+C+S ($n = 10$)		x	x		x	
	MCAO+V+EE ($n = 10$)	x			x	x	
	MCAO+C+EE ($n = 11$)		x		x	x	
	MCAO+V7+EE ($n = 8$)	x			x		x
	MCAO+C7+EE ($n = 7$)		x		x		x

SHAM, sham-operated; MCAO, middle cerebral artery occlusion; V, 2 d vehicle; C, 2 d cell infusion; V7, 7 day vehicle; C7, 7 day cell infusion; S, standard housing; EE, enriched environment.

every second day. Altogether, 8–9 animals were housed per cage. The animals in the non-rehabilitation group were housed in groups of three rats in standard cages (53 × 32.5 × 20 cm).

Behavioral Testing

All behavioral tests were carried out in a blinded manner 1 day before the occlusion procedure and on post-operative days (between 9 and 12 a.m.) as shown in **Figure 1**. Behavioral impairment was assessed using the Rogers', cylinder and sticky label tests. Two animals were excluded from the sticky label test analysis due to problems with their teeth and behavioral peculiarities.

Rogers' Test

The Rogers' functional evaluation scale was used to assess the gross behavioral impairment, including reflexes, sensory responses, and simple motor functions (17). It consists of a 7-point behavioral rating scale: score 0—no functional deficit; score 1—failure to extend left forepaw fully; score 2—decreased grip of the left forelimb while tail gently pulled; score 3—spontaneous movement in all directions, contralateral circling only if pulled by the tail; score 4—circling or walking to the left; score 5—walking only when stimulated; score 6—unresponsive to stimulation with a depressed level of consciousness; and score 7—dead.

Cylinder Test

The cylinder test was used to measure spontaneous forelimb use and imbalance between the non-impaired and impaired forelimbs (48). In this test, the rat was placed in a transparent plastic cylinder (Ø 20 cm) and video-recorded (5 min) through a mirror placed under the cylinder. The videotaped exploratory activity in the cylinder was analyzed for 1 to 3 min using a program with slow motion capabilities. The number of contacts on cylinder by either the impaired or the non-impaired forelimb or both forelimbs was counted (minimum 30 contacts). The imbalance in forelimb use was calculated as: [(use of impaired forelimb + 0.5 × use of both forelimbs) ÷ (total contacts)] × 100%.

Sticky Label Test

The sticky label test was used to evaluate sensory function and motor learning, and was performed as previously described (48). Before testing, the animals were familiarized with handling and the testing cage. In the test, a white colored circular label (Ø 9 mm, Tough-Spots, Diversified Biotech) was placed on the distal-radial region of both wrists and rat was moved to a test cage. The time for the first contact to the label and time to remove the label were measured. A maximum time of 120 s was set if the rat was not able to contact or remove the label.

Histology

After the behavioral assessment, rats were perfused on post-operative day 44 (**Figure 1**) with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were carefully removed from the skull, post-fixed, and cryoprotected. Brain sections (35 um) were cut using a sliding microtome and stored in antifreeze solutions at −20°C. Systematically sampled sections with a random start covering the entire infarct were selected for staining and analysis. One series of sections was stained with the angiogenesis marker, anti-RECA-1 antibody, and another series with the gliosis marker, anti-GFAP antibody.

RECA-1 Immunohistochemistry

The sampled sections were stained with anti-RECA-1 antibody to visualize blood vessels. Briefly, free-floating sections were washed in 0.1 M phosphate buffer (PB) (3 × 15 min) and kept overnight in a cold room. They were then rinsed with 0.5 M Tris buffered saline + Triton (TBS-T) at pH 7.6 (3 × 5 min) before treatment with the primary antibody mouse anti-rat RECA-1 (AbD Serotec, Bio-Rad) at 1:2000 in TBS-T at pH 7.6 for 18 h on a shaker table in the dark at room temperature. Afterwards, the sections were rinsed in TBS-T at pH 7.6 (3 × 5 min) and then treated with secondary antibody biotinylated anti-mouse IgG (Vector) made in goat at 1:500 in TBS-T at pH 7.6 for 2 h on a shaker table, room temperature. Subsequently, the sections were rinsed in TBS-T at pH 7.6 (3 × 5 min), followed by treatment with Streptavidin-horseradish peroxidase conjugate (GE Healthcare UK Limited) at 1:1000 in TBS-T at pH 7.6 for 2 h on a shaker table at room temperature. The sections were again rinsed in TBS-T at pH 7.6 (3 × 5 min) and then carefully developed with filtered nickel-intensified DAB (Sigma) for ∼3 min. Excess DAB was rinsed with PB (3 × 4 min) before the sections were mounted and kept at 37°C overnight. The mounted sections were then washed in PB for 5 min before they were counterstained with thionin to reveal the neuroanatomy and delineate the perilesional area. They were then cleared in xylene (2 × 5 min) and coverslips were mounted using Depex.

GFAP Immunohistochemistry

Other sets of sampled sections were stained with anti-GFAP antibody to visualize astrocytes. Briefly, free-floating sections were washed in PB (3 × 15 min) and kept overnight in a cold

room. They were then washed in TBS-T at pH 8.6 (2 × 5 min) before incubating in primary antibody mouse anti-GFAP (Sigma) at 1:1000 in TBS-T at pH 8.6 for 18 h on a shaker table in the dark at room temperature. The sections were again rinsed in TBS-T at pH 8.6 (3 × 5 min) and then treated with secondary antibody goat anti-mouse IgG-HRP conjugated (Invitrogen) at 1:500 in TBS-T at pH 8.6 for 2 h on a shaker table at room temperature. Afterwards, the sections were rinsed in TBS-T at pH 8.6 (3 × 5 min) and then carefully developed with filtered nickel-intensified DAB for ∼3 min. Excess DAB was rinsed with PBS (3 × 4 min) before the sections were mounted and kept at 37°C overnight. Finally, the mounted sections were cleared in xylene (2 × 5 min) and coverslips were mounted using Depex.

Histological Analysis

The analysis was done with the aid of Stereo Investigator software (MicroBrightField, Inc., VT, USA) attached to an ECLIPSE E600 microscope (Nikon, Japan) via a 3-Chip CCD color video camera (QImaging, Canada). A motorized stage with a microcator (Heidenhain EXE 610C) attachment (providing a 0.1 μm resolution in the Z axis) was mounted on the microscope.

In the assessment of angiogenesis, the perilesional tissue 200 μm from the ischemic border around the lesion was first outlined under 4x magnification (N.A. 0.06) and thereafter blood vessels were counted under 20x magnification (N.A. 0.75). To determine the vessel density in sections stained with RECA-1, we used the virtual sphere method (49). A three-dimensional sampling hemisphere ("space ball") with a radius of 20 μm was placed within a sampling box with known dimensions (x = 200 μm, y = 200 μm, and z = 20 μm) and focused through the section thickness. The x-y steps giving the distance between sampling areas was 400 μm (x-axis) by 400 μm (y-axis), aimed at generating counts of about 300 vessel intersections per animal. In order to calculate the total length of blood vessels, the following equation was used: $L_{total} = \Sigma Q \times 2 \times 1/ssf \times 1/asf \times 1/tsf \times [v/a]$, where Q is the number of intersections between vessels and the probe, ssf (section sampling fraction) is 1/15, asf (area sampling fraction) is 0.03, tsf (tissue sampling fraction) is 1, and v/a is 19.2 μm [defined as the ratio of the volume (v) of the counting frame (sampling box) to the surface area (a) of the hemisphere probe (space ball)]. The vessel density was counted as the ratio of measured length and the total volume of perilesional area.

The optical fractionator technique was used to measure the glial scar and to assess the total number of GFAP labeled cells in the perilesional area (50). The glial scar was defined as glial cell aggregation. The perilesional zone was defined as a 200 μm wide cortical zone directly surrounding the scar. The cut thickness of the tissue was 35 μm and the average mounted thickness was 20 μm. The size of the counting frame was 100 × 100 μm, with the height of the dissector cube of 20 μm and a grid of 200 × 200 μm. The perilesional area and the scar area were traced using a 2x objective (N.A. 0.10) and the number of GFAP positive cells was counted using a 20x objective (N.A. 0.75). A GFAP positive cell was counted when the cell soma did not intersect with the uppermost focal plane (exclusion plane) and the lateral exclusion boundaries of the counting frame. The perilesional area reference volume was determined by adding the traced perilesional area for each section multiplied by the distance between sections sampled. For the total number of GFAP positive cells, the following equation was used: $N_{total} = \Sigma Q \times 2 \times 1/ssf \times 1/asf \times 1/tsf$. The number of GFAP labeled cells was then related to a total perilesional volume. The enclosed volume of the scar was acquired from the contour summary provided by Neurolucida software (MicroBrightField, Inc.).

Statistical Analysis

Statistical analyses were performed using SPSS software for Windows (version 25). One-way analysis of variance (ANOVA), followed by the LSD *post-hoc* test if necessary, was used to analyze the statistical differences between groups in infarct volume, RECA-1, and GFAP staining. The Kruskal-Wallis H test, followed by the Mann-Whitney *U*-test if necessary, was used to compare the neurological scores. Repeated measures ANOVA, followed by the LSD *post-hoc* test if necessary, was used to analyze behavioral data from the cylinder and sticky label tests. Spearman and Pearson correlations were used to examine the relationship between behavioral impairment and infarct size, angiogenesis, and gliosis. Data are expressed as mean ± standard deviation (SD).

RESULTS

Intravenous ADMSC Infusion Was Not Associated With Mortality or Adverse Effects

To assess the safety of ADMSC treatment, we carefully monitored the rats during the follow-up. There was no mortality after infusion of either cells or vehicle. Weight gain was similar in all experimental groups (see **Supplemental Table 1**). Safety was also evaluated using the Rogers' scale on post-operative days 1, 2, 3, 5, 7, 21, and 42 (see **Supplemental Table 2**). There was a statistically significant difference ($P < 0.001$) in neurological scores at all time points after the occlusion procedure, demonstrated by the better performance in the sham-operated groups compared to the MCAO groups. No statistically significant differences were found between vehicle- and cell-treated groups at all post-operative time points (data not shown).

Infarct Size Was Not Affected by ADMSCs

There were no differences in the cortical infarct size between the experimental groups at 24 h after operation (**Figure 2A**). In order to determine whether the therapeutic effect would be related to delayed neuroprotection, the infarct size was measured also at the end of the follow-up (**Figure 2B**). A variable maturation of infarct was observed, in many cases leading to a liquid-filled cyst. However, the infarct size did not differ between control and cell-treated groups on post-operative day 43.

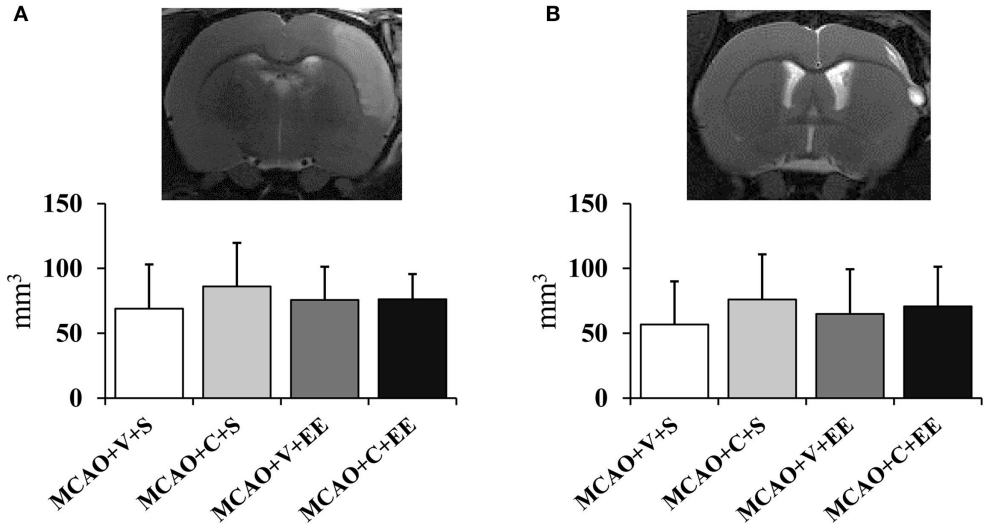


FIGURE 2 | Infarct size and location. Infarct size 24 h **(A)** after MCAO in rats and at the end of the follow-up on day 43 **(B)**. MRI images show the location of a typical infarct in the sensorimotor cortex at these time points.

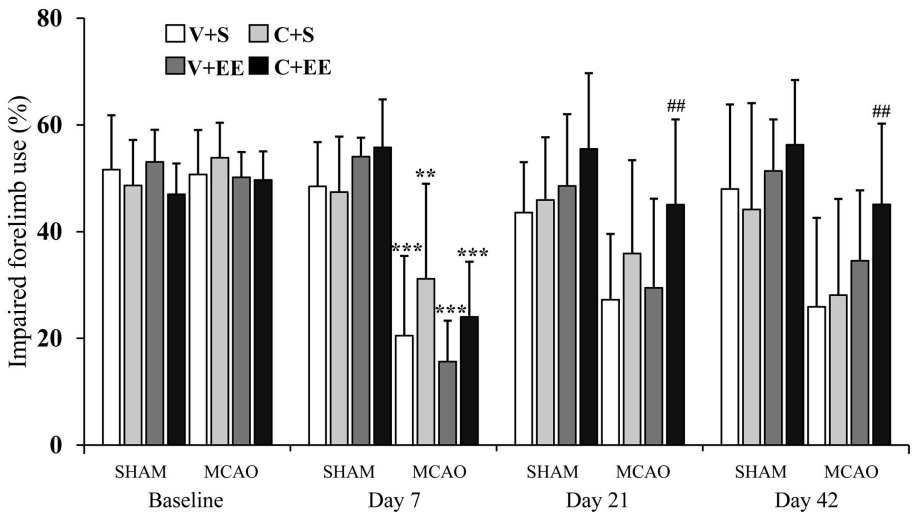


FIGURE 3 | Cylinder test. Impaired forelimb use during the vertical exploration in the cylinder test was improved by ADMSC treatment and housing in an enriched environment in MCAO rats. Statistical significance: $^{**}P < 0.01$; $^{***}P < 0.001$ (compared to SHAM+V+S), $\#\#P < 0.01$ (compared to MCAO+V+S). SHAM, sham-operated; MCAO, middle cerebral artery occlusion; V, 2 d vehicle; C, 2 d cell infusion; S, standard housing; EE, enriched environment.

ADMSC Infusion and EE Improved Spontaneous Forelimb use in MCAO Rats

Treatment effects were assessed by the performance of the animals in the cylinder and sticky label tests on post-operative days 7, 21, and 42.

Cylinder Test

The imbalance in the spontaneous forelimb use during vertical exploration was assessed in the cylinder test (**Figure 3**). There was a significant overall group effect ($P < 0.001$) and time × group interaction ($P < 0.001$). In the more detailed analysis, significant time × group interactions were found between standard-housed vs. EE-housed ($P < 0.01$) as well as with vehicle-treated vs. ADMSCs-treated ($P < 0.05$) animals, showing that recovery of the impaired forelimb was different between the groups. All MCAO groups were different from SHAM+V+S on post-operative day 7. The MCAO+V+S group displayed a slower recovery on post-operative day 21. MCAO+V+S control rats were different from the MCAO+C+EE group on post-operative days 21 and 42 ($P < 0.01$). The amount of spontaneous forelimb use at the end of the follow-up correlated with infarct size on post-operative day 1 ($r = -0.440$, $P < 0.01$), blood vessel density

TABLE 2 | Correlations of behavioral outcome at the end of the follow-up with infarct size (24 h), angiogenesis and gliosis.

	Rogers' test	Cylinder test	Sticky label test	
			Time to first contact	Time to remove
Infarct size (24 h)	$r = 0.311$; $P < 0.05$	$r = -0.440$; $P < 0.01$	NS	NS
Blood vessel density	NS	$r = -0.278$; $P < 0.01$	NS	NS
Number of glial cells	$r = 0.530$; $P < 0.001$	$r = -0.550$; $P < 0.001$	NS	NS
Glial scar volume	NS	NS	NS	NS

NS, not significant.

($r = -0.278$; $P < 0.01$), and number of glial cells ($r = -0.550$; $P < 0.001$) (**Table 2**).

Sticky Label Test

The sticky label test was performed to evaluate sensorimotor function and motor learning (**Figure 4**). With respect to the impaired forelimb, there were no significant overall group effect ($P = 0.055$) or any time × group interaction ($P = 0.138$) on the time for the first contact with the label (**Figure 4A**). With respect to the time to remove the label, there was a significant overall group effect ($P < 0.01$) and a time × group interaction ($P < 0.01$). MCAO+V+S rats used more time to remove the label as compared to SHAM+V+S ($P < 0.01$) or animals in the other MCAO groups ($P < 0.01$) on post-operative day 21. At the end of the behavioral follow-up, MCAO+V+S controls were different from SHAM+V+S ($P < 0.01$), MCAO+C+S ($P < 0.05$), and MCAO+C+EE ($P < 0.01$) groups (**Figure 4B**). With respect to the non-impaired forelimb, there were no significant overall group effects and time × group interactions for the time to the first contact with the label or for the time required to remove the label (data not shown).

Cell Infusion on Post-operative Day 7 Was Not Effective as When Cells Infused on Day 2

The optimal therapeutic time window for combined therapy was assessed by comparing treatment starting on days 2 and 7. There were no significant overall group effects or time × group interactions in the cylinder test (**Figure 5A**) or sticky label test (**Figures 5B,C**), when cell infusion on days 2 and 7 were compared. However, a trend toward a better recovery was observed when cells were delivered earlier.

The Extent of Angiogenesis or Gliosis Was Not Related to the Behavioral Recovery

Perilesional cortex undergoes a major reorganization after cerebral ischemia, a phenomenon thought to be related to the behavioral recovery. Here, we measured the formation of new blood vessels as a mechanism behind the behavioral recovery and we also assessed the extent of glial scar formation that might hinder the recovery process.

Angiogenesis

The overall distribution of RECA-1 stained blood vessels in the cortex is shown for a sham-operated rat (**Figure 6B**) and for a MCAO rat (**Figure 6C**). **Figure 6A** shows how the perilesional cortex was defined. There was a significant difference ($P < 0.01$) in the blood vessel density in the cortex due to the difference between sham-operated and MCAO animals. However, there was no significant difference between the MCAO groups (**Figure 6D**).

Glial Cells and Glial Scar

Figure 7A shows GFAP staining for resting glial cells in a sham-operated rat with **Figure 7B** revealing the change in the phenotype into hypertrophic reactive astrocytes and scar-forming astrocytes in the perilesional cortex after ischemia. There was a significant overall effect in the number of glial cells in perilesional cortex ($P < 0.001$) (**Figure 7C**); this was due to an increase in the number of glial cells in MCAO animals. However, there was no significant difference between the MCAO groups nor was there any significant difference between MCAO groups in the glial scar volume (**Figure 7D**).

DISCUSSION

In this experimental model of stroke, we investigated whether the therapeutic effect of human ADMSCs could be further enhanced by rehabilitation. We found that cell therapy or rehabilitation alone improved the functional recovery of the impaired forelimb, when treatment was started 2 days after the induction of ischemia. Combined therapy further improved the behavioral outcome. Delayed neuroprotection, angiogenesis or altered gliosis did not explain the behavioral improvement.

Methodological Issues

ADMSCs are easy to obtain in large quantities (7), have no ethical concerns (10, 11), and have an excellent safety profile (9, 17). The same cell product is being used in the ongoing RESSTORE clinical trial (NCT03570450). The MCAO stroke model was selected according to recent SRRR guidelines (51). It produces a consistent infarct in the sensorimotor cortex causing a moderate behavioral impairment and partial spontaneous recovery in the long-term follow-up. The somewhat large variability in infarct size, which was also reflected in behavioral scores, and thus complicating the statistical analysis, is possibly due to the rat strain. The selected outcome measures are sensitive at identifying treatment effects and are not affected by repeated testing, but combined treatment effects can be missed by the presence of spontaneous recovery and of a ceiling effect.

Our study also has some methodological limitations. Young male rats were used, although the main unmodifiable risk factor for stroke is aging, which may impair brain repair including angiogenesis (52), and also decrease the therapeutic effect of cells (53). Thus, aging as well as co-morbidities such as hypertension and diabetes (54) should be addressed despite the fact that they further complicate the study design. As far as we are aware, this is the first time that stereology has been used to evaluate the extent of brain repair in a stroke model. However, since the RECA-1 antibody stains all blood vessels, perhaps CD31 (PECAM-1) staining would have revealed post-stroke angiogenesis (52). The

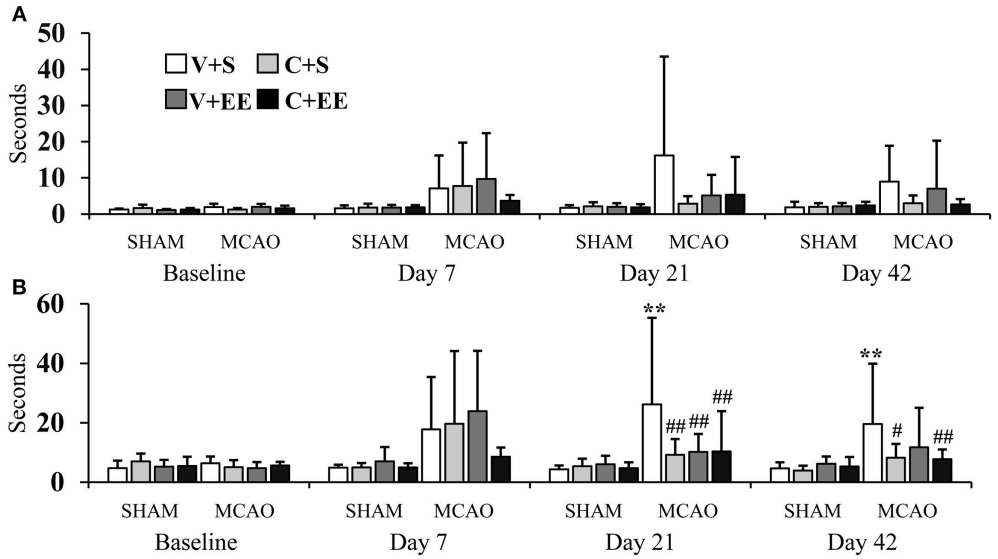


FIGURE 4 | Sticky label test. Time needed before the first contact was not different between the experimental groups **(A)**. The time to removal of sticky label from impaired forelimb was increased in MCAO rats, an effect partially reversed by ADMSCs an EE **(B)**. Statistical significance: $^{**}P < 0.01$ (compared to SHAM+V+S); $\#P < 0.05$, $\#\#P < 0.01$ (compared to MCAO+V+S). SHAM, sham-operated; MCAO, middle cerebral artery occlusion; V, 2 d vehicle; C, 2 d cell infusion; S, standard housing; EE, enriched environment.

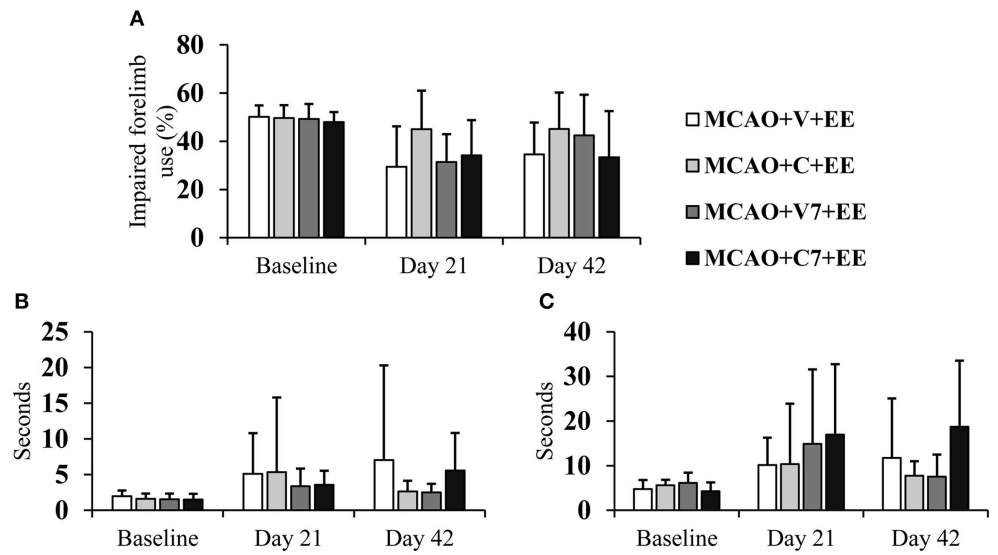


FIGURE 5 | Timing of cell delivery. Early ADMSC delivery (48 h) in MCAO rats seemed to improve forelimb use when compared to delayed delivery (7 d) as assessed in the cylinder test **(A)** and sticky label test: time to touch **(B)**, time to remove **(C)**. MCAO, middle cerebral artery occlusion; V, 2 d vehicle; C, 2 d cell infusion; V7, 7 day vehicle; C7, 7 day cell infusion; EE, enriched environment.

only reliable way to differentiate between new and old blood vessels would be to conduct BrdU/RECA-1 double staining.

Intravenous ADMSC Delivery Is Safe

The safety of new therapies is of the utmost importance (55); however, it is often overlooked unless demanded by the regulatory authorities. In the present study, we did not observe mortality due to pulmonary embolism or any other reasons; weight gain was similar in all groups; the gross neurological evaluation revealed no abnormalities in sham-operated or ischemic rats after cell treatment; and MRI images were clean with no sign of bleeding. These all support the concept that the used cell product, dose and delivery route were safe in our rat stroke model. However, regulatory safety studies will need to be carried out before embarking on patient trials.

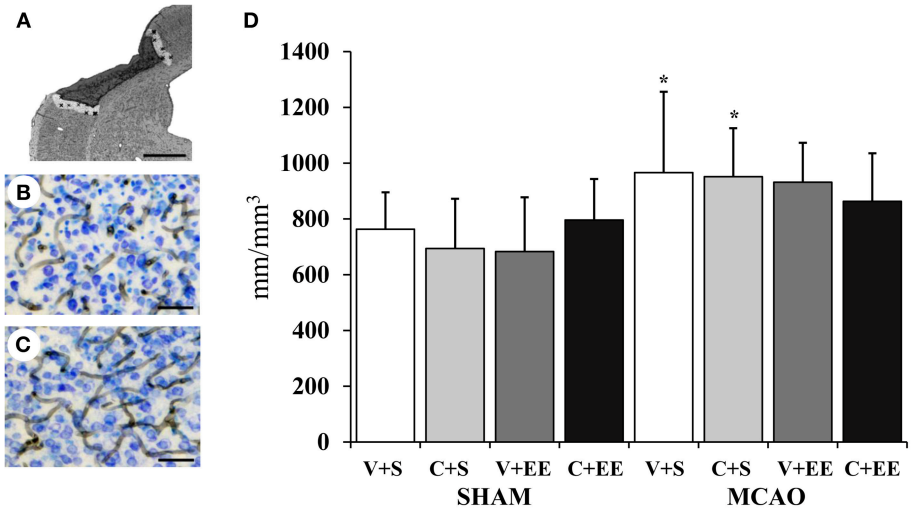


FIGURE 6 | Quantification of perilesional angiogenesis. Definition of perilesional cortex 200 μm from the border of ischemic core **(A)**. Representative RECA-1 staining with thionin counterstaining in sham-operated **(B),** and MCAO **(C)** rats. Stereological analysis showed ischemia-induced increase in blood vessel density in perilesional cortex **(D)**. Scale 1 mm **(A)**, 50 μm **(B,C)**. Statistical significance: *$P < 0.05$ (compared to SHAM+V+S). SHAM, sham-operated; MCAO, middle cerebral artery occlusion; V, 2 d vehicle; C, 2 d cell infusion; S, standard housing; EE, enriched environment.

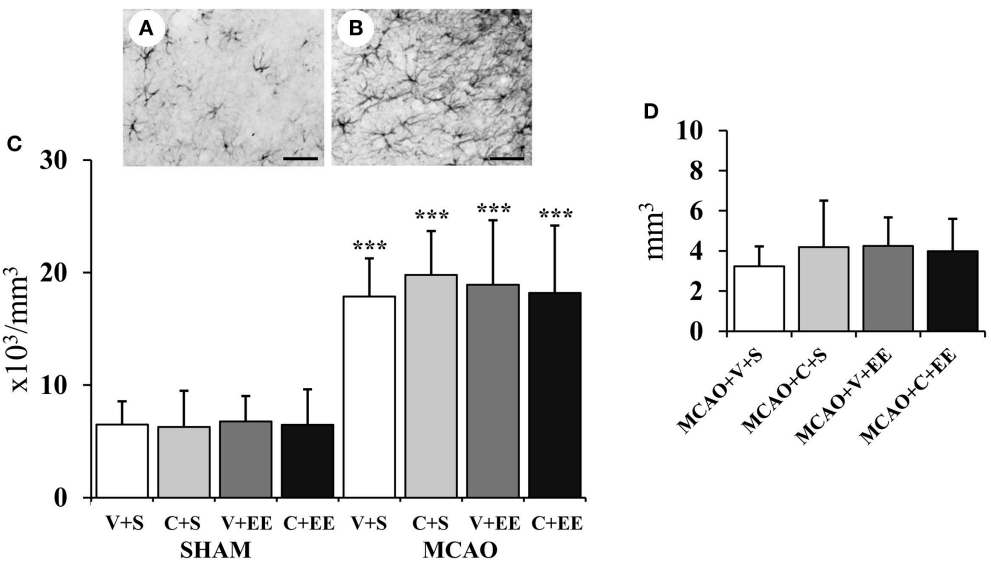


FIGURE 7 | Quantification of perilesional gliosis. Representative staining of GFAP-stained sections from a sham-operated rat **(A)** and an MCAO rat **(B)**. Stereological analysis revealed an ischemia-induced increase in the number of glial cells in the perilesional cortex **(C)**. Glial scar volume did not differ between the MCAO groups **(D)**. Scale 50 μm. Statistical significance: ***$P < 0.001$ (compared to SHAM+V+S). SHAM, sham-operated; MCAO, middle cerebral artery occlusion; V, 2 d vehicle; C, 2 d cell infusion; S, standard housing; EE, enriched environment.

Early ADMSC Transplantation Improves Sensorimotor Recovery

Promising behavioral improvements have been reported in experimental stroke models following IV infusion of MSCs (12, 13), including ADMSCs (15, 19, 20, 27). Consistent with these studies, we observed that the spontaneous use of the impaired forelimb in the cylinder test had been improved by the infusion of the ADMSCs. In addition, 2 d post-stroke delivery of ADMSCs to the MCAO rats resulted in a significant decrease in the time needed to contact and particularly, to remove sticky labels from the impaired forelimb.

The critical time window during which the brain is most responsive to cell therapy is not known. In most of the experimental studies, cells have been infused within 24 h after

brain ischemia (Cui et al. in press), although later time points have also been evaluated (7, 56). Here we found a trend toward a better recovery when the cells were delivered earlier. While early delivery might provide neuroprotection, that hypothesis was however not supported by the present data. Furthermore, the recent phase II MASTERS study in stroke patients supports the efficacy of early delivery (57). In addition, there is accumulating evidence suggesting that intravenously delivered cells modulate peripheral immune systems within another specific time window i.e., 24–48 h post-stroke, improving functional recovery (58). This is the target in the ongoing phase III MASTERS-2 study in stroke patients (NCT03545607).

The relatively modest behavioral improvement in our study could be due to the loss of cells after xenogenic transplantation. The differences in the clearances of human and rat bone marrow-derived MSCs after systemic infusion support this proposal (59). However, both allogenic and xenogenic delivery of ADMSCs have been effective in experimental stroke (7, 15, 17, 26, 27, 56). Cell entrapment in lungs might also affect the efficacy of the treatment, although it is still not clear whether the cells have to enter the brain parenchyma to play a role in behavioral recovery (60). Lastly, when robust study quality criteria are applied (e.g., randomization, blind assessment), the preclinical results are not so convincing and are more in line with the relatively modest evidence of efficacy emerging from small patient studies.

EE Results in a Modest Improvement in Sensorimotor Functions

Experimental stroke rehabilitation is an emerging research area. The available data does not support the belief that one particular approach is superior to the others (61). In our study, we examined the benefits of an enriched environment, which provides spatial, sensory, motor, and social stimuli for rodents. This is known to be one of the most powerful forms of experimental rehabilitation (39), improving not only gross neurological and sensorimotor functions (38, 62–64), but also spatial learning and memory after cerebral ischemia (40, 65). In our study, housing in the enriched environment, when started on post-operative day 2, improved spontaneous forelimb use in the cylinder test and reduced the removal time in the sticky label test.

A major problem with enriched environment is that the overall stimulation varies and is dependent on the activity of the animal. Thus, it remains to be determined whether intensive or forced physical training or a more controllable and task-specific exercise such as skilled forelimb reaching to supplement the enriched environment would have been more effective (66). One way to increase treatment contrast would be to house control rats in single cages. In the present study, three rats were housed in the same cage as demanded by the animal ethics committee.

Combined Therapy Seems to Further Improve Sensorimotor Functions

The wide therapeutic time windows for both cell therapy and rehabilitation allow their combination. Although the prospect of combining different neurorestorative approaches to maximize the therapeutic effect is theoretically very interesting (41), to date, very few studies have utilized this research strategy (12, 13, 37, 42–45). In our study, we found that combining ADMSC at 2 d cell delivery with EE in MCAO animals increased the spontaneous use of the impaired forelimb during vertical exploration. The impaired forelimb had almost completely recovered by the end of the follow-up. In addition, the time needed to remove sticky labels from their impaired forelimb was significantly reduced. However, the spontaneous recovery, complex study design and challenging statistics did not make it possible to discriminate the add-on therapeutic effect. To achieve this, multicenter preclinical trials with greater statistical power will be needed (67).

Cell treatment and housing in EE was started at the same time but it is not known whether this is the most optimal approach. Interestingly, there is recent evidence that a timed sequence of treatments could maximize the therapeutic effects in experimental stroke animals (68). In the case of cell therapy, stabilization with nonselective stimulation such as an enriched environment might be preferable after the initiation of the brain plasticity by the infused cells.

Neuroprotection may also be a prerequisite for delayed cortical plasticity and functional recovery. In the elegant work of Fernández-García et al. (69), transplantation of mesenchymal stem cells alone into stroke mice was not effective as these animals showed permanent sensorimotor deficits in the grid walking test. However, when cells where encapsulated in silk fibroin hydrogel, significant cortical neuroprotection was observed, leading to delayed remapping of forelimb representations and a behavioral recovery similar to that associated with rehabilitation.

Perilesional Angiogenesis or Gliosis Does not Explain the Behavioral Recovery

The brain repair mechanisms underlying spontaneous or therapy-induced recovery after stroke are still poorly understood (70). However, there is emerging evidence that in particular the perilesional tissue undergoes a major remodeling to foster restitution of function in damaged areas (71).

Angiogenesis, i.e., the formation of new capillaries, is restricted to the border of the infarct and it is claimed to aid in cleansing the necrotic brain tissue (72) as well as providing a site for neuroblast migration (73). MSCs further promote angiogenesis and this has been associated with improved behavioral outcome (74). Consistent with previous studies, we could confirm the ischemia-induced increase of angiogenesis in MCAO rats but were not able to demonstrate any treatment effect. Previous studies that reported increased angiogenesis and improved behavioral performance employed a follow-up period between 14 and 21 days (14, 17, 18). In our study, it may be that ADMSCs only transiently promoted angiogenesis during the early behavioral recovery and this was missed when evaluated after a relatively long follow-up. A temporal monitoring of the extent of angiogenesis at different time points until the end of the follow-up could have been helpful. Furthermore, as previously stated, perhaps a BrdU/RECA-1 double labeling to differentiate old and new blood vessels would have better revealed a treatment effect.

The glial cell response to brain ischemia and glial scar formation are also involved in perilesional remodeling and functional recovery (75). The presence of a glial scar has been claimed to impair repair processes (76). MSCs including ADMSCs decrease perilesional GFAP labeling, a marker for glial cells (17). We detected an increase in the number of glial cells in the perilesional cortex, but no differences between the MCAO groups. In addition, there were no differences in glial scar volume between the MCAO groups. Nonetheless, possible temporal changes in glial cell phenotype attributable to either the ADMSCs and/or the enriched environment cannot be excluded.

Clinical Implications

Most stroke patients receive some form of rehabilitation. Nonetheless, there is still no agreement about which modalities should be used nor how and when they should be applied. Rehabilitation is a major confounding factor in clinical stem cell trials and it should be taken into account or carefully controlled. Our data strongly support this view. Indeed, the importance of rehabilitation has already been included into the STEPS 3 recommendations (47). However, complex study designs are needed to discriminate add-on and stand-alone therapeutic effects, although these might not be feasible in experimental studies and even less so in clinical trials.

CONCLUSIONS

The combination of multiple regenerative treatments to improve stroke recovery is an attractive strategy. Here we demonstrated that intravenous delivery of ADMSCs and housing in an enriched environment is a safe treatment and improved the behavioral recovery of MCAO rats with a further improvement associated with the combined treatment. The treatment effect was not associated with neuroprotection or altered perilesional angiogenesis or the extent of glial scar formation, but this does not exclude the possibility that there was altered neuronal excitability or axonal sprouting. Further studies with greater statistical power to cope with complex study designs will be needed to determine the optimal protocol and to reveal the true value of combination therapies in stroke.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

JM and EO conducted behavioral testing. PK and LC carried out MCAO operations. MJ and SM were responsible for preparation of cells. AB performed histological staining and statistical analysis. MM and SZ were responsible for stereological analysis. AB and JJ drafted the manuscript. All authors approved the final manuscript.

FUNDING

This work was supported by RESSTORE project (www.resstore.eu) funded by the European Commission under the Horizon2020 program (grant number 681044), VTR funding from Kuopio University Hospital and partly supported by the Academy of Finland, Business Finland and Competitive State Research Financing of the Expert Responsibility area of Tampere University Hospital.

ACKNOWLEDGMENTS

The authors thank Anaick Moisan for cell production; Anne Kankkunen, Jenni Niiranen, and Jenni Matilainen for their excellent technical assistance. Giedrius Kalesnykas and Xavier Ekolle Ndode-Ekane are acknowledged for help in planning the stereological analysis. We also want to thank RESSTORE Steering Committee members: Olivier Detante, Exuperio Díez-Tejedor, Blanca Fuentes, Marc Hommel, Assia Jaillard, Robert Mikulik, Anaick Moisan, Francisco Moniche, Joan Montaner, Alejandro Bustamante, Keith Muir, Heikki Numminen, David Koubi, Claire Bollart and Tuija Keinonen for their important support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.00235/full#supplementary-material

REFERENCES

1. Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, et al. Heart disease and stroke statistics-2017 update: a report from the american heart association. *Circulation*. (2017) 135:e146–603. doi: 10.1161/CIR.0000000000000485
2. Krishnamurthi RV, Moran AE, Feigin VL, Barker-Collo S, Norrving B, Mensah GA, et al. Stroke prevalence, mortality and disability-adjusted life years in adults aged 20-64 years in 1990-2013: data from the global burden of disease 2013 study. *Neuroepidemiology*. (2015) 45:190–202. doi: 10.1159/000441098
3. World Health Organization. *The Atlas of Heart Disease and Stroke* [Internet]. Geneva: World Health Organization (2004). Available online at: http://apps.who.int/iris/handle/10665/43007 (accessed Dec 12, 2018).
4. Teasell R, Rice D, Richardson M, Campbell N, Madady M, Hussein N, et al. The next revolution in stroke care. *Expert Rev Neurother*. (2014) 14:1307–14. doi: 10.1586/14737175.2014.968130
5. Jovin TG, Chamorro A, Cobo E, de Miquel MA, Molina CA, Rovira A, et al. Thrombectomy within 8 hours after symptom onset in ischemic stroke. *N Engl J Med*. (2015) 372:2296–306. doi: 10.1056/NEJMoa1503780
6. Grossman AW, Broderick JP. Advances and challenges in treatment and prevention of ischemic stroke. *Ann Neurol*. (2013) 74:363–72. doi: 10.1002/ana.23993
7. Zhou F, Gao S, Wang L, Sun C, Chen L, Yuan P, et al. Human adipose-derived stem cells partially rescue the stroke syndromes by promoting spatial learning and memory in mouse middle cerebral artery occlusion model. *Stem Cell Res Ther*. (2015) 6:92. doi: 10.1186/s13287-015-0078-1
8. Patrikoski M, Sivula J, Huhtala H, Helminen M, Salo F, Mannerström B, et al. Different culture conditions modulate the immunological

properties of adipose stem cells. *Stem Cells Transl Med*. (2014) 3:1220–30. doi: 10.5966/sctm.2013-0201
9. Ra JC, Shin IS, Kim SH, Kang SK, Kang BC, Lee HY, et al. Safety of intravenous infusion of human adipose tissue-derived mesenchymal stem cells in animals and humans. *Stem Cells Dev*. (2011) 20:1297–308. doi: 10.1089/scd.2010.0466
10. Gutiérrez-Fernández M, Rodríguez-Frutos B, Otero-Ortega L, Ramos-Cejudo J, Fuentes B, Díez-Tejedor E. Adipose tissue-derived stem cells in stroke treatment: from bench to bedside. *Discov Med*. (2013) 16:37–43.
11. Mezey E. The therapeutic potential of bone marrow-derived stromal cells. *J Cell Biochem*. (2011) 112:2683–7. doi: 10.1002/jcb.23216
12. Sasaki Y, Sasaki M, Kataoka-Sasaki Y, Nakazaki M, Nagahama H, Suzuki J, et al. Synergic effects of rehabilitation and intravenous infusion of mesenchymal stem cells after stroke in rats. *Phys Ther*. (2016) 96:1791–8. doi: 10.2522/ptj.20150504
13. Zhang Y-X, Yuan M-Z, Cheng L, Lin L-Z, Du H-W, Chen R-H, et al. Treadmill exercise enhances therapeutic potency of transplanted bone mesenchymal stem cells in cerebral ischemic rats via anti-apoptotic effects. *BMC Neurosci*. (2015) 16:56. doi: 10.1186/s12868-015-0196-9
14. Leu S, Lin Y-C, Yuen C-M, Yen C-H, Kao Y-H, Sun C-K, et al. Adipose-derived mesenchymal stem cells markedly attenuate brain infarct size and improve neurological function in rats. *J Transl Med*. (2010) 8:63. doi: 10.1186/1479-5876-8-63
15. Oh S-H, Choi C, Chang D-J, Shin D-A, Lee N, Jeon I, et al. Early neuroprotective effect with lack of long-term cell replacement effect on experimental stroke after intra-arterial transplantation of adipose-derived mesenchymal stromal cells. *Cytotherapy*. (2015) 17:1090–103. doi: 10.1016/j.jcyt.2015.04.007
16. Chi L, Huang Y, Mao Y, Wu K, Zhang L, Nan G. Tail vein infusion of adipose-derived mesenchymal stem cell alleviated inflammatory response and improved blood brain barrier condition by suppressing endoplasmic reticulum stress in a middle cerebral artery occlusion rat model. *Med Sci Monit*. (2018) 24:3946–57. doi: 10.12659/MSM.907096
17. Gutiérrez-Fernández M, Rodríguez-Frutos B, Ramos-Cejudo J, Otero-Ortega L, Fuentes B, Vallejo-Cremades MT, et al. Comparison between xenogeneic and allogeneic adipose mesenchymal stem cells in the treatment of acute cerebral infarct: proof of concept in rats. *J Transl Med*. (2015) 13:46. doi: 10.1186/s12967-015-0406-3
18. Gutiérrez-Fernández M, Rodríguez-Frutos B, Ramos-Cejudo J, Teresa Vallejo-Cremades M, Fuentes B, Cerdán S, et al. Effects of intravenous administration of allogenic bone marrow- and adipose tissue-derived mesenchymal stem cells on functional recovery and brain repair markers in experimental ischemic stroke. *Stem Cell Res Ther*. (2013) 4:11. doi: 10.1186/scrt159
19. Otero-Ortega L, Gutiérrez-Fernández M, Ramos-Cejudo J, Rodríguez-Frutos B, Fuentes B, Sobrino T, et al. White matter injury restoration after stem cell administration in subcortical ischemic stroke. *Stem Cell Res Ther*. (2015) 6:121. doi: 10.1186/s13287-015-0111-4
20. Huang H, Lin F, Jiang J, Chen Y, Mei A, Zhu P. Effects of intra-arterial transplantation of adipose-derived stem cells on the expression of netrin-1 and its receptor DCC in the peri-infarct cortex after experimental stroke. *Stem Cell Res Ther*. (2017) 8:223. doi: 10.1186/s13287-017-0671-6
21. Shen LH, Li Y, Chen J, Zhang J, Vanguri P, Borneman J, et al. Intracarotid transplantation of bone marrow stromal cells increases axon-myelin remodeling after stroke. *Neuroscience*. (2006) 137:393–9. doi: 10.1016/j.neuroscience.2005.08.092
22. Hansen TM, Moss AJ, Brindle NPJ. Vascular endothelial growth factor and angiopoietins in neurovascular regeneration and protection following stroke. *Curr Neurovasc Res*. (2008) 5:236–45. doi: 10.2174/156720208786413433
23. Slevin M, Krupinski J, Slowik A, Kumar P, Szczudlik A, Gaffney J. Serial measurement of vascular endothelial growth factor and transforming growth factor-beta1 in serum of patients with acute ischemic stroke. *Stroke*. (2000) 31:1863–70. doi: 10.1161/01.STR.31.8.1863
24. Cramer SC, Nelles G, Benson RR, Kaplan JD, Parker RA, Kwong KK, et al. A functional MRI study of subjects recovered from hemiparetic stroke. *Stroke*. (1997) 28:2518–27. doi: 10.1161/01.STR.28.12.2518
25. Jiang W, Liang G, Li X, Li Z, Gao X, Feng S, et al. Intracarotid transplantation of autologous adipose-derived mesenchymal stem cells significantly improves neurological deficits in rats after MCAo. *J Mater Sci Mater Med*. (2014) 25:1357–66. doi: 10.1007/s10856-014-5157-9
26. Liu X-L, Zhang W, Tang S-J. Intracranial transplantation of human adipose-derived stem cells promotes the expression of neurotrophic factors and nerve repair in rats of cerebral ischemia-reperfusion injury. *Int J Clin Exp Pathol*. (2014) 7:174–83.
27. Chen K-H, Chen C-H, Wallace CG, Yuen C-M, Kao G-S, Chen Y-L, et al. Intravenous administration of xenogenic adipose-derived mesenchymal stem cells (ADMSC) and ADMSC-derived exosomes markedly reduced brain infarct volume and preserved neurological function in rat after acute ischemic stroke. *Oncotarget*. (2016) 7:74537–56. doi: 10.18632/oncotarget.12902
28. Díez-Tejedor E, Gutiérrez-Fernández M, Martínez-Sánchez P, Rodríguez-Frutos B, Ruiz-Ares G, Lara ML, et al. Reparative therapy for acute ischemic stroke with allogeneic mesenchymal stem cells from adipose tissue: a safety assessment: a phase II randomized, double-blind, placebo-controlled, single-center, pilot clinical trial. *J Stroke Cerebrovasc Dis*. (2014) 23:2694–700. doi: 10.1016/j.jstrokecerebrovasdis.2014.06.011
29. Langhorne P, Bernhardt J, Kwakkel G. Stroke rehabilitation. *Lancet*. (2011) 377:1693–702. doi: 10.1016/S0140-6736(11)60325-5
30. Gabriel-Salazar M, Morancho A, Rodriguez S, Buxó X, García-Rodríguez N, Colell G, et al. Importance of angiogenin and endothelial progenitor cells after rehabilitation both in ischemic stroke patients and in a mouse model of cerebral ischemia. *Front Neurol*. (2018) 9:508. doi: 10.3389/fneur.2018.00508
31. Ke Z, Yip SP, Li L, Zheng X-X, Tong K-Y. The effects of voluntary, involuntary, and forced exercises on brain-derived neurotrophic factor and motor function recovery: a rat brain ischemia model. *PLoS ONE*. (2011) 6:e16643. doi: 10.1371/journal.pone.0016643
32. Zhang C, Zou Y, Li K, Li C, Jiang Y, Sun J, et al. Different effects of running wheel exercise and skilled reaching training on corticofugal tract plasticity in hypertensive rats with cortical infarctions. *Behav Brain Res*. (2018) 336:166–72. doi: 10.1016/j.bbr.2017.09.002
33. El Amki M, Baumgartner P, Bracko O, Luft AR, Wegener S. Task-specific motor rehabilitation therapy after stroke improves performance in a different motor task: translational evidence. *Transl Stroke Res*. (2017) 8:347–50. doi: 10.1007/s12975-016-0519-x
34. Jones TA, Chu CJ, Grande LA, Gregory AD. Motor skills training enhances lesion-induced structural plasticity in the motor cortex of adult rats. *J Neurosci*. (1999) 19:10153–63. doi: 10.1523/JNEUROSCI.19-22-10153.1999
35. Spalletti C, Lai S, Mainardi M, Panarese A, Ghionzoli A, Alia C, et al. A robotic system for quantitative assessment and poststroke training of forelimb retraction in mice. *Neurorehabil Neural Repair*. (2014) 28:188–96. doi: 10.1177/1545968313506520
36. Vigaru B, Lambercy O, Graber L, Fluit R, Wespe P, Schubring-Giese M, et al. A small-scale robotic manipulandum for motor training in stroke rats. *IEEE Int Conf Rehabil Robot*. (2011) 2011:5975349. doi: 10.1109/ICORR.2011.5975349
37. Hicks AU, Lappalainen RS, Narkilahti S, Suuronen R, Corbett D, Sivenius J, et al. Transplantation of human embryonic stem cell-derived neural precursor cells and enriched environment after cortical stroke in rats: cell survival and functional recovery. *Eur J Neurosci*. (2009) 29:562–74. doi: 10.1111/j.1460-9568.2008.06599.x
38. Zhang X, Chen X-P, Lin J-B, Xiong Y, Liao W-J, Wan Q. Effect of enriched environment on angiogenesis and neurological functions in rats with focal cerebral ischemia. *Brain Res*. (2017) 1655:176–85. doi: 10.1016/j.brainres.2016.11.001
39. Janssen H, Bernhardt J, Collier JM, Sena ES, McElduff P, Attia J, et al. An enriched environment improves sensorimotor function post-ischemic stroke. *Neurorehabil Neural Repair*. (2010) 24:802–13. doi: 10.1177/1545968310372092
40. Yu K, Wu Y, Zhang Q, Xie H, Liu G, Guo Z, et al. Enriched environment induces angiogenesis and improves neural function outcomes in rat stroke model. *J Neurol Sci*. (2014) 347:275–80. doi: 10.1016/j.jns.2014.10.022
41. Sommer CJ, Schäbitz W-R. Fostering poststroke recovery: towards combination treatments. *Stroke*. (2017) 48:1112–9. doi: 10.1161/STROKEAHA.116.013324
42. Zhao K, Li R, Bi S, Li Y, Liu L, Jia Y-L, et al. Combination of mild therapeutic hypothermia and adipose-derived stem cells for ischemic brain injury. *Neural Regen Res*. (2018) 13:1759–70. doi: 10.4103/1673-5374.238617

43. de Fátima Dos Santos Sampaio M, Marcilio FDS, Giraldi-Guimarães A. Does treatment with bone marrow mononuclear cells recover skilled motor function after focal cortical ischemia? Analysis with a forelimb skilled motor task in rats. *Brain Res*. (2013) 1492:130–9. doi: 10.1016/j.brainres.2012.11.026
44. Kim J-M, Lee S-T, Chu K, Jung K-H, Song E-C, Kim S-J, et al. Systemic transplantation of human adipose stem cells attenuated cerebral inflammation and degeneration in a hemorrhagic stroke model. *Brain Res*. (2007) 1183:43–50. doi: 10.1016/j.brainres.2007.09.005
45. Hicks AU, Hewlett K, Windle V, Chernenko G, Ploughman M, Jolkkonen J, et al. Enriched environment enhances transplanted subventricular zone stem cell migration and functional recovery after stroke. *Neuroscience*. (2007) 146:31–40. doi: 10.1016/j.neuroscience.2007.01.020
46. Diamandis T, Borlongan CV. One, two, three steps toward cell therapy for stroke. *Stroke*. (2015) 46:588–91. doi: 10.1161/STROKEAHA.114.007105
47. Savitz SI, Cramer SC, Wechsler L, STEPS 3 Consortium. Stem cells as an emerging paradigm in stroke 3: enhancing the development of clinical trials. *Stroke*. (2014) 45:634–9. doi: 10.1161/STROKEAHA.113.003379
48. Mitkari B, Nitzsche F, Kerkelä E, Kuptsova K, Huttunen J, Nystedt J, et al. Human bone marrow mesenchymal stem/stromal cells produce efficient localization in the brain and enhanced angiogenesis after intra-arterial delivery in rats with cerebral ischemia, but this is not translated to behavioral recovery. *Behav Brain Res*. (2014) 259:50–9. doi: 10.1016/j.bbr.2013.10.030
49. West MJ. Space balls revisited: stereological estimates of length with virtual isotropic surface probes. *Front Neuroanat*. (2018) 12:49. doi: 10.3389/fnana.2018.00049
50. Huusko N, Römer C, Ndode-Ekane XE, Lukasiuk K, Pitkänen A. Loss of hippocampal interneurons and epileptogenesis: a comparison of two animal models of acquired epilepsy. *Brain Struct Funct*. (2015) 220:153–91. doi: 10.1007/s00429-013-0644-1
51. Corbett D, Carmichael ST, Murphy TH, Jones TA, Schwab ME, Jolkkonen J, et al. Enhancing the alignment of the preclinical and clinical stroke recovery research pipeline: consensus-based core recommendations from the stroke recovery and rehabilitation roundtable translational working group. *Neurorehabil Neural Repair*. (2017) 31:699–707. doi: 10.1177/1545968317724285
52. Buga AM, Margaritescu C, Scholz CJ, Radu E, Zelenak C, Popa-Wagner A. Transcriptomics of post-stroke angiogenesis in the aged brain. *Front Aging Neurosci*. (2014) 6:44. doi: 10.3389/fnagi.2014.00044
53. Popa-Wagner A, Buga A-M, Doeppner TR, Hermann DM. Stem cell therapies in preclinical models of stroke associated with aging. *Front Cell Neurosci*. (2014) 8:347. doi: 10.3389/fncel.2014.00347
54. Ergul A, Hafez S, Fouda A, Fagan SC. Impact of comorbidities on acute injury and recovery in preclinical stroke research: focus on hypertension and diabetes. *Transl Stroke Res*. (2016) 7:248–60. doi: 10.1007/s12975-016-0464-8
55. Boltze J, Arnold A, Walczak P, Jolkkonen J, Cui L, Wagner D-C. The dark side of the force - constraints and complications of cell therapies for stroke. *Front Neurol*. (2015) 6:155. doi: 10.3389/fneur.2015.00155
56. Cho YJ, Song HS, Bhang S, Lee S, Kang BG, Lee JC, et al. Therapeutic effects of human adipose stem cell-conditioned medium on stroke. *J Neurosci Res*. (2012) 90:1794–802. doi: 10.1002/jnr.23063
57. Hess DC, Wechsler LR, Clark WM, Savitz SI, Ford GA, Chiu D, et al. Safety and efficacy of multipotent adult progenitor cells in acute ischaemic stroke (MASTERS): a randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet Neurol*. (2017) 16:360–8. doi: 10.1016/S1474-4422(17)30046-7
58. Mays RW, Savitz SI. Intravenous cellular therapies for acute ischemic stroke. *Stroke*. (2018) 49:1058–65. doi: 10.1161/STROKEAHA.118.018287
59. Khabbal J, Kerkelä E, Mitkari B, Raki M, Nystedt J, Mikkonen V, et al. Differential clearance of rat and human bone marrow-derived mesenchymal stem cells from the brain after intra-arterial infusion in rats. *Cell Transplant*. (2015) 24:819–28. doi: 10.3727/096368914X679336
60. Borlongan CV, Hadman M, Sanberg CD, Sanberg PR. Central nervous system entry of peripherally injected umbilical cord blood cells is not required for neuroprotection in stroke. *Stroke*. (2004) 35:2385–9. doi: 10.1161/01.STR.0000141680.49960.d7
61. Schmidt A, Wellmann J, Schilling M, Strecker J-K, Sommer C, Schäbitz W-R, et al. Meta-analysis of the efficacy of different training strategies in animal models of ischemic stroke. *Stroke*. (2014) 45:239–47. doi: 10.1161/STROKEAHA.113.002048
62. Madinier A, Quattromani MJ, Sjölund C, Ruscher K, Wieloch T. Enriched housing enhances recovery of limb placement ability and reduces aggrecan-containing perineuronal nets in the rat somatosensory cortex after experimental stroke. *PLoS ONE*. (2014) 9:e93121. doi: 10.1371/journal.pone.0093121
63. Nygren J, Wieloch T. Enriched environment enhances recovery of motor function after focal ischemia in mice, and downregulates the transcription factor NGFI-A. *J Cereb Blood Flow Metab*. (2005) 25:1625–33. doi: 10.1038/sj.jcbfm.9600157
64. Johansson BB, Ohlsson AL. Environment, social interaction, and physical activity as determinants of functional outcome after cerebral infarction in the rat. *Exp Neurol*. (1996) 139:322–7. doi: 10.1006/exnr.1996.0106
65. Puurunen K, Jolkkonen J, Sirviö J, Haapalinna A, Sivenius J. Selegiline combined with enriched-environment housing attenuates spatial learning deficits following focal cerebral ischemia in rats. *Exp Neurol*. (2001) 167:348–55. doi: 10.1006/exnr.2000.7563
66. Biernaskie J, Corbett D. Enriched rehabilitative training promotes improved forelimb motor function and enhanced dendritic growth after focal ischemic injury. *J Neurosci*. (2001) 21:5272–80. doi: 10.1523/JNEUROSCI.21-14-05272.2001
67. Dirnagl U, Fisher M. REPRINT: international, multicenter randomized preclinical trials in translational stroke research: it is time to act. *Stroke*. (2012) 43:1453–4. doi: 10.1161/STROKEAHA.112.653709
68. Wahl AS, Omlor W, Rubio JC, Chen JL, Zheng H, Schröter A, et al. Neuronal repair. Asynchronous therapy restores motor control by rewiring of the rat corticospinal tract after stroke. *Science*. (2014) 344:1250–5. doi: 10.1126/science.1253050
69. Fernández-García L, Pérez-Rigueiro J, Martinez-Murillo R, Panetsos F, Ramos M, Guinea GV, et al. Cortical reshaping and functional recovery induced by silk fibroin hydrogels-encapsulated stem cells implanted in stroke animals. *Front Cell Neurosci*. (2018) 12:296. doi: 10.3389/fncel.2018.00296
70. Cassidy JM, Cramer SC. Spontaneous and therapeutic-induced mechanisms of functional recovery after stroke. *Transl Stroke Res*. (2017) 8:33–46. doi: 10.1007/s12975-016-0467-5
71. Seitz RJ, Donnan GA. Recovery potential after acute stroke. *Front Neurol*. (2015) 6:238. doi: 10.3389/fneur.2015.00238
72. Crafts TD, Jensen AR, Blocher-Smith EC, Markel TA. Vascular endothelial growth factor: therapeutic possibilities and challenges for the treatment of ischemia. *Cytokine*. (2015) 71:385–93. doi: 10.1016/j.cyto.2014.08.005
73. Thored P, Wood J, Arvidsson A, Cammenga J, Kokaia Z, Lindvall O. Long-term neuroblast migration along blood vessels in an area with transient angiogenesis and increased vascularization after stroke. *Stroke*. (2007) 38:3032–9. doi: 10.1161/STROKEAHA.107.488445
74. Chopp M, Li Y. Treatment of neural injury with marrow stromal cells. *Lancet Neurol*. (2002) 1:92–100. doi: 10.1016/S1474-4422(02)00040-6
75. Hermann DM, Chopp M. Promoting brain remodelling and plasticity for stroke recovery: therapeutic promise and potential pitfalls of clinical translation. *Lancet Neurol*. (2012) 11:369–80. doi: 10.1016/S1474-4422(12)70039-X
76. Becerra-Calixto A, Cardona-Gómez GP. The role of astrocytes in neuroprotection after brain stroke: potential in cell therapy. *Front Mol Neurosci*. (2017) 10:88. doi: 10.3389/fnmol.2017.00088

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.

Copyright © 2019 Mu, Bakreen, Juntunen, Korhonen, Oinonen, Cui, Myllyniemi, Zhao, Miettinen and Jolkkonen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Neurology

ORIGINAL RESEARCH
published: 05 April 2019
doi: 10.3389/fneur.2019.00335

Persistent Quantitative Vitality of Stem Cell Graft Is Necessary for Stabilization of Functional Brain Networks After Stroke

Claudia Green[1], *Anuka Minassian*[1], *Stefanie Vogel*[1], *Michael Diedenhofen*[1], *Dirk Wiedermann*[1] *and Mathias Hoehn*[1,2,3]*

[1] *In-vivo-NMR Laboratory, Max Planck Institute for Metabolism Research, Cologne, Germany,* [2] *Department of Radiology, Leiden University Medical Center, Leiden, Netherlands,* [3] *Percuros B.V., Enschede, Netherlands*

Stem cell treatment after stroke has demonstrated substantial outcome improvement. However, monitoring of stem cell fate *in vivo* is still challenging and not routinely performed, yet important to quantify the role of the implanted stem cells on lesion improvement; in several studies even mortality of the graft has been reported. Resting state functional magnetic resonance imaging (rs-fMRI) is a highly sensitive imaging modality to monitor the brain-wide functional network alterations of many brain diseases *in vivo*. We monitor for 3 months the functional connectivity changes after intracortical stem cell engraftment in large, cortico-striatal ($n = 9$), and in small, striatal ($n = 6$) ischemic lesions in the mouse brain with non-invasive rs-fMRI on a 9.4T preclinical MRi scanner with GE-EPI sequence. Graft vitality is continuously recorded by bioluminescence imaging (BLI) roughly every 2 weeks after implantation of 300 k neural stem cells. In cortico-striatal lesions, the lesion extension induces graft vitality loss, in consequence leading to a parallel decrease of functional connectivity strength after a few weeks. In small, striatal lesions, the graft vitality is preserved for the whole observation period and the functional connectivity is stabilized at values as in the pre-stroke situation. But even here, at the end of the observation period of 3 months, the functional connectivity strength is found to decrease despite preserved graft vitality. We conclude that quantitative graft viability is a necessary but not sufficient criterion for functional neuronal network stabilization after stroke. Future studies with even longer time periods after stroke induction will need to identify additional players which have negative influence on the functional brain networks.

Keywords: stem cell graft, graft vitality, stroke, functional neuronal networks, resting state fMRI, bioluminescence imaging

OPEN ACCESS

Edited by:
Johannes Boltze, Fraunhofer-Institut für Zelltherapie und Immunologie (IZI), Germany

Reviewed by:
Zaal Kokaia, Lund Stem Cell Center, Sweden
Alfonso Mastropietro, Institute of Bioimaging and Molecular Physiology, Italy

***Correspondence:**
Mathias Hoehn
mathias@sf.mpg.de

Specialty section:
This article was submitted to Stroke, a section of the journal Frontiers in Neurology

Received: *09 January 2019*
Accepted: *19 March 2019*
Published: *05 April 2019*

Citation:
Green C, Minassian A, Vogel S, Diedenhofen M, Wiedermann D and Hoehn M (2019) Persistent Quantitative Vitality of Stem Cell Graft Is Necessary for Stabilization of Functional Brain Networks After Stroke. Front. Neurol. 10:335. doi: 10.3389/fneur.2019.00335

INTRODUCTION

Ischemic stroke is the third most common cause of death and the leading cause for disabilities worldwide (1). There is still no clinically accepted therapy available except for the FDA-approved thrombolysis, which is only available to a small proportion of patients due to a limited time window for treatment and serious risks for side effects (2). Therefore, new therapeutic approaches

are urgently required. With the enormous progress in stem cell biology during the past several years application of stem cells for regenerative therapy has rapidly gained high interest. Several experimental studies on rodent models of stroke have reported outcome improvements after stem cell implantations (3–8). Most of these studies focused on the stem cell effect on lesion volume or relied on behavioral assays for the read-out of outcome improvement (7–11). Other reports concentrated mainly on the structural and functional integration of the stem cell grafts in the host tissue (12, 13).

A novel approach based on resting state functional magnetic resonance imaging (rs-fMRI) accesses the functional networks of the whole brain and thereby allows to reveal functional network alterations during brain diseases (14–17). Applying rs-fMRI to stroke we (18) and others (19–21) have reported on the whole-brain network deficits after stroke, characterized by substantial decrease of the functional connectivity strength and by the far reaching extension of this decrease to both hemispheres, irrespective of localization of the ischemic tissue.

In the first report on response of functional networks to stem cell implantation after induction of large cortico-striatal ischemic lesion we described an early paracrine effect (18). This paracrine effect was reflected by the early and complete stabilization of the functional connectivity by the stem cell graft. However, in that report, this stabilization was lost after a few weeks when the graft viability was substantially decreasing by ∼70% of its original level (18), as monitored by bioluminescence imaging (BLI).

In the present investigation we compare functional networks in large cortico-striatal lesions, in correspondence with our previous study (18), with those of animals with lesion extension limited to the striatum. This comparison is of specific interest, as it can provide insight on the effect of later expansion of the ischemic territory on the cortical graft. We hypothesized that graft vitality in the striatal lesion group will persist long-term and that in consequence the functional connectivity stabilization will continue, thus leading to an enduring therapeutic effect. To that aim, we monitored in all animals the graft viability by BLI (22–24) and repetitively recorded rs-fMRI up to 3 months after stroke induction. We compared the results of both groups, with small striatal and with large cortico-striatal lesions, and looked for connections between graft viability and successful functional connectivity stabilization for therapeutic assessment.

MATERIALS AND METHODS

Experimental Animals and Experimental Design

All animal experiments were carried out in accordance with the guidelines of the German Animal Welfare Act and approved by the local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen). Animals were socially housed under a fixed 12:12 h light/darkness cycle with *ad libitum* access to food and water.

A total of 15 adult, male NMRI-*Foxn1*$^{nu/nu}$ mice (12–14 weeks old, 32–37 g; Janvier, France) were used in this study. All surgical experiments were performed under anesthesia with 1.5–2% Isoflurane in a mixture of 70/30% N_2O/O_2 atmosphere. Analgetic treatment included subcutaneous injection of 4 mg/kg Carprofen (Rimadyl, Zoetis, Berlin, Germany) twice a day, for 3 days following surgery. Two days after stroke induction by the filament occlusion method (18, 23), the animals received an intracortical stem cell implantation on the ipsilateral hemisphere. Resting state fMRI was performed 1 week before, as well as 1, 2, 4, 8, and 12 weeks after stroke induction and cell implantation (2 days after stroke induction), while vitality of the transgenic graft was monitored by BLI at weeks 2, 3, 4, 5, 7, 9, 10, and 12 after stroke.

Transient Middle Cerebral Artery Occlusion

After MRI baseline measurements, focal ischemic stroke was induced by transient occlusion of the middle cerebral artery (MCA) with an intraluminal filament as described previously (23, 25). In brief, mice were anesthetized with Isoflurane and the right common carotid artery (CCA), the external and the internal carotid artery (ICA) were exposed. A rubber-coated filament (length of 20 mm, diameter of 170 μm at the tip; Doccol Corp., Sharon, USA) was inserted into the ICA until blockage of the MCA. After 30 min occlusion time, the filament was removed and the CCA was ligated permanently. Analgesic medication was started directly before surgery and further provided during 3 days thereafter. Location and size of the ischemic damage was assessed at 48 h after stroke induction by MRI.

Intracortical Implantation and Monitoring of Transgenic Neural Stem Cells

The commercially available human neural stem cell line H9-NSC (WA09, Life Technologies) was lentivirally transduced with the construct EF1α-Luc2-T2A-eGFP to express the light emitting imaging reporter firefly luciferase (*Luc2*) and an enhanced green fluorescence protein (*eGFP*) under the constitutive promotor of the elongation factor 1 alpha (EF1α), as described earlier (26).

Two days after stroke induction and before cell implantation, all animals received a T2-weighted MRI scan to determine successful stroke induction and, based on MRI results, were divided into cortico-striatal and striatal lesion group, respectively. For cell implantation, NMRI-*Foxn1*$^{nu/nu}$ mice were anesthetized and the head was fixed in a stereotaxic frame (Stoelting, Dublin, Ireland). A small hole was drilled into the skull above the planned injection site, and a Hamilton syringe (26 G needle) was slowly inserted ipsilesionally into the somatosensory cortex (S1) at the injection site with AP: +0.5 mm, DV: −0.5 mm according to bregma. The lateral coordinate had previously been selected individually based on the respective T2-weighted MRI at 48 h and was set to implant the cells adjacent to but outside the lesion for each individual animal, at approximately L: 1–2 mm.

Nine animals which had a cortico-striatal stroke and six animals with a striatal stroke were injected with a total of 2 μl of H9-EF1α-Luc2-T2A-eGFP cells at a concentration of 150,000 cells/μl, resuspended in Hank's balanced salt solution (HBSS, Life Technologies), at a flow rate of 150 nl/min. After injection, the needle was kept in place for 5 min before withdrawal.

Cell vitality was monitored via BLI, which was recorded once every 1–2 weeks for the duration of the study. For BLI measurements the mice were intraperitoneally injected with 300 mg/kg D-Luciferin sodium salt (Synchem, Felsberg, Germany) solved in Dulbecco's phosphate-buffered saline (Life Technologies) and subsequently anesthetized with a mixture of 2% Isoflurane in 70/30% N_2O/O_2 atmosphere (24). Photon Emission (PE) was recorded for 30 min with the Photon Imager IVIS SPECTRUM CT (Perkin-Elmer, Waltham, MA, USA) under Isoflurane anesthesia.

Structural and Functional MRI Data Acquisition

All experiments were conducted on a small animal 9.4 T horizontal MRI system with a 20 cm bore diameter and actively shielded gradient coils (BGA12S2, >660 mT/m, Bruker BioSpin, Ettlingen, Germany). RF excitation and signal reception were performed with a 1H quadrature cryogenic surface coil (CryoProbe, Bruker BioSpin). Image acquisition was executed with ParaVision 5.1 software (Bruker BioSpin GmbH). Physiological parameters were monitored with the SA Instruments 1025T System (SA Instruments, NY, USA) and recorded with DASYlab Software (Measurement Computing, Norton, USA). Body temperature was measured via a fiber optic rectal probe (SA Instruments, NY, USA) and kept constant at 37°C ± 1.0°C by a water circulating system (medres, Cologne, Germany). Animals were anesthetized with 2% Isoflurane in a mixture of 70/30% N_2/O_2 and the head was fixated in the animal cradle with ear bars and a tooth bar in a nose cone with continuous gas flow. At the beginning of the imaging session, Isoflurane was reduced to 1.5%.

Functional activity was acquired as resting state functional MRI. All MRI sessions were preceded by a three-plane scout scan (Tripilot), adjustment of the RF signal receiver gain, and a FieldMap with consecutive local shim to optimize magnetic field homogeneity and image quality. An anatomical reference scan was acquired with a T2-weighted TurboRARE sequence with a field of view (FOV) of 17.5 × 17.5 mm^2, 48 contiguous slices of 0.2 mm slice thickness, matrix dimension of 256 × 256, repetition time (TR) = 5,500 ms, echo time (TE) = 32.5 ms, and a RARE factor of 8 with two averages. Moreover, a T2-weighted spin echo sequence was recorded at 48 h after stroke induction, directly before the stem cell grafting, with equivalent parameters.

A bolus of 0.1 mg/kg Medetomidine (Domitor®, Elanco), suspended in 250 µl NaCl, was administered subcutaneously 15–20 min prior to the functional imaging scan, with subsequent reduction of Isoflurane level to 0.5%, following an earlier reported protocol (18, 27). A gradient-echo echo-planar imaging (GE-EPI) sequence was used for rs-fMRI with the following parameters: FOV: 17.5 × 17.5 mm^2, matrix size: 96 × 96, in-plane resolution: 182 × 182 μm^2, TR = 2,840 ms, and TE = 18 ms. One hundred and five image sets were acquired with 16 slices each, with slice thickness of 0.5 mm and inter-slice gap of 0.1 mm, recorded non-interleaved and covering the whole forebrain, starting only after a minimal time of 10 min on reduced Isoflurane levels.

rs-fMRI Data Processing

All datasets were brain extracted, slice-wise motion corrected with FSL (FMRIB Software Library; http://www.fmrib.ox.ac.uk/fsl) and linearly detrended (28). To allow for comparison of different imaging sessions for analysis of specific Regions of Interest (ROIs), we co-registered our datasets to publicly available mouse brain atlases of the whole brain (29) and the neocortex (30) through the following multi-step process: (1) First, an in-house made MRI mouse brain template was warped to both brain atlases with FSL. (2) The acquired anatomical reference scan from each individual session was linearly co-registered to the in-house mouse brain template and (3) the functional datasets to the anatomical datasets via rigid-body transformation. (4) Finally, the co-registration results themselves were discarded but the transformation matrices from all steps were concatenated, inverted, and then applied to the mouse brain atlas to fit the atlas to the individual original data, thus avoiding deformation bias of the raw data. Six ROIs, or nodes, were extracted from the sensorimotor network, separate for each hemisphere: The primary and secondary motor cortex (M1/M2), the primary somatosensory cortex excluding the limb regions (S1 w/o limbs), the S1 fore- and hindpaw limb regions (S1 limbs), the secondary somatosensory cortex (S2), the thalamus (Th), and the caudate putamen (CPu) (**Supplementary Figure 1**). Bilateral ROIs were only analyzed until week 2 after stroke induction for accuracy purposes of ROI extraction. For longer periods after stroke, due to brain swelling and/or atrophy, only the contralateral hemisphere was analyzed.

Regression of physiological noise was performed by regressing out the recorded respiratory signals, motion parameters, and drifts up to the second order (28). Furthermore, the data were spatially smoothed in-plane with a Gaussian filter of FWHM = 0.3 mm. To limit contributions from low-frequency signal drifts and high-frequency physiological noise, the time series signal was bandpass-filtered to 0.01–0.08 Hz and normalized. Group-wise full Pearson correlation between pairs of ROIs (i.e., nodes) of the average time series was calculated with FSLNets (v0.6; www.fmrib.ox.ac.uk/fsl) and compiled in matrix form. All correlation values were transformed to z-values by taking the Fisher transformation of the r-values prior to averaging.

Statistical Analysis

Statistical analysis was performed with IBM SPSS 24 software (IBM Corporation, New York, NY, USA), Matlab 2014b (The MathWorks Inc., Natick, MA, USA) and GraphPad Prism v.8.0.2 for Windows (GraphPad Software, La Jolla California USA). Statistical significance levels were set to $^{*}p < 0.05$, $^{**}p < 0.005$.

Functional Connectivity

Univariate repeated measures ANOVA was used for the statistical analysis of the cross-correlation, within-group results and mixed ANOVA for the cross-correlation between group effects with *post-hoc* Bonferroni corrections, respectively. Most of the comparisons did not survive the most conservative statistical correction for multiple comparisons. We refrained from the less accurate statistical analysis but decided to present the changes

over time and the difference between groups as strong trends, unless where statistical significance is explicitly given.

Bioluminescence Imaging

A mixed-effects model with restricted maximum likelihood was applied to the BLI data to calculate statistical within-group effects of consecutive time points. The alpha level was set to 0.05, a Greenhouse-Geisser correction was enabled for non-spherical data and a Sidak's *post-hoc* test corrected for multiple comparison.

RESULTS

Ischemic Lesion Types

Forty eight hours after intraluminal filament occlusion of the MCA, all animals received T2-weighted MRI to confirm successful stroke induction. Two types of ischemic lesions were found on the MRI: a small lesion restricted to the striatum ($n = 6$), and a large lesion encompassing the striatum plus a large part of the cortex ($n = 9$). **Figure 1** presents the ischemic lesion on T2-weighted MRI of a representative mouse of both lesion types.

Vitality of the Stem Cell Graft During 12 Weeks

Forty eight hours after stroke induction, 300,000 neural stem cells, previously transduced to express luciferase under constitutive control, were implanted into the S1 cortex. BLI was performed every 1–2 weeks, starting at week 2 post MCA occlusion. At the time point two weeks after implantation, BLI signal intensity was strong and indistinguishable between both lesion groups. In the group of animals with striatal lesion, BLI signal remained high with little non-significant [$F_{(7,30)} = 4.02$] changes throughout the 12 weeks observation. In the cortico-striatal lesion group, BLI signal started to decrease, non-significantly [$F_{(7,59)} = 0.67$] between consecutive weeks, shortly after 2 weeks after cell implantation (**Figure 2A**). The quantitative analysis of the BLI signal intensity of the cell grafts is given in **Figure 2B**, clearly showing the persistent high level of stable graft viability for the striatal lesion group (black line). The strong signal decrease in the cortico-striatal lesion group to approximately 30% on average of the original average value indicated a vitality loss of a large graft fraction within the first weeks after implantation (red line).

Functional Neuronal Network Changes

Both Hemispheres During the First 2 Weeks After Stroke

Presentation of the functional connectivity strength of the sensorimotor networks in matrix form is schematically presented in **Figure 3** (top left), with values of both lesion groups combined in one matrix to better visualize the differences between them. Before stroke induction, the functional connectivity values (z-score values; **Figure 3**, top right) show equal correlation strength in both hemispheres within each lesion group, as presented in the two triangular segments of the matrix triangle for the respective group. Furthermore, comparison of the matrices of both groups does not show statistically significant differences before stroke induction (**Figure 3**, top right). At week 1 after stroke induction, a small, but non-significant increase of connectivity strength is noted in the cortico-striatal group across the whole brain, irrespective of the hemispheric side (**Figure 3**, bottom left; upper matrix triangle), while the functional connectivity values remained widely unchanged in the striatal group (**Figure 3** bottom left; lower matrix triangle). Interestingly, in the striatal group, the *intra*-hemispheric cross-correlations of both hemispheres remained indistinguishable among each other and from pre-stroke condition, while parts of the *inter*-hemispheric cross-correlations show a clearly demarcated rectangle with reduced values (**Figure 3**, bottom left, blue field in lower matrix triangle). After 2 weeks, the functional connectivity values have renormalized in both groups, with the *inter*-hemispheric connectivity field with reduced values of the

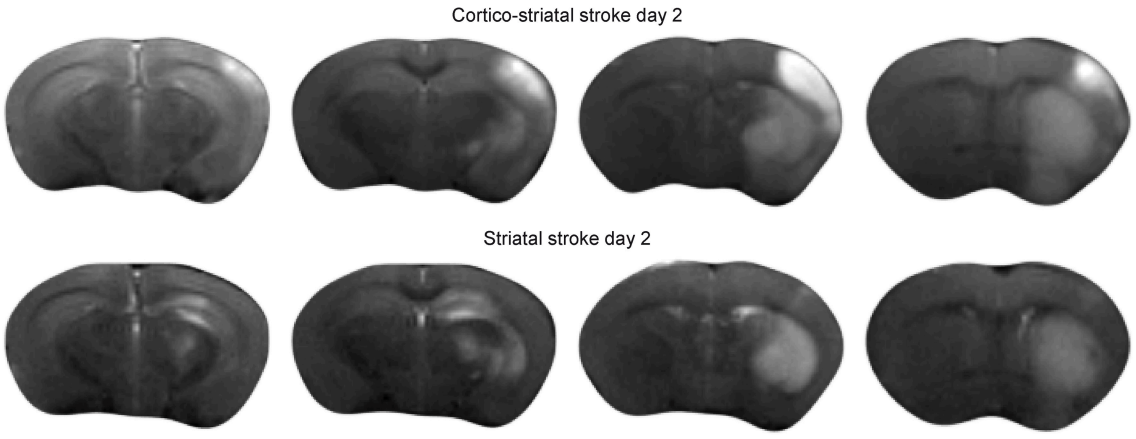


FIGURE 1 | Acute ischemic stroke lesion on T2-weighted MRI. Multislice T2-weighted MR images at 48 h after stroke induction, directly before implantation of neural stem cells. The hyperintensity on the MR images clearly demarcates the extent of the ischemia-affected tissue: the upper row shows the spread of the lesion across the cortical and sub-cortical tissue areas of a representative animal with cortico-striatal lesion. The lower row presents the situation for a representative mouse of the striatal lesion group.

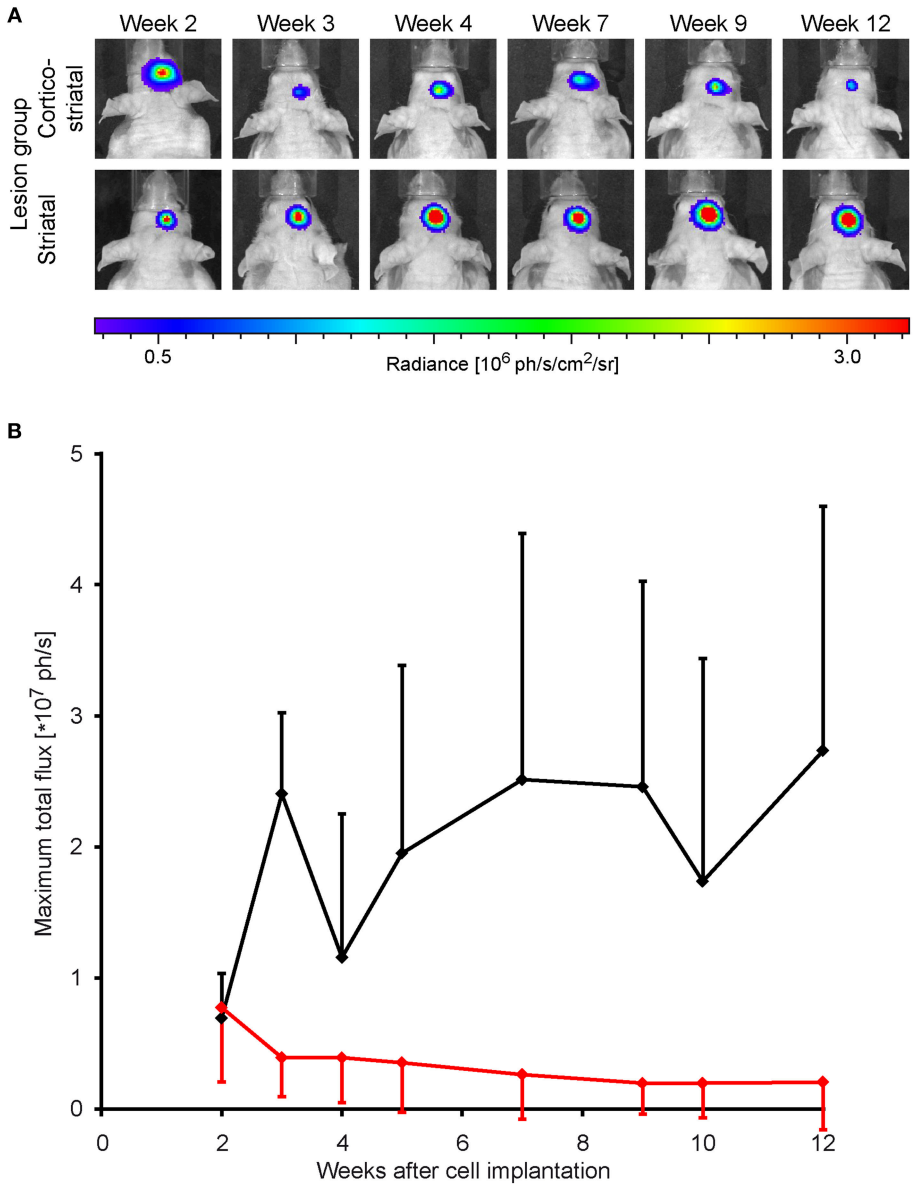


FIGURE 2 | Stem cell graft viability during 3 months after implantation. Bioluminescence imaging of the luciferase-expressing neural stem cells was recorded over 3 months every 1 or 2 weeks after implantation into the cortex of the ischemic hemisphere. **(A)** Representative animals of the cortico-striatal and striatal lesion group at various time points after implantation. The BLI intensity of the cortico-striatal lesion animal shows a continuous decrease over time, while the BLI intensity of the mouse with striatal lesion remains stable over the whole 3 months. **(B)** Quantitative analysis of BLI intensity of both lesion groups with in-group standard deviations: the striatal lesion group (black line) shows an overall stable BLI signal intensity on the pre-stroke level. The BLI signal intensity of the cortico-striatal group (red line) presents a rapid decrease with time, leveling off at ∼30% of the pre-stroke value.

striatal group being less pronounced and showing a clear trend toward normalization (**Figure 3**, bottom right). Interestingly, of all six calculated interhemispheric homotopic connectivties, thalamic connectivity decreases the most in both groups, with an even higher reduction in the striatal group (−76% vs. −40.9% for the cortico-striatal group).

The Contralateral Hemisphere During 12 Weeks After Stroke

Brain swelling followed by later brain atrophy prohibited the reliable co-registration of the ipsilateral, ischemic hemisphere with the assigned regions of the anatomic brain atlas to determine corresponding functional connectivities at times later than 2 weeks post stroke induction. Therefore, the connectivity matrices of the sensorimotor networks were limited to the healthy hemisphere after week 2 (**Figure 4**). The matrices of the contralateral hemisphere up to week 2 are already included in the whole brain matrices in **Figure 3**. Analysis of the functional connectivity changes on the ipsilateral hemisphere during the chronic phase, though also highly interesting, will have to await future development of more complex co-registration strategies of the rs-fMRI data with the mouse

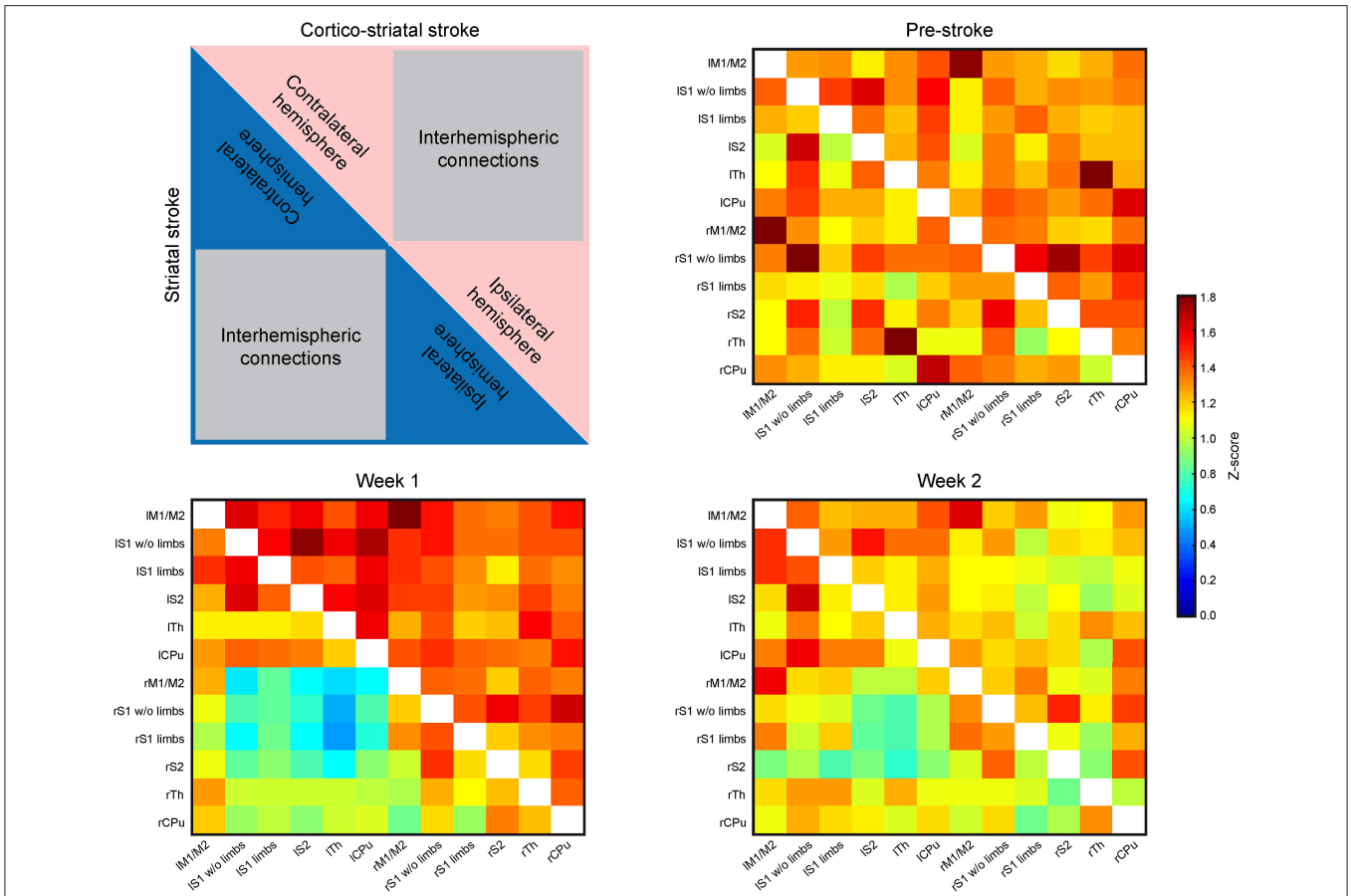


FIGURE 3 | Functional connectivity matrices of the whole brain. The matrices contain the connectivity values (*z*-score) of selected brain regions. The distributions of values within the matrices are laid out in the schematic at the upper left. The upper, red triangle stands for the cortico-striatal group, the lower, blue triangle stands for the striatal group. The regions representing *intra*-hemispheric connectivities of ipsi- and contralateral hemisphere, and the *inter*-hemispheric connectivities are marked. The three matrices show the ipsi- and contralateral brain connectivity values at pre-stroke time (upper right), at week 1 post-stroke (lower left) and at week 2 post-stroke induction (lower right). During the control time point (pre-stroke) both groups show closely equal connectivity strength across the whole brain (upper right). At week 1 post-stroke induction, the cortico-striatal group shows a slight hyperconnectivity, while the connectivity values of the striatal group remain unaffected by stroke induction, with the exception of a small field of reduced connectivities within the *inter*-hemispheric connections. At week 2 post-stroke (lower right), both groups show values close to the pre-stroke condition in the *intra*-hemispheric parts. The *inter*-hemispheric parts show a reduction relative to the pre-stroke condition.

brain atlas, assuring correct anatomical alignment under severe brain deformations.

The functional connectivity strength of the cortico-striatal group at week 2 is still closely similar to the pre-stroke situation. However, a substantial reduction of connectivity strength is noted beginning at week 4. This reduction becomes very pronounced at week 8, remaining at similarly low values at week 12 (**Figure 4**; upper matrix triangles) with the strongest reduction noted for the connection between the primary somatosensory cortex wo limbs and the secondary somatosensory cortex (−67.2% reduction in z-score value between pre-stroke and 12 weeks post MCA occlusion), as well as the caudate putamen with the S1 wo limbs (−59.1%) and S2 (−66.9%).

On the contrary, in the striatal group functional connectivity values remain high until week 8, similar to the pre-stroke situation. Only in week 12, this group shows distinctly lowered connectivity strengths, approximating the state of low cross-correlations of the cortico-striatal group. For the striatal group highest differences are observed between the lS1 wo limbs with the thalamus (−44.9% from before stroke, to 12 weeks after), and with the caudate putamen (−46.7%). Some correlations in the striatal group even increase slightly between pre-stroke measurement and the last time point. This is specifically observed for anatomically adjacent regions, such as the lS1 limbs and lS1 wo limbs (+13.4%), and the lM with the lS1 limbs (+11%). On average the functional connectivity strength measured in *z*-score correlation coefficient reduces between the pre-stroke situation and 12 weeks after stroke induction for the cortico-striatal group by 39.4 ± 15.3%, whilst the striatal-only group only shows a reduction by 15.0 ± 21.4% averaged over all connection pairs.

Scatterplot Analysis of Functional Connectivity Changes Over Time

Additional to the matrix presentation of individual correlations between specific ROIs above, we also aimed to analyze the overall functional differences over time after stem cell grafting

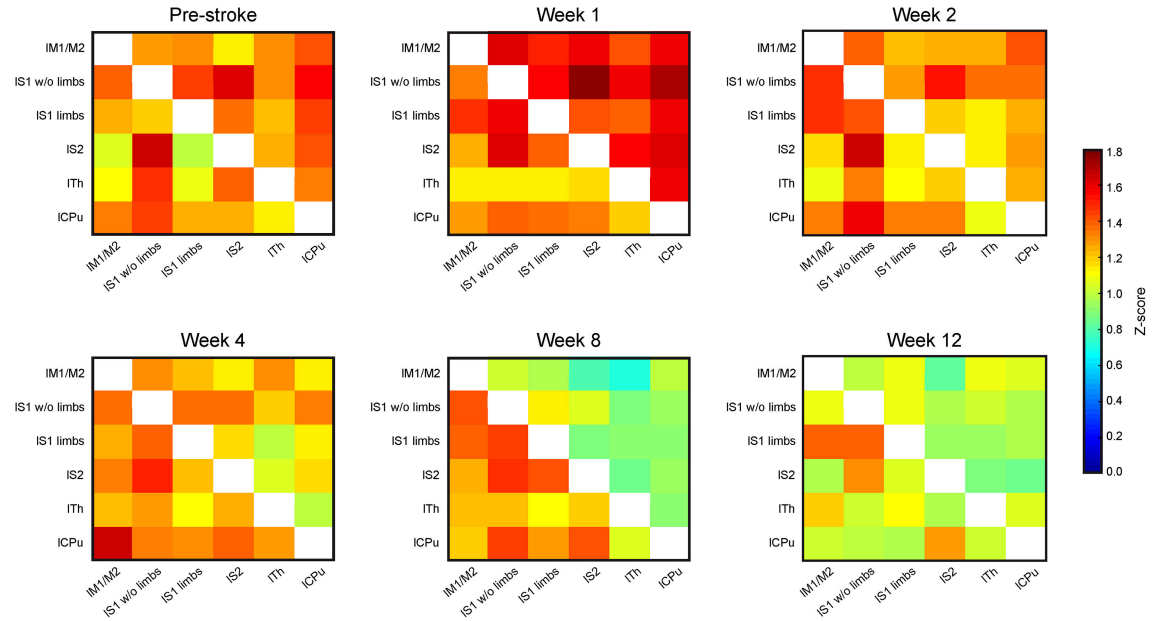


FIGURE 4 | Connectivity matrices of the contralesional hemisphere over 3 months. The connectivity matrix of the contralateral hemisphere for the cortico-striatal group in the upper triangle, and for the striatal group in the lower triangle, with data presented for pre-stroke, weeks 1, 2, 4, 8, and 12 post stroke induction and neural stem cell implantation. The *z*-score values of the sensorimotor functional network of the cortico-striatal lesion group present a connectivity decrease, being substantial from week 4 on, and reaching its minimum at week 8. In the striatal lesion group, *z*-score values remain without clear changes to the pre-stroke situation during the first 8 weeks. Only at the last time point, at week 12 post implantation, the connectivity values decrease approximating those of the cortico-striatal group.

and stroke. Scatter plots were generated plotting all functional connectivity matrix elements at the pre-stroke condition against the ones of three time points after grafting. The values at week 1 were chosen for the acute time window and the values at weeks 8 and 12, respectively, for the late changes. In the case of no changes of the individual matrix elements between two time points, all matrix elements would be expected to lie on the central diagonal through zero with a slope of 1 (identity line). Deviation of the fitted slope from the central diagonal (slope = 1.000, red line in **Figure 5**) hereby indicates the overall increase or decrease of the *z*-score values from the pre-stroke condition to the selected second time point.

In the cortico-striatal lesion group (**Figure 5**, upper row) the slope at week 1 is only slightly increased ($slope_{wk1;\ cortico-striatal} = 1.144$) indicating little change from the baseline condition. At weeks 8 and 12, the slope has substantially deviated from the identity diagonal, with strongly decreased values ($slope_{wk8;\ cortico-striatal} = 0.681$; $slope_{wk12;\ cortico-striatal} = 0.717$).

In contrast, for the striatal lesion group (**Figure 5**, lower row), the slope at week 1 and still at week 8 is indistinguishable from the identity diagonal ($slope_{week1;\ striatal} = 1.026$; $slope_{week8;\ striatal} = 1.016$). Only at the end of the monitoring period, at week 12, the slope has decreased from the identity diagonal, reaching a low value at $slope_{week12;\ striatal} = 0.869$. However, this deviation from the identity diagonal remained still less than the corresponding value of the cortico-striatal lesion group.

DISCUSSION

We have combined the two imaging modalities, resting state functional MRI and BLI to (i) observe modulations of the functional neuronal network changes after stroke by stem cell grafts and to (ii) monitor the viability of the stem cells during 3 months following stroke induction. Our present results indicate that stabilization of the functional connectivity of the sensorimotor networks after stroke requires persistent and quantitative viability of the stem cell graft.

Quantitative Determination of Graft Survival

Many investigations have been concerned with functional improvement of the brain after stem cell implantations (4–6, 12, 13, 31). They mostly relied on behavioral evaluations of the functional deficit and improvement, while histological analysis was used for demonstration of existing stem cell grafts and their potential neuronal differentiation (7–11, 32). However, these histological studies are commonly qualitative assessments of the existence of implanted stem cells, without quantification of the fraction of stem cells survived at the end of the observation period and in correspondence to the functional status. This means that those studies missed a clear temporal relationship between the functional improvement and the persistent survival of the graft. This is, however, of high importance as several studies had reported high mortality of engrafted stem cells (33–39). Rather than on qualitative histological assessment, here, we have relied on the well-established quantitative BLI as a tool to quantitatively

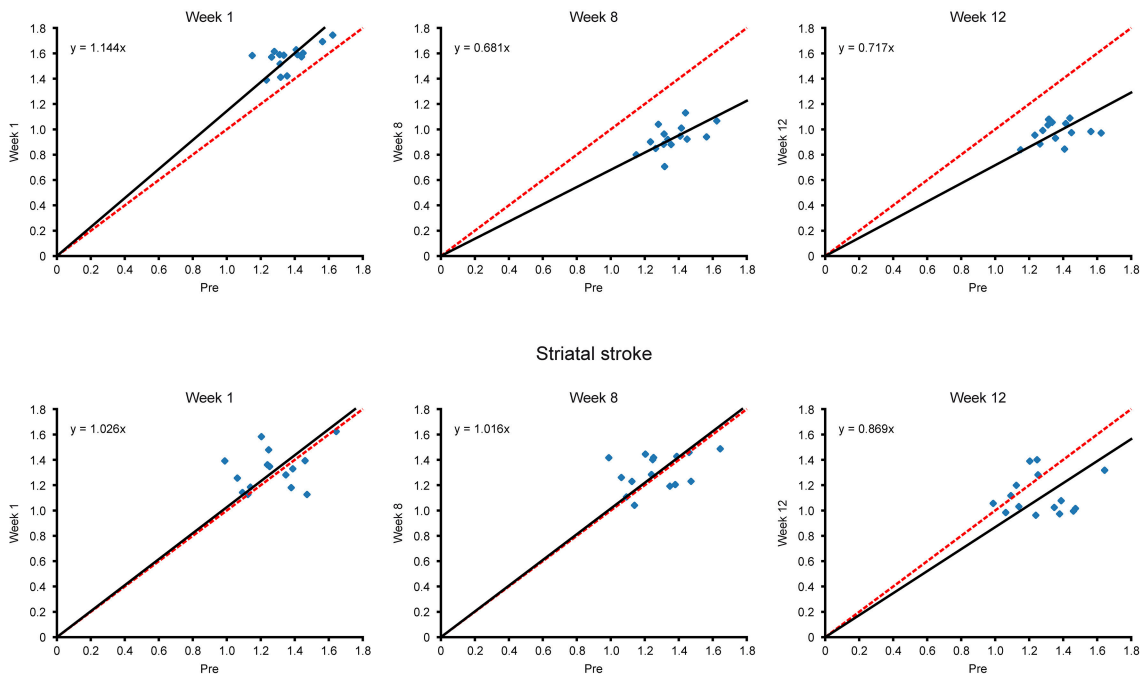


FIGURE 5 | Scatterplots of matrix elements between two time points. The *z*-score values of all connectivities of the contralateral hemisphere, contained in the matrices of **Figure 4**, are compared for different time points with their respective values before stroke induction, as presented in scatter plot diagrams. Deviation of the scatterplots from the identity line is presented for the two time points in each diagram. The identity line indicating no changes for the individual matrix elements between two time points is marked in red, while the fit through the scatter plot and passing through zero is given in black. The slope of the fit, in deviation from slope = 1.000 for the identity line, indicates the overall increase or decrease of the *z*-score values from the pre-stroke time point to the second selected time point. For the cortico-striatal group (upper row diagrams), the diagram at week 1 shows the slight slope increase ($slope_{wk1;\ cortico-striatal} = 1.144$) for the hyperconnectivity; at weeks 8 and 12, the slopes have substantially decreased ($slope_{wk8;\ cortico-striatal} = 0.681$, $slope_{wk12;\ cortico-striatal} = 0.717$), demonstrating the weakening of the sensorimotor functional network at those times. For the striatal group (lower row diagrams), complete superposition with the identity line is preserved for week 1 and also at the late chronic period at week 8, indicating complete stabilization of the pre-stroke connectivity strength by the neural stem cells ($slope_{wk1;\ striatal} = 1.026$, $slope_{wk8;\ striatal} = 1.016$). Only at week 12, here the slope has noticeably decreased to $slope_{wk12} = 0.869$, which, however, remains still above the slope of the cortico-striatal group at this time point.

determine cell viability. As the firefly luciferase which we used as imaging reporter to follow vitality of our engrafted stem cells depends on the presence of ATP and oxygen for the photon generation, the BLI signal intensity is directly proportional to the number of luciferase positive stem cells (40). It should be noted though, that in an earlier report, we had described differentiation of the exact same stem cells, used in the present study, into neurons 3 months after implantation into the corticostriatal lesion model reporting HuNu/NeuN double-positive cells in a qualitative immunohistochemical analysis (18).

We have demonstrated that correct placement of the graft, sufficiently distant to the ischemic lesion, allows long-term and fully quantitative preservation of the graft viability. However, in the case of our cortico-striatal stroke lesion, the stem cells had been implanted too close to the acute lesion rim, resulting in a later substantial loss of viability as the lesion expansion jeopardized the survival of the stem cell majority (18). This was demonstrated by the severe reduction of BLI signal intensity of the stem cell graft. In contrast, in the case of the striatal lesions, the cortical graft preserved its early full level of vitality over the 3 months observation time, as demonstrated with the steady BLI signal over 3 months following engraftment. This finding is in full agreement with earlier reports of the same stem cell line, H9, in NMRI-*Foxn1*$^{nu/nu}$ mice. There, a stable quantitative preservation of cortical graft viability in the healthy mouse brain (26, 41) and also in a different cortical stroke model (41) was seen with robust and stable BLI signal intensity for 7 weeks (26) and even for 3 months (41).

Functional Connectivity of the Sensorimotor Networks

Persistent strong reduction of the functional connectivity strength of both hemispheres had been shown by us (18) and others (19–21) for stroke situation without cell treatment. In correspondence with our earlier study (18), we found a transient stabilization of the functional connectivity after cortical stem cell engraftment in animals with large cortico-striatal lesions. The stabilization remained for ∼4 weeks when the connectivity strength decreased to approach that of animals with stroke only. Those reductions in functional connectivity affected also the

contralateral hemisphere showing the massive far-range effect of the stroke lesion on the neuronal networks. In contrast, our data on the striatal lesions showed a robust stabilization of the functional connectivity by the stem cell graft, remaining stable beyond the 2 month time point, and still being stronger at week 12 than in the case of cortico-striatal lesions. Interestingly, also the selective intermittent inter-hemispheric connectivity decrease, seen in the striatal lesions with stem cells, showed a clear tendency for re-normalization within 2 weeks.

Correlation Between Graft Vitality and Functional Network Stabilization

In the large cortico-striatal lesions, the lesion expanded into the adjacent graft location, thereby jeopardizing the graft vitality. When the graft vitality is reduced, the connectivity stabilization is weakened in consequence and is finally lost, here after about 4 weeks. However, some cells were still surviving and had been shown earlier to differentiate into neurons (18). In contrast, in the small striatal lesion, the graft is not threatened by expansion of the ischemic territory into the cortical cell location and vitality of the graft remains stable over the whole observation period of 12 weeks. In consequence, functional connectivity strength remains at the level of control before stroke at least till week 8. Surprisingly, at week 12 the connectivity strength is also substantially weakened in the striatal lesion group, approximating the lower values of the cortico-striatal group—despite persistent vitality. From our findings we conclude that vitality of the stem cell graft is a necessary, but not a sufficient condition for stabilization of functional neuronal networks.

From our present results it becomes plausible that full quantitative graft vitality is required for functional connectivity stabilization. Previously, we had concluded from comparison of the functional connectivity strength of animals after stroke with and without stem cell engraftment that a paracrine effect must be the responsible mechanism for the stem cell mediated functional network stabilization (18). The here observed much delayed functional connectivity weakening at week 12 post-stroke induction and engraftment points to additional effects that further influence the functional networks stability. Late rejection of the graft can be excluded because of persistent high BLI signal intensities. However, interaction of the graft with other mechanisms must be sought that modulate the paracrine effect, which is therapeutically active during the first 2 months. Here, a hot candidate with time dependent activity are the immune cells of which a strong interaction with stem cells has been discussed (42). Microglia and macrophages have been shown to be of anti-inflammatory, protective polarization phenotype in the acute stroke phase and switch to a pro-inflammatory, aggravating polarization phenotype at later time points (43). Although little is known about the immune cells' activity in the chronic phase of 3 months, it may be speculated that the immune cell activity may affect the paracrine effect of the graft by modulating the stem cell mediated secretion of cytokines and growth factors. Then, external modulation of the polarization state of the immune cells, which has previously been demonstrated to have a protective neuronal effect (44, 45) may also contribute to the recovery of the graft's therapeutically active paracrine effect. Future studies may follow this interaction between stem cell graft and immune cells by multicolor BLI (46), allowing simultaneous monitoring of the graft viability and of the immune cell polarization (47), while assessing the effect of polarization status modulation on the functional brain networks over time.

CONCLUSIONS

Our present investigations on functional connectivity after stroke have contributed further evidence for a paracrine effect of stem cells stabilizing the functional neuronal networks already directly after implantation. Further, we have shown here that this paracrine effect of the stem cells requires quantitative preservation of graft vitality. Finally, the quantitative conservation of vitality is a necessary, but not a sufficient condition for the long-term therapeutic effect of functional network stabilization. Additional influencers, indicated to become of increasing importance in the chronic phase, such as immune cell activity, will need to be analyzed in future for complete and enduring preservation of functional networks.

ETHICS STATEMENT

All animal experiments were carried out in accordance with the guidelines of the German Animal Welfare Act and approved by the local authorities (Landesamt für Natur, Umwelt, und Verbraucherschutz Nordrhein-Westfalen).

AUTHOR CONTRIBUTIONS

CG designed the study, performed the MRI experiments, and wrote the manuscript. AM performed the infarct surgery. SV analyzed the BLI experiments. MD performed data analysis of the rs-fMRI data. DW analyzed the rs-fMRI data and wrote the manuscript. MH designed the study, analyzed the data, and wrote the manuscript.

FUNDING

This work was financially supported by grants from the EU-FP7 programs TargetBraIn (HEALTH-F2-2012-279017) and BrainPath (PIAPP-GA-2013-612360) and by a grant from the German Research Foundation DFG (AS-464/1-1).

ACKNOWLEDGMENTS

The excellent support of Ulla Uhlenküken for graphical art work and of Andreas Beyrau for BLI measurements is gratefully acknowledged.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.00335/full#supplementary-material

Supplementary Figure 1 | Overlay of regions of interest on MR images. The six regions analyzed on each hemisphere for analysis of functional network changes are overlaid on coronal RARE MR images of a representative healthy mouse brain. This was achieved by co-registration of the mouse brain atlas with the MRI mouse brain template which again was co-registered with the individual RARE MRI data set. This procedure is described in detail in the Methods section. The four coronal images presented here cover the range of the mouse brain for all relevant sensorimotor cortex areas and the thalamus, at the rostral-caudal axis, equidistantly from −1.55 mm to +1.05 mm relative to bregma.

REFERENCES

1. Mackay J, Mensah GA. *The Atlas of Heart Disease and Stroke*. World Health Organization and Center for Disease Control And Prevention (2013). Available online at: http://www.who.int/cardiovascular_diseases/resources/atlas/en (accessed on February 14, 2013).
2. Tobin MK, Bonds JA, Minshall RD, Pelligrino DA, Testai FD, Lazarov O. Neurogenesis and inflammation after stroke: what is known and where we go from here. *J Cereb Blood Flow Metab*. (2014) 34:1573–84. doi: 10.1038/jcbfm.2014.130
3. Bacigaluppi M, Pluchino S, Peruzzotti L, Kilic E, Kilic U, Salani G, et al. Delayed postischemic neuroprotection following systemic neural stem cell transplantation involves multiple mechanisms. *Brain*. (2009) 132:2239–51. doi: 10.1093/brain/awp174
4. Cui LL, Nitsche F, Pryazhnikov E, Tibeykina M, Tolppanen L, Rytkönen J, et al. Integrin alpha4 overexpression on rat mesenchymal stem cells enhances transmigration and reduces cerebral embolism after intracarotid injection. *Stroke*. (2017) 48:2895–900. doi: 10.1161/STROKEAHA.117.017809
5. Doeppner TR, Kaltwasser B, Teli MK, Sanchez-Mendoza EH, Kilic E, Bär M, et al. Post-stroke transplantation of subventricular zone derived neural progenitor cells - a comprehensive analysis of cell delivery routes and their underlying mechanisms. *Exp Neurol*. (2015) 273:45–56. doi: 10.1016/j.expneurol.2015.07.023
6. Doeppner TR, Traut V, Heidenreich A, Kaltwasser B, Bosche B, Bähr M, et al. Conditioned medium derived from neural progenitor cells induces long-term post-ischemic neuroprotection, sustained neurological recovery, neurogenesis, and angiogenesis. *Mol Neurobiol*. (2017) 54:1531–40. doi: 10.1007/s12035-016-9748-y
7. Ramos-Cabrer P, Justicia C, Wiedermann D, Hoehn M. Stem cell mediation of functional recovery after stroke in the rat. *PloS ONE*. (2010) 5:e12779. doi: 10.1371/journal.pone.0012779
8. Tornero D, Wattananit S, Madsen MG, Koch P, Wood J, Tatarishvili J, et al. Human induced pluripotent stem cell-derived cortical neurons integrate in stroke-injured cortex and improve functional recovery. *Brain*. (2013) 136:3561–77. doi: 10.1093/brain/awt278
9. Chen J, Sanberg PR, Li Y, Wang L, Lu M, Willing AE, et al. Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. *Stroke*. (2001) 32:2682–8. doi: 10.1161/hs1101.098367
10. Doeppner TR, Kaltwasser B, Bär M, Hermann DM. Effects of neural progenitor cells on post-stroke neurological impairment - a detailed and comprehensive analysis of behavioral tests. *Front Cell Neurosci*. (2014) 8:e338. doi: 10.3389/fncel.2014.00338
11. Patkar S, Tate R, Modo M, Plevin R, Carswell HV. Conditionally immortalized neural stem cells promote functional recovery and brain plasticity after transient focal cerebral ischaemia in mice. *Stem Cell Res*. (2012) 8:14–25. doi: 10.1016/j.scr.2011.07.001
12. Oki K, Tatrishvili J, Wood J, Koch P, Wattananit S, Mine Y, et al. Human-induced pluripotent stem cells form functional neurons and improve recovery after grafting in stroke-damaged brain. *Stem Cells*. (2012) 30:1120–33. doi: 10.1002/stem.1104
13. Tornero D, Tsupykov O, Granmo M, Rodriguez C, Gronning-Hansen M, Thelin J, et al. Synaptic inputs from stroke-injured brain to grafted human stem cell-derived neurons activated by sensory stimuli. *Brain*. (2017) 140:692–706. doi: 10.1093/brain/aww347
14. Grandjean J, Azzinnari D, Seuwen A, Sigrist H, Seifritz E, Pryce CR, et al. Chronic psychosocial stress in mice leads to brain functional connectivity and metabolite levels comparable to human depression. *Neuroimage*. (2016) 15:544–52. doi: 10.1016/j.neuroimage.2016.08.013
15. Grandjean J, Derungs R, Kulic L, Welt T, Henkelman M, Nitsch RM, et al. Complex interplay between brain function and structure during cerebral amyloidosis in APP transgenic mouse strains revealed by multiparametric MRI comparison. *Neuroimage*. (2016) 134:1–11. doi: 10.1016/j.neuroimage.2016.03.042
16. Shah D, Blockx I, Guns PJ, De Deyn PP, Van Dam D, Jonckers E, et al. Acute modulation of the cholinergic system in the mouse brain detected by pharmacological resting state functional MRI. *Neuroimage*. (2015) 109:151–9. doi: 10.1016/j.neuroimage.2015.01.009
17. Shah D, Praet J, Latif Hernandez A, Höfling C, Anckerts C, Bard F, et al. Early pathologic amyloid induces hypersynchronyof BOLD resting state networks in transgenic mice and provides an early therapeutic window before amyloid plaque deposition. *Alzheimers Dement*. (2016) 12:964–76. doi: 10.1016/j.jalz.2016.03.010
18. Green C, Minassian A, Vogel S, Diedenhofen M, Beyrau A, Wiedermann D, et al. Sensorimotor functional and structural networks after intracerebral stem cell grafts in the ischemic mouse brain. *J Neurosci*. (2018) 38:1648–61. doi: 10.1523/JNEUROSCI.2715-17.2018
19. van Meer MPA, Otte WM, van der Marel K, Nijboer CH, Kavelaars A, van der Sprenkel JW, et al. Extent of bilateral neuronal network reorganization and functional recovery in relation to stroke severity. *J Neurosci*. (2012) 32:4495–507. doi: 10.1523/JNEUROSCI.3662-11.2012
20. van Meer MPA, van der Marel K, Otte WM, Berkelbach van der Sprenkel JW, Dijkhuizen RM. Correspondence between altered functional and structural connectivity in the contralesional sensorimotor cortex after unilateral stroke in rats: a combined resting-state functional MRI and manganese-enhanced MRI. Journal of Cerebral *Blood Flow Metab*. (2010) 30:1707–11. doi: 10.1038/jcbfm.2010.124
21. van Meer MPA, van der Marel K, Wang K, Otte WM, el Bouazati S, Roeling TAP, et al. Recovery of sensorimotor function after experimental stroke correlates with restoration of resting-state interhemispheric functional connectivity. *J Neurosci*. (2010) 30:3964–72. doi: 10.1523/JNEUROSCI.5709-09.2010
22. Adamczak J, Aswendt M, Kreutzer C, Rotheneicher P, Riou A, Selt M, et al. Neurogenesis upregulation on the healthy hemisphere after stroke enhances compensation for age-dependent decrease of basal neurogenesis. *Neurobiol Dis*. (2017) 99:47–57. doi: 10.1016/j.nbd.2016.12.015
23. Adamczak JM, Schneider G, Nelles M, Que I, Suidgeest E, van der Weerd L, et al. *In vivo* bioluminescence imaging of vascular remodeling after stroke. *Front Cell Neurosci*. (2014) 8:274. doi: 10.3389/fncel.2014.00274
24. Aswendt M, Adamczak J, Couillard-Despres S, Hoehn M. Boosting bioluminescence neuroimaging: an optimized protocol for brain studies. *PloS ONE*. (2013) 8:e55662. doi: 10.1371/journal.pone.0055662
25. Hamzei Taj S, Kho W, Riou A, Wiedermann D, Hoehn M. MiRNA-124 induces neuroprotection and functional improvement after focal cerebral ischemia. *Biomaterials*. (2016) 91:151–65. doi: 10.1016/j.biomaterials.2016.03.025
26. Tennstaedt A, Aswendt M, Adamczak J, Collienne U, Selt M, Schneider G, et al. Human neural stem cell intracerebral grafts show spontaneous early neuronal differentiation after several weeks. *Biomaterials*. (2015) 44:143–54. doi: 10.1016/j.biomaterials.2014.12.038
27. Grandjean J, Schroeter A, Batata I, Rudin M. Optimization of anesthesia protocol for resting-state fMRI in mice based on differential effects of anesthetics on functional connectivity patterns. *Neuroimage*. (2014) 102(Pt 2):838–47. doi: 10.1016/j.neuroimage.2014.08.043

28. Kalthoff D, Seehafer JU, Po C, Wiedermann D, Hoehn M. Functional connectivity in the rat at 11.7T: impact of physiological noise in resting state fMRI. *NeuroImage*. (2011) 54:2828–39. doi: 10.1016/j.neuroimage.2010.10.053
29. Dorr AE, Lerch JP, Spring S, Kabani N, Henkelman RM. High resolution three-dimensional brain atlas using an average magnetic resonance image of 40 adult C57Bl/6J mice. *Neuroimage*. (2008) 42:60–9. doi: 10.1016/j.neuroimage.2008.03.037
30. Ullmann JF, Watson C, Janke AL, Kurniawan ND, Reutens DC. A segmentation protocol and MRI atlas of the C57BL/6J mouse neocortex. *Neuroimage*. (2013) 78:196–203. doi: 10.1016/j.neuroimage.2013.04.008
31. Bacigaluppi M, Russo GL, Peruzotti-Jametti L, Rossie S, Sandrone S, Butti E, et al. Neural stem cell transplantation induces stroke recovery by upregulating glutamate transporter GLT-1 in astrocytes. *J Neurosci*. (2016) 36:10529–44. doi: 10.1523/JNEUROSCI.1643-16.2016
32. Hicks AU, Lappalainen RS, Narkilahti S, Suuronen R, Corbett D, Sivenius J, et al. Transplantation of human embryonic stem cell-derived neural precursor cells and enriched environment after cortical stroke in rats: cell survival and functional recovery. *Eur J Neurosci*. (2009) 29:562–74. doi: 10.1111/j.1460-9568.2008.06599.x
33. Boehm-Sturm P, Aswendt M, Minassian A, Michalk S, Mengler L, Adamczak J, et al. A multi-modality platform to image stem cell graft survival in the naive and stroke-damaged mouse brain. *Biomaterials*. (2014) 35:2218–26. doi: 10.1016/j.biomaterials.2013.11.085
34. Kallur T, Farr TD, Böhm-Sturm P, Kokaia Z, Hoehn M. Spatio-temporal dynamics, differentiation and viability of human neural stem cells after implantation into neonatal rat brain. *Eur J Neurosci*. (2011) 34:382–93. doi: 10.1111/j.1460-9568.2011.07759.x
35. Kim DE, Tsuji K, Kim YR, Mueller FJ, Eom HS, Snyder EY, et al. Neural stem cell transplant survival in brains of mice: assessing the effect of immunity and ischemia by using real-time bioluminescent imaging. *Radiology*. (2006) 241:822–30. doi: 10.1148/radiol.2413050466
36. Reekmans K, De Vocht N, Praet J, Fransen E, Le Blon D, Hoornaert C, et al. Spatiotemporal evolution of early innate immune responses triggered by neural stem cell grafting. *Stem Cell Res Ther*. (2012) 3:56. doi: 10.1186/scrt147
37. Sher F, van Dam G, Boddeke E, Copray S. Bioluminescence imaging of Olig2-neural stem cells reveals improved engraftment in a demyelination mouse model. *Stem Cells*. (2009) 27:1582–91. doi: 10.1002/stem.76
38. Tennstaedt A, Mastropietro A, Nelles M, Beyrau A, Hoehn M. *In vivo* fate imaging of intracerebral stem cell grafts in mouse brain. *PloS ONE*. (2015) 10:e144262. doi: 10.1371/journal.pone.0144262
39. Vogel S, Aswendt M, Nelles M, Henn N, Schneider G, Hoehn M. Initial graft size and not the innate immune response limit survival of engrafted neural stem cells. *J Tissue Eng Regener Med*. (2018) 12:784–93. doi: 10.1002/term.2497
40. Talmadge JE. Bioluminescence in drug development for cancer: shedding light on therapeutic efficacy. *Cancer Biol Ther*. (2011) 11:846–8. doi: 10.4161/cbt.11.9.15405
41. Minassian A. *Stem Cell Therapy for Stroke*. Cologne: University at Cologne (2018).
42. Kokaia Z, Martino G, Schwartz M, Lindvall O. Cross-talk between neural stem cells and immune cells. The key to better brain repair? *Nat Neurosci*. (2012) 15:1078–87. doi: 10.1038/nn.3163
43. Hu X, Li P, Guo Y, Wang H, Leak RK, Chen S, et al. Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. *Stroke*. (2012) 43:3063–70. doi: 10.1161/STROKEAHA.112.659656
44. Hamzei Taj S, Kho W, Aswendt M, Collmann FM, Green C, Adamczak J, et al. Dynamic modulation of microglia/macrophage polarization by miR-124 after focal cerebral ischemia. *J Neuroimmune Pharmacol*. (2016) 11:733–48. doi: 10.1007/s11481-016-9700-y
45. Hamzei Taj S, Le Blon D, Hoornaert C, Daans J, Quarta A, Praet J, et al. Targeted intracerebral delivery of the anti-inflammatory cytokine IL13 promotes alternative activation of both microglia and macrophages after stroke. *J Neuroinflamm*. (2018) 15:e174. doi: 10.1186/s12974-018-1212-7
46. Mezzanotte L, Aswendt M, Tennstaedt A, Hoeben R, Hoehn M, Löwik C. Evaluating reporter genes of different luciferases for optimized *in vivo* bioluminescence imaging of transplanted neural stem cells in the brain. *Contrast Media Mol Imaging*. (2013) 8:505–13. doi: 10.1002/cmmi.1549
47. Collmann FM, Pijnenburg R, Schneider G, Schafer C, Folz-Donahue K, Kukat C, et al. Imaging reporter strategy to monitor gene activation of microglia polarisation states under stimulation. *J Neuroimmune Pharmacol*. (2018) 13:371–82. doi: 10.1007/s11481-018-9789-2

Conflict of Interest Statement: MH was employed part time by Percuros B.V.

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.

Copyright © 2019 Green, Minassian, Vogel, Diedenhofen, Wiedermann and Hoehn. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Neurology

REVIEW
published: 09 April 2019
doi: 10.3389/fneur.2019.00332

Cell-Based Therapies for Stroke: Promising Solution or Dead End? Mesenchymal Stem Cells and Comorbidities in Preclinical Stroke Research

*Fernando Laso-García†, Luke Diekhorst†, Mari Carmen Gómez-de Frutos, Laura Otero-Ortega, Blanca Fuentes, Gerardo Ruiz-Ares, Exuperio Díez-Tejedor and María Gutiérrez-Fernández**

Neuroscience and Cerebrovascular Research Laboratory, Department of Neurology and Stroke Center, La Paz University Hospital, Neuroscience Area of IdiPAZ Health Research Institute, Autonoma University of Madrid, Madrid, Spain

OPEN ACCESS

Edited by:
Paulo Henrique Rosado-de-Castro, Instituto D'Or de Pesquisa e Ensino (IDOR), Brazil

Reviewed by:
Yuka Ikegame, Kizawa Memorial Hospital, Japan
Catriona Cunningham, University of Manchester, United Kingdom

***Correspondence:**
María Gutiérrez-Fernández
mgutierrezfernandez@salud.madrid.org

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Stroke, a section of the journal Frontiers in Neurology

Received: *30 November 2018*
Accepted: *19 March 2019*
Published: *09 April 2019*

Citation:
Laso-García F, Diekhorst L, Gómez-de Frutos MC, Otero-Ortega L, Fuentes B, Ruiz-Ares G, Díez-Tejedor E and Gutiérrez-Fernández M (2019) Cell-Based Therapies for Stroke: Promising Solution or Dead End? Mesenchymal Stem Cells and Comorbidities in Preclinical Stroke Research. Front. Neurol. 10:332. doi: 10.3389/fneur.2019.00332

Stroke is a major health problem worldwide. It has been estimated that 90% of the population attributable risk of stroke is due to risk factors such as aging, hypertension, hyperglycemia, diabetes mellitus and obesity, among others. However, most animal models of stroke use predominantly healthy and young animals. These models ignore the main comorbidities associated with cerebrovascular disease, which could be one explanation for the unsuccessful bench-to-bedside translation of protective and regenerative strategies by not taking the patient's situation into account. This lack of success makes it important to incorporate comorbidities into animal models of stroke in order to study the effects of the various therapeutic strategies tested. Regarding cell therapy, the administration of stem cells in the acute and chronic phases has been shown to be safe and effective in experimental animal models of stroke. This review aims to show the results of studies with promising new therapeutic strategies such as mesenchymal stem cells, which are being tested in preclinical models of stroke associated with comorbidities and in elderly animals.

Keywords: aging, hypertension, diabetes, hyperglycemia, obesity, comorbidity, mesenchymal, stroke

INTRODUCTION

Stroke is still the most common cause of permanent disability in adults and the second leading cause of death in the world (1). The pathology of stroke is poorly understood; however, it has been shown that the majority of patients with stroke have at least one comorbidity (2). The contribution of various risk factors to worldwide stroke burden is unknown. The INTERSTROKE study has demonstrated that five risk factors accounted for more than 80% of the global risk for all strokes (either ischemic stroke or intracerebral hemorrhage [ICH]): hypertension, current smoking, abdominal obesity, diet and physical activity (3). Furthermore, stroke incidence rises with increased in age (4). This high prevalence of comorbidities in stroke patients indicates the need for therapies in preclinical studies that take these comorbidities into account (**Figure 1**).

Of 502 experimental therapies for acute focal ischemic stroke, only 10% were tested in animals with hypertension. Hypertensive animals have larger infarct sizes and reduced efficacy with

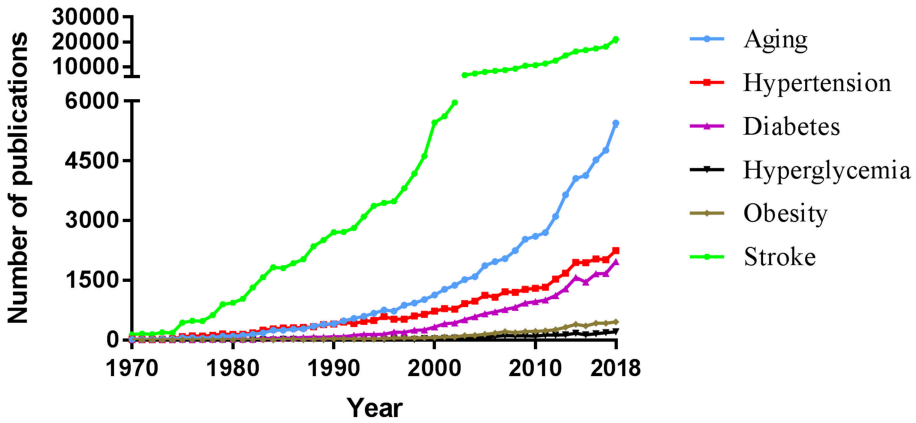


FIGURE 1 | Comparative evolution of the evaluation of the effect of various comorbidities in animal models of stroke as well as in clinical research. An advanced search was performed in PubMed in December 2018 to find, for each year of publication, all articles using the text word stroke with the given term as text word: age or aging; hypertension or hypertensive or high blood pressure; diabetes or diabetes mellitus; hyperglycemia or hyperglycemic or high blood sugar or high glucose levels; or obesity or overweight. Year of final publication (and not advanced online date) of articles in English (and not other languages) was taken into account. Manual elimination of articles describing a non-comorbid stroke association were excluded.

therapeutic intervention (5, 6). Even fewer preclinical studies assess the effects of diabetes or acute hyperglycemia on the response to therapeutic intervention (7). The majority of preclinical studies for novel therapies use young healthy animal models and this may play a role in the fact that of 1,026 treatments tested on animal models, only one has been effective in clinical trials (8).

In particular, stem cell therapy has been proven to be effective mostly in healthy animals. Various types of stem cells have been used in preclinical stroke models: embryonic stem cells, neural stem cells, induced pluripotent stem cells, mesenchymal stem cells (MSCs) and hematopoietic stem cells (9). Cell therapy has been shown to promote functional recovery, participating in processes such as immunomodulation, neurogenesis, synaptogenesis, oligodendrogenesis, axonal connectivity, and myelin formation, improvement in blood brain barrier (BBB) integrity, neovascularization and reduced lesion size, showing efficacy not only in grey matter, but also white matter injury (10–17). However, its mechanisms of action has not yet been clarified. Recent evidence has suggested that it might be related to long-distance cell-to-cell communication by paracrine function through secretory factors in the extracellular environment. Intercellular communication between stem cells and the damaged organ was thought to be regulated via the release of free molecules that transmit the signal by binding to a receptor. These molecules could in part be trophic factors, inflammation modulators and even exosomes. In order to avoid previous translation failure in stem cell therapy, STAIR guidelines suggest that further studies should be performed on animals with comorbid conditions such as hypertension and diabetes in order to improve the quality of preclinical studies of purported stroke therapies (18).

This review is focused on MSC therapies being tested in preclinical models of stroke with the most common comorbidities (hypertension, hyperglycemia, diabetes, obesity), as well as in elderly animals. We intend to provide insight into the viability of this new strategy, which could lead to an improved translation of cell therapy from bench to bedside.

MESENCHYMAL STEM CELLS IN PRECLINICAL STUDIES

Aging in Stroke

Age is the most important risk factor for developing a stroke. Age has been proven to be a predictive factor for recovery after stroke, independent of stroke severity, characteristics and complications (19). Stroke incidence is also strongly but not solely correlated with an increase in age, in addition to the patient's general fitness (4). As previously stated, patient data shows a direct correlation between age and the occurrence of stroke. For these reasons, the impact of age should be considered carefully in preclinical studies given that the mechanisms of stroke and response to drugs can be very different in the developing, juvenile, adult and elderly brain (20). However, despite being the most important risk factor, there are currently few studies (only 7) on aged animals that evaluate the effect of administering MSC after ischemic stroke (**Table 1**). In this regard, systemic administration of bone marrow mononuclear cells (BMMNCs) in the acute phase after stroke reduced neurological deficits (21, 22) and reduced infarct volume, modulating post-ischemic inflammatory cytokines within the brain in older rats (21). Also in the acute phase, intravenous administration of human umbilical tissue-derived cells improved recovery of neurological function in aged rats after stroke and was associated with activation of repair processes (23). This beneficial effect of cell therapy is not only observed in the short

Abbreviations: ICH, intracerebral hemorrhage; MSCs, mesenchymal stem cells; BBB, blood-brain barrier; BMMNCs, bone marrow mononuclear cells; BMSCs, bone marrow stromal cells; G-CSF, granulocyte colony-stimulating factor; BMMSCs, bone marrow mesenchymal stem cells; SHRs, spontaneously hypertensive rats; SHR-SPs, stroke-prone SHRs; HUCBCs, human umbilical cord blood cells; DM-BMSCs, BMSCs derived from type I diabetes rats.

term, but also in the long term. In one study, intra-arterial administration of bone marrow stromal cells (BMSCs) at 1 day after ischemic stroke had long-lasting beneficial effects on recovery of neurological functional (24). One interesting strategy for reducing ischemic damage to the brain would be based on a combination of therapies to act in different steps of the ischemic cascade. In this sense, the combination the granulocyte colony-stimulating factor (G-CSF) with bone marrow mesenchymal stem cells (BMMSCs) increased neurogenesis and improved microvessel recovery and density in the aged brain (25). However, another study demonstrated that the combination of G-CSF and bone marrow mononuclear cells (BMMNCs) did not further improve post-stroke recovery (26).

Currently, all studies with MSCs in elderly animals have been performed on ischemic stroke, none on hemorrhagic stroke. Also, no standardized protocols were used in the above cell therapy studies for observing the various routes of administration and doses used. The number of studies is still very limited and further preclinical research is needed to determine the efficacy of MSCs in aged animal models. In view of the results, however, the aged brain retains the capacity for repair in response to cell therapy.

Hypertension in Stroke

Hypertension is considered one of the most common and important vascular risk factors for stroke (3) and is responsible for approximately 52% of strokes (5); it is also closely correlated with stroke severity. Hypertension has numerous effects, such as reducing BBB integrity and promoting white matter damage and post-stroke edema (5).

In order to test a new therapeutic strategy, a good experimental animal model must first be selected. Several models have been used to induce hypertension in animals. In the past, dogs were used in experiments as a hypertension model. Currently, the rat has become the common model for research as a cost-effective alternative. There are various ways to induce hypertension in rats; for example, spontaneously hypertensive rats (SHRs), stroke-prone SHRs (SHR-SPs), endocrine hypertension by deoxycorticosterone acetate administration, angiotensin II administration and hypertension induced by stress (47–49). Based on consistent reproducibility, the SHR is probably the best model with which to observe hypertension.

Regarding cell therapy, MSCs have been used in hypertensive rats to evaluate the efficacy of functional recovery in animals (**Table 1**).

In cerebral ischemia, intracerebral transplantation of BMMSCs has been shown to decrease apoptotic neurons in the neocortex and ameliorate brain damage by decreasing cell death (27). This has been shown to play a primary role in brain protection. BMMSCs also act on vasculogenesis in SHR rats. Thus, the cells significantly increased the number of microvessels and their reactivity to collagen IV in the neocortex, which indicates protection of the neurovascular unit and improvement of vascular integrity (27). In this regard, BMMSCs also increase of levels of the antiapoptotic B-cell lymphoma 2 (Bcl-2) gene and decrease superoxide, demonstrating that MSC has antioxidant potential and a protective effect in SHR rats with stroke (28). Also, intravenously administered dual transplantation of human maternal or fetal placenta MSCs produces increased density of glial fibrillary acidic protein-positive cells in the area adjacent to the infarct border which may increase survival rates of regenerative astrocytes, leading to a decrease in infarct volume on day 60, triggering functional improvement in SHRs (29). However, not all studies have reported favorable functional outcomes after ischemic stroke with hypertension SHRs. BMMNCs or cryopreserved human umbilical cord blood mononuclear cells given intravenously did not show a beneficial effect on infarct volume, behavioral outcomes or inflammatory response (30, 31). Pösel et al. performed a study to determine a possible synergistic effect of G-CSF and BMMNCs after stroke in SHR rats, in which they found administration of G-CSF improved long-term functional recovery. However, this effect was negated by cotransplantation of BMMNCs as provoked splenic accumulation of granulocytes and transplanted cells, accompanied by a significant rise in circulating granulocytes and infiltration in the ischemic brain, which was detrimental to stroke outcome (32).

In addition, the Framingham Study clearly demonstrated the relevance of age and high blood pressure for lifetime risk of stroke (50), indicating the need to mimic these risk factors in preclinical stroke studies. Along these lines, animals transplanted with intravenous bone marrow cells from young SHR-SPs displayed an increase in microvasculature density in the peri-infarction zone which led to reduced ischemic brain damage and improved neurological function (33). However, in the same study BM cells led to a significant increase in levels of cytokines such as interleukin 1β (IL-1β) and monocyte chemoattractant protein 1(MCP-1) in the brain and a decrease of IL-6 levels in serum. These results suggest that modulation in the expression of inflammatory cytokines (i.e., favoring recovery/decreased inflammatory profile) did not occur and, therefore, is not likely to explain the beneficial effect of the response to cerebral ischemia observed in older SHR-SPs transplanted with BM cells from young SHR-SPs (33). Additionally, further studies reported negative results, such as the study by Wagner et al. which evaluated the therapeutic efficacy of intravenously transplanted young and aged BMMNCs in aged hypertensive rats. The authors concluded that BMMNCs from both juvenile and elderly donors failed to decrease lesion volume and functional recovery was not improved (34).

There are currently two studies in SHRs with ICH. In both studies, intravenously (35) or intracerebrally (36) transplanted BMMSCs improved neurological function and integrity of the BBB by preventing extravasation of blood through the endothelium (35, 36), resulting in improvements such as reduced brain edema and decreased cell apoptosis (36).

Although several different doses and administration routes have been used for treatment in hypertensive animals post-stroke, contradictory results have been found between different research groups using MSCs in hypertensive animals (**Table 1**). More studies should be performed to evaluate whether MSC therapy is effective not only in brain protection, but also in brain repair in the treatment of stroke in hypertensive animals.

TABLE 1 | Original studies evaluating the effect of MSC administration in ischemic stroke models using aged animals, hypertension- and diabetes-induced stroke models.

References	Species	Stroke type	Cell type	N° Cells	Administration Route	Recovery	Comorbidity
Brenneman et al. (21)	Long Evans	MCAO	BMMNC	4×10^6	Intra-arterial	Improved	Aging
Coelho et al. (22)	Wistar	Focal cortical ischemia	BMMNC	3×10^7	Intravenous	Improved	Aging
Zhang et al. (23)	Wistar	MCAO	HUTC	1×10^7/kg	Intravenous	Improved	Aging
Shen et al. (24)	Wistar	MCAO	BMSC	2×10^6	Intra-arterial	Improved	Aging
Balseanu et al. (25)	Sprague-Dawley	MCAO	G-CSF + BMMSC	50 μg/kg + 1×10^6/kg	Intravenous	Improved	Aging
Buga et al. (26)	Sprague-Dawley	MCAO	G-CSF + BMMNC	50μg/kg + 1×10^6/kg	Intravenous	Improved	Aging
Ito et al. (27)	SHR	Stroke prone	BMSC	5×10^5	Intracranial	Not evaluated	Hypertension
Calió et al. (28)	SHR	Stroke prone	BMMSC	1×10^6	Intracranial	Not evaluated	Hypertension
Kranz et al. (29)	SHR	MCAO	MSC from maternal or fetal placenta	1×10^6	Intravenous	Improved	Hypertension
Minnerup et al. (30)	SHR	MCAO	BMMNC	$1/5/20 \times 10^6$	Intravenous	Did not improve	Hypertension
Weise et al. (31)	SHR	MCAO	HUCBMNC	8×10^6/kg	Intravenous	Did not improve	Hypertension
Pösel et al. (32)	SHR	MCAO	G-CSF + BMMNC	50 μg/kg + 1.5×10^7/kg	Intravenous	Did not improve	Hypertension
Taguchi et al. (33)	SHR	Focal cortical ischemia	BMMNC	5×10^5	Intravenous and intraosseous	Improved	Hypertension
Wagner et al. (34)	SHR	MCAO	BMMNC	8×10^6/kg	Intravenous	Did not improve	Hypertension
Wang et al. (35)	SHR	Intracerebral hemorrhage	BMMSC	1×10^6	Intravenous	Improved	Hypertension
Ding et al. (36)	SHR	Intracerebral hemorrhage	BMSC	1×10^6	Intracranial	Improved	Hypertension
Yan et al. (37)	Wistar	MCAO	HUCBC	5×10^6	Intravenous	Improved	Diabetes Type I
Cui et al. (38)	Wistar	MCAO	BMSC	5×10^6	Intravenous	Improved	Diabetes Type I
Chen et al. (39)	Wistar	MCAO	BMSC	3×10^6	Intravenous	Did not improve	Diabetes Type I
Yan et al, (40)	Wistar	MCAO	BMSC + Niaspan	5×10^6 + 40 mg/kg	Intravenous	Did not improve	Diabetes Type I
Ye et al. (41)	Wistar	MCAO	BMSC + Niaspan	5×10^6 + 40 mg/kg	Intravenous	Not evaluated	Diabetes Type I
Yan et al. (42)	Wistar	MCAO	HUCBC	5×10^6	Intravenous	Improved	Diabetes Type II
Ding et al. (43)	Wistar	MCAO	BMSC	5×10^7	Intravenous	Improved	Diabetes Type II
Hu et al. (44)	Wistar	MCAO	BMSC	5×10^6	Intravenous	Improved	Diabetes Type II
Xiang et al. (45)	Wistar	MCAO	BMSC-CM	10 ml/kg	Intravenous	Improved	Diabetes Type II
Yan et al. (46)	Wistar	MCAO	BMSC	5×10^6	Intravenous	Improved	Diabetes Type II

MCAO, middle cerebral artery occlusion; BMMNC, bone marrow mononuclear cell; HUTC, human umbilical tissue-derived cell; BMSC, bone marrow stromal cell; G-CSF, granulocyte colony-stimulating factor; BMMSC, bone marrow mesenchymal stem cell; SHR, spontaneously hypertensive rats; G-CSF, granulocyte colony-stimulating factor; HUCBMNC, human umbilical cord blood mononuclear cell; HUCBC, human umbilical cord blood cell; BMSC-CM, bone marrow stromal cell conditioned medium.

Diabetes in Stroke

Diabetes is divided into two types: type 1, in which the beta-cells of the pancreas are damaged and affected people need external administration of insulin; and type 2, which is peripheral insulin resistance, and is present in 85% of the patients with diabetes (51, 52). Diabetes causes several metabolic and pathological changes that lead to stroke including arterial stiffness, systematic inflammation, endothelial dysfunction and heart failure (53). In addition, stroke in diabetes patients increases hospital mortality (54).

There are many methods to try to mimic type 1 and 2 diabetes in rats (55). One of the most commonly used models for diabetes type 1 are the Biobreeding rats; these rats develop diabetes spontaneously or it is induced by a virus (55). Recently, however, injection of chemicals to destroy beta-cells in the islets of Langerhans has been growing in importance. Alloxan has been used for some time as a good diabetic model but it has problems such as spontaneous recovery from the diabetic condition or renal toxicity. To solve this, another beta-cytotoxic agent, streptozotocin, is used. One advantage of this chemical is that the damage is dose-dependent, which allows researchers to control the severity of that animals' hyperglycemia (56).

To replicate type 2 diabetes, various spontaneous models are used in laboratories such as the spontaneously diabetic tori rat (57), due to gradual beta-cell degeneration, or Goto-Kakizaki rats, which develop peripheral insulin resistance after 56 days (58). Another option is induction by a high fat diet and intraperitoneal streptozotocin administration, in which the rats develop hyperinsulinemia, obesity and a reduction in beta-cells (59). In addition, administration of nicotinamide intraperitoneally prior to administration of a low dose of

streptozotocin protects the cells by attenuating the effect of streptozotocin (60).

Regarding MSC therapy, human umbilical cord blood cells (HUCBCs) and BMSCs have been shown to contribute to an increase in phosphorylated neurofilament marker SMI-31 and synaptophysin expression. These markers are involved in axonal and synaptic plasticity that promotes white matter remodeling in the ischemic brain (37, 38, 41). Vascular remodeling was revealed by an increase in the expression of smooth muscle actin (α-SMA) and Von Willebrand Factor (vWF) (37) in the ischemic brain which led to an improvement in functional outcomes (37, 38). In addition, BMSCs decreased miR-145 expression, which reduces endothelial cell proliferation, contributing to increased functional cells and restorative effects in type 1 diabetic rats (38). However, contradictory results have been reported by other authors. BMSC treatment by tail vein starting 24h after middle cerebral artery occlusion in diabetes type 1 rats resulted in increased brain hemorrhage, BBB leakage and higher expression of angiogenin. This causes accelerated cerebral arteriosclerosis and prevents improvement in functional outcomes (39).

In subsequent studies, however, the harmful effects of BMSC administration were negated when the treatment was administered in combination with Niaspan. Despite the combination, BMSC and Niaspan treatment for stroke did not improve functional outcomes. However, it did decrease BBB leakage and atherosclerotic-like changes (40) and promoted white matter remodeling in type 1 diabetes rats after stroke (41) (**Table 1**).

Regarding type 2 diabetes, all experimental animal studies have shown a beneficial effect of MSCs. Independent of treatment with HUCBCs, BMSCs or bone marrow stromal cell-conditioned medium initiated at 24 h or 3 days after stroke via intravenous administration improved functional recovery, promoted restorative effects and reduced BBB disruption after stroke in type 2 diabetes rats (42–46) (**Table 1**). As in type 1 diabetes, MSC therapy is also associated with white matter remodeling in type 2 diabetes (42, 46), participating in axonal regeneration, sprout and remyelination which led to improved long-term functional outcomes (46). MSC therapy after stroke also contributes to vascular remodeling in type 2 diabetes. Specifically, BMSC-CM treatment enhanced expression of angiopoietin 1 (Ang1), tyrosine-protein kinase receptor Tie-2 (45), α-SMA, and vWF (39, 43), which indicates higher cerebral artery and vascular density (42). Ang1 also seems to be related with a reduction in BBB leakage and promotes vascular stabilization in the ischemic brain. Moreover, it plays a role in white matter remodeling (37, 41), which may improve functional outcome. Regarding the immune system, several authors defend the idea that MSC treatment can also regulate pro-inflammatory factors. This has been shown by a decrease in expression of the receptor for advanced glycation end-products (RAGE) after HUCBC (42) and BMSC (44) treatment in diabetic rats. This indicates a decrease in inflammation, neuronal death, vascular injury and brain damage following ischemia in type 2 diabetic rats. HUCBC and BMSC treatment of type 2 diabetic stroke rats also had an effect on macrophage polarization, promoting decreases inflammation (42, 46), decreased the expression of the proinflammatory protein toll-like receptor 4 (TLR4) (42), increasing brain platelet-derived growth factor (PDGF) expression in the ischemic brain, contributing to restoration (46) and promoting functional improvement after stroke (42).

In summary, with regard to type 1 diabetes, although the experimental studies are homogeneous in terms of the route of administration, cell dose and stroke location, the results are contradictory with no good functional recovery observed in any of them. This reveals the need for further research to increase understanding of the interaction between type 1 diabetes mellitus and cell-based therapy with MSCs. Although all the studies performed thus far reveal that treatment of type 2 diabetes with MSCs in ischemic stroke models can be successful, to our knowledge no studies have been conducted to test the efficacy of MSCs in hemorrhagic stroke. The meager interest aroused could be due to the fact that the prevalence of diabetes is higher in patients with ischemic compared with hemorrhagic stroke, as recently demonstrated by a meta-analysis (61).

Hyperglycemia in Stroke

Hyperglycemia plays an important role in stroke and is associated with poorer functional recovery and an increase in mortality in ischemic and hemorrhagic stroke (62, 63). It has been observed that after stroke, hyperglycemia is present in over 50% of patients (64). Given the high percentage of patients who develop post-stroke hyperglycemia, it is important to conduct research in hyperglycemic animal models. Two options have been used to reproduce hyperglycemic situations in preclinical stroke models, depending on the researchers' requirements: acute hyperglycemia after anesthesia can be mimicked in animals with intravenous infusion of glucose (65); or intravenous administration of streptozotocin and continuous administration of insulin, in which the animals develop hyperglycemia after 1 week (66). To our knowledge, no data have been published on how MSC-based therapy affects stroke associated with hyperglycemia.

Obesity in Stroke

Obesity, especially in the abdominal zone (67), is an important risk factor at all ages. An increase in body mass index (BMI) significantly increases the risk of stroke. Compared with healthy weight individuals, the obese population has a 64% greater probability of experiencing an ischemic stroke (68). The association between BMI and ischemic stroke is linear, without differences between sex or race (69). Other studies also linked obesity with hemorrhage, in which people with a high BMI have a 37% higher incidence (70).

Various animal models have been established to induce obesity in rats (71). Yet despite the high prevalence of obesity in stroke, no research has been performed on how MSC therapy affects the pathology of stroke associated with obesity. In conclusion, studies should be conducted to show the interactions between obesity and MSC treatment after stroke.

Take Home Message

Most of the studies carried out on this subject indeed favor BMMSCs. However, compared to other cell types, adipose tissue-derived mesenchymal stem cells (ADMSCs) have

several advantages in clinical applications for neurological disorders (72). ADMSCs are derived from adipose tissue and thus are abundant, accessible and easy to obtain using lipoaspiration techniques. Moreover, they provide proliferation and differentiation potential (73) without adverse side effects (12, 74, 75) and they can be administered without ethical concerns. All of these advantages mean that ADMSCs present a great opportunity for the treatment of diseases such as comorbidities in stroke. Further studies should take this into consideration.

It should be emphasized that patients present not only one but also several associated comorbidities at a time. Therefore, it is clear that there is a need for multimodelling for successful translation of preclinical research to the clinic (6). In this sense, not only modifiable factors, but also non-modifiable risk factors such as age and sex, are important to include in animal model studies (6). Moreover, animal models with co-morbidities show higher variability in outcome measures and therefore, higher sample sizes should be estimated with the specific disease model in mind (6). Also, aged animals take longer to recover after stroke, but eventually recovered to the same degree as young mice, making clear the importance of implementing long-term studies (76).

Adequate selection of the experimental model for stroke and comorbidity induction is important to reduce mortality, as it is often higher in models with preexisting comorbid conditions. This strategy leads to decreased costs. Besides, outcome measures should be optimized and adequate for these studies as there is variability in outcomes compared to healthy animals (6).

CONCLUSION

The high prevalence of comorbidities in patients with stroke indicates the need for therapies in preclinical studies that take into account these comorbidities in order to avoid failures in translation to the patient. Preclinical studies are beginning to evaluate the efficacy of MSC treatment in stroke associated with comorbidities, especially hypertension, for ischemic and hemorrhagic stroke. Regarding aging and diabetes, only ischemic stroke studies have been performed. For the moment, few studies have been performed and contradictory results are being reported. These contradictory results may be due to the use of different stroke and comorbidity models, and to the use of different protocols for administering cell-based therapies. This situation indicates a further need to promote standardization of cell concentration and administration routes. Obesity and hyperglycemia have been completely ignored, although they are frequently present in patients with stroke. For this reason, the role of comorbidities should have a more prominent place in preclinical stroke studies. This will hopefully improve bench-to-bedside translation and identify viable therapeutic options.

AUTHOR CONTRIBUTIONS

FL-G and LD wrote the first draft of the manuscript. MG-dF and LO-O wrote sections of the manuscript. BF, GR-A, and ED-T contributed to manuscript revision and read and approved the submitted version. MG-F contributed to the conception of the study, wrote and revised the manuscript, and approved the submitted version.

ACKNOWLEDGMENTS

Supported by research grants PI16/01052, INVICTUS PLUS (RD16/0019/0005) (Spanish Neurovascular Network) from the Carlos III Research Institute, Spanish Ministry of Science and Innovation, the European Regional Development Fund (FEDER Funding) and the RESSTORE project (www.resstore.eu), funded by the European Commission under the H2020 program (grant number 681044). We greatly appreciate the support of Morote for editing assistance.

REFERENCES

1. Donnan GA, Fisher M, Macleod M, Davis SM, Royal S, Macleod UKM. Stroke. *Lancet.* (2018) 371:1612–23. doi: 10.1016/S0140-6736(08)60694-7
2. Karatepe AG, Gunaydin R, Kaya T, Turkmen G. Comorbidity in patients after stroke: Impact on functional outcome. *J Rehabil Med.* (2008) 40:831–5. doi: 10.2340/16501977-0269
3. O'Donnell MJ, Denis X, Liu L, Zhang H, Chin SL, Rao-Melacini P, et al. Risk factors for ischaemic and intracerebral haemorrhagic stroke in 22 countries (the INTERSTROKE study): A case-control study. *Lancet*. (2010) 376:112–23. doi: 10.1016/S0140-6736(10)60834-3
4. Soriano-Tárraga C, Giralt-Steinhauer E, Mola-Caminal M, Ois A, Rodríguez-Campello A, Cuadrado-Godia E, et al. Biological age is a predictor of mortality in ischemic stroke. *Sci Rep.* (2018) 8:4148. doi: 10.1038/s41598-018-22579-0
5. O'Collins VE, Donnan GA, Macleod MR, Howells DW. Hypertension and experimental stroke therapies. *J Cereb Blood Flow Metab.* (2013) 33:1141–7. doi: 10.1038/jcbfm.2013.88
6. Ergul A, Hafez S, Fouda A, Fagan SC. Impact of comorbidities on acute injury and recovery in preclinical stroke research: focus on hypertension and diabetes. *Transl Stroke Res.* (2016) 7:248–60. doi: 10.1007/s12975-016-0464-8
7. Kelly-Cobbs AI, Prakash R, Li W, Pillai B, Hafez S, Coucha M, et al. Targets of vascular protection in acute ischemic stroke differ in type 2 diabetes. *Am J Physiol Heart Circ Physiol.* (2013) 304:806–15. doi: 10.1152/ajpheart.00720.2012
8. O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp BH, Howells DW. 1,026 experimental treatments in acute stroke. *Ann Neurol.* (2006) 59:467–77. doi: 10.1002/ana.20741
9. Liu X, Ye R, Yan T, Yu SP, Wei L, Xu G, et al. Cell based therapies for ischemic stroke: from basic science to bedside. *Prog Neurobiol.* (2014) 115:92–115. doi: 10.1016/j.pneurobio.2013.11.007
10. Marei HE, Hasan A, Rizzi R, Althani A, Afifi N, Cenciarelli C, et al. Potential of stem cell-based therapy for ischemic stroke. *Front Neurol.* (2018) 9:34. doi: 10.3389/fneur.2018.00034
11. George PM, Steinberg GK. Novel stroke therapeutics: unraveling stroke pathophysiology and its impact on clinical treatments. *Neuron.* (2015) 87:297–3097. doi: 10.1016/j.neuron.2015.05.041
12. Gutiérrez-Fernández M, Rodríguez-Frutos B, Ramos-Cejudo J, Vallejo-Cremades MT, Fuentes B, Cerdán S, et al. Effects of intravenous administration of allogenic bone marrow- and adipose tissue-derived mesenchymal stem cells on functional recovery and brain repair markers in experimental ischemic stroke. *Stem Cell Res Ther.* (2013) 4:11. doi: 10.1186/scrt159
13. Gutierrez-Fernandez M, Rodríguez-Frutos B, Ramos-Cejudo J, Otero-Ortega L, Fuentes B, Vallejo-Cremades MT, et al. Comparison between xenogeneic

and allogeneic adipose mesenchymal stem cells in the treatment of acute cerebral infarct: Proof of concept in rats. *J Transl Med.* (2015) 13:4–13. doi: 10.1186/s12967-015-0406-3
14. Leu S, Lin YC, Yuen CM, Lin YC, Yuen CM, Yen CH, et al. Adipose-derived mesenchymal stem cells markedly attenuate brain infarct size and improve neurological function in rats. *J Transl Med.* (2010) 8:63. doi: 10.1186/1479-5876-8-63
15. Gutiérrez-Fernández M, Rodríguez-Frutos B, Alvarez-Grech J, Vallejo-Cremadesa MT, Expósito-Alcaidea M, Merino J, et al. Functional recovery after hematic administration of allogenic mesenchymal stem cells in acute ischemic stroke in rats. *Neuroscience.* (2011) 175:394–405. doi: 10.1016/j.neuroscience.2010.11.054
16. Chopp M, Li Y. Treatment of neural injury with marrow stromal cells. *Lancet Neurol.* (2002) 1:92–100.doi: 10.1016/S1474-4422(02)00040-6
17. Schu S, Nosov M, O'Flynn L, Shaw G, Treacy O, Barry F, et al. Immunogenicity of allogeneic mesenchymal stem cells. *J Cell Mol Med.* (2012) 16:2094–103. doi: 10.1111/j.1582-4934.2011.01509.x
18. Fisher M, Feuerstein G, Howells DW, Hurn PD, Kent TA, Savitz SI, et al. Update of the stroke therapy academic industry roundtable preclinical recommendations. *Stroke.* (2009) 40:2244–50. doi: 10.1161/STROKEAHA.108.541128
19. Knoflach M, Matosevic B, Rücker M, Furtner M, Mair A, Wille G, et al. Functional recovery after ischemic stroke - a matter of age: data from the austrian stroke unit registry. *Neurology.* (2012) 78:279–85. doi: 10.1212/WNL.0b013e31824367ab
20. Bosetti F, Koenig JI, Ayata C, Back SA, Becker K, Broderick JP, et al. Translational stroke research: vision and opportunities. *Stroke.* (2017) 48:2632–7. doi: 10.1161/STROKEAHA.117.017112
21. Brenneman M, Sharma S, Harting M, Strong R, Cox CS, Aronowski J, et al. Autologous bone marrow mononuclear cells enhance recovery after acute ischemic stroke in young and middle-aged rats. *J Cereb Blood Flow Metab.* (2010) 30:140–9. doi: 10.1038/jcbfm.2009.198
22. Coelho BP, Giraldi-Guimarães A. Effect of age and gender on recovery after stroke in rats treated with bone marrow mononuclear cells. *Neurosci Res.* (2014) 88:67–3. doi: 10.1016/j.neures.2014.08.007
23. Zhang L, Yi L, Chopp M, Kramer BC, Romanko M, Gosiewska A, et al. Intravenous administration of human umbilical tissue-derived cells improves neurological function in aged rats after embolic stroke. *Cell Transplant.* (2013) 22:1569–76. doi: 10.3727/096368912X658674
24. Shen LH, Li Y, Chen J, Cui Y, Zhang C, Kapke A, et al. One-year follow-up after bone marrow stromal cell treatment in middle-aged female rats with stroke. *Stroke.* (2007) 38:2150–6. doi: 10.1161/STROKEAHA.106.481218
25. Balseanu AT, Buga AM, Catalin B, Wagner DC, Boltze J, Zagrean AM, et al. Multimodal approaches for regenerative stroke therapies: combination of granulocyte colony-stimulating factor with bone marrow mesenchymal stem cells is not superior to G-CSF alone. *Front Aging Neurosci.* (2014) 6:130. doi: 10.3389/fnagi.2014.00130
26. Buga AM, Scheibe J, Moller K, Ciobanu O, Posel C, Boltze J, et al. Granulocyte colony-stimulating factor and bone marrow mononuclear cells for stroke treatment in the aged brain. *Curr Neurovasc Res.* (2015) 12:155–62. doi: 10.2174/1567202612666150311112550
27. Ito M, Kuroda S, Sugiyama T, Maruichi K, Kawabori M, Nakayama N, et al. Transplanted bone marrow stromal cells protect neurovascular units and ameliorate brain damage in stroke-prone spontaneously hypertensive rats. *Neuropathology.* (2012) 32:522–33. doi: 10.1111/j.1440-1789.2011.01291.x
28. Calió ML, Marinho DS, Ko GM, Ribeiro RR, Carbonel AF, Oyama LM, et al. Transplantation of bone marrow mesenchymal stem cells decreases oxidative stress, apoptosis, and hippocampal damage in brain of a spontaneous stroke model. *Free Radic Biol Med.* (2014) 70:141–54. doi: 10.1016/j.freeradbiomed.2014.01.024
29. Kranz A, Wagner DC, Kamprad M, Scholz M, Schmidt UR, Nitzsche F, et al. Transplantation of placenta-derived mesenchymal stromal cells upon experimental stroke in rats. *Brain Res.* (2010) 1315:128–36. doi: 10.1016/j.brainres.2009.12.001
30. Minnerup J, Wagner D-C, Strecker J-K, Pösel C, Sevimli-Abdis S, Schmidt A, et al. Bone marrow-derived mononuclear cells do not exert acute neuroprotection after stroke in spontaneously hypertensive rats. *Front Cell Neurosci.* (2014) 7:288. doi: 10.3389/fncel.2013.00288
31. Weise G, Lorenz M, Pösel C, Maria Riegelsberger U, Störbeck V, Kamprad M, et al. Transplantation of cryopreserved human umbilical cord blood mononuclear cells does not induce sustained recovery after experimental stroke in spontaneously hypertensive rats.*J Cereb Blood Flow Metab.* (2014) 34:e1–9. doi: 10.1038/jcbfm.2013.185
32. Pösel C, Scheibe J, Kranz A, Bothe V, Quente E, Fröhlich W, et al. Bone marrow cell transplantation time-dependently abolishes efficacy of granulocyte colony-stimulating factor after stroke in hypertensive rats. *Stroke.* (2014) 45:2431–7. doi: 10.1161/STROKEAHA.113.004460
33. Taguchi A, Zhu P, Cao F, Kikuchi-Taura A, Kasahara Y, Stern DM, et al. Reduced ischemic brain injury by partial rejuvenation of bone marrow cells in aged rats. *J Cereb Blood Flow Metab.* (2011) 31:855–67. doi: 10.1038/jcbfm.2010.165
34. Wagner DC, Bojko M, Peters M, Lorenz M, Voigt C, Kaminski A, et al. Impact of age on the efficacy of bone marrow mononuclear cell transplantation in experimental stroke. *Exp Transl Stroke Med.* (2012) 4:17. doi: 10.1186/2040-7378-4-17
35. Wang C, Fei Y, Xu C, Zhao Y, Pan Y. Bone marrow mesenchymal stem cells ameliorate neurological deficits and blood-brain barrier dysfunction after intracerebral hemorrhage in spontaneously hypertensive rats. *Int J Clin Exp Pathol.* (2015) 8:4715–24.
36. Ding R, Lin C, Wei S, Zhang N, Tang L, Lin Y, et al. Therapeutic benefits of mesenchymal stromal cells in a rat model of hemoglobin-induced hypertensive intracerebral hemorrhage. *Mol Cells.* (2017) 40:133–42. doi: 10.14348/molcells.2017.2251
37. Yan T, Venkat P, Ye X inchun, Chopp M, Zacharek A, Ning R, et al. HUCBCs increase angiopoietin 1 and induce neurorestorative effects after stroke in T1DM rats. *CNS Neurosci Ther.* (2014) 20:935–44. doi: 10.1111/cns.12307
38. Cui C, Ye X, Chopp M, Venkat P, Zacharek A, Yan T, et al. miR-145 regulates diabetes-bone marrow stromal cell-induced neurorestorative effects in diabetes stroke rats. *Stem Cells Transl Med.* (2016) 5:1656–67. doi: 10.5966/sctm.2015-0349
39. Chen J, Ye X, Yan T, Zhang C, Yang XP, Cui X, et al. Adverse effects of bone marrow stromal cell treatment of stroke in diabetic rats. *Stroke.* (2011) 42:3551–8. doi: 10.1161/STROKEAHA.111.627174
40. Yan T, Ye X, Chopp M, Zacharek A, Ning R, Venkat P, et al. Niaspan attenuates the adverse effects of bone marrow stromal cell treatment of stroke in type one diabetic rats. *PLoS ONE.* (2013) 8: e0081199. doi: 10.1371/journal.pone.0081199
41. Ye X, Yan T, Chopp M, Zacharek A, Ning R, Venkat P, et al. Combination BMSC and Niaspan treatment of stroke enhances white matter remodeling and synaptic protein expression in diabetic rats. *Int J Mol Sci.* (2013) 14:22221–32. doi: 10.3390/ijms141122221
42. Yan T, Venkat P, Chopp M, Zacharek A, Ning R, Cui Y, et al. Neurorestorative therapy of stroke in type two diabetes rats treated with human umbilical cord blood cells. *Stroke.* (2015) 46:2599–606. doi: 10.1161/STROKEAHA.115.009870
43. Ding G, Chen J, Chopp M, Li L, Yan T, Li Q, et al. Cell treatment for stroke in type two diabetic rats improves vascular permeability measured by MRI. *PLoS ONE.* (2016) 11:e0149147. doi: 10.1371/journal.pone.0149147
44. Hu J, Liu B, Zhao Q, Jin P, Hua F, Zhang Z, et al. Bone marrow stromal cells inhibits HMGB1-mediated inflammation after stroke in type 2 diabetic rats. *Neuroscience.* (2016) 324:11–9. doi: 10.1016/j.neuroscience.2016.02.058
45. Xiang J, Hu J, Shen T, Liu B, Hua F, Zan K, et al. Bone marrow mesenchymal stem cells-conditioned medium enhances vascular remodeling after stroke in type 2 diabetic rats. *Neurosci Lett.* (2017) 644:62–6. doi: 10.1016/j.neulet.2017.02.040
46. Yan T, Venkat P, Chopp M, Zacharek A, Ning R, Roberts C, et al. Neurorestorative responses to delayed human mesenchymal stromal cells treatment of stroke in type 2 diabetic rats. *Stroke.* (2016) 47:2850–8. doi: 10.1161/STROKEAHA.116.014686
47. Leong XF, Ng CY, Jaarin K. Animal models in cardiovascular research: hypertension and atherosclerosis. *Biomed Res Int.* (2015) 2015:1–7. doi: 10.1155/2015/528757

48. Diz DI, Baer PG, Nasjletti A. Angiotensin II-induced hypertension in the rat. Effects on the plasma concentration, renal excretion, and tissue release of prostaglandins. *J Clin Invest*. (1983) 72:466–77. doi: 10.1172/JCI110994
49. Bailey EL, Smith C, Sudlow CL, Wardlaw JM. Is the spontaneously hypertensive stroke prone rat a pertinent model of sub cortical ischemic stroke? A systematic review. *Int J Stroke*. (2011) 6:434–44. doi: 10.1111/j.1747-4949.2011.00659.x
50. Seshadri S, Beiser A, Kelly-Hayes M, Kase CS, Au R, Kannel WB, et al. The lifetime risk of stroke: estimates from the framingham study. *Stroke*. (2006)37:345–50. doi: 10.1161/01.STR.0000199613.38911.b2
51. Bruno A, Liebeskind D, Hao Q, Raychev R. Diabetes mellitus, acute hyperglycemia, and ischemic stroke. *Curr Treat Options Neurol*. (2010) 12:492–503. doi: 10.1007/s11940-010-0093-6
52. Rom S, Zuluaga-Ramirez V, Gajghate S, Seliga A, Winfield M, Heldt NA, et al. Hyperglycemia-driven neuroinflammation compromises BBB leading to memory loss in both diabetes mellitus (DM) Type 1 and Type 2 mouse models. *Mol Neurobiol*. (2019) 56:1883–96. doi: 10.1007/s12035-018-1195-5
53. Chen R, Ovbiagele B, Feng W. Diabetes and stroke: epidemiology, pathophysiology, pharmaceuticals and outcomes. *Am J Med Sci*. (2016) 351:380–6. doi: 10.1016/j.amjms.2016.01.011
54. Arboix A, Rivas A, García-Eroles L, de Marcos L, Massons J, Oliveres M. Cerebral infarction in diabetes: clinical pattern, stroke subtypes, and predictors of in-hospital mortality. *BMC Neurology*. (2005) 5:9. doi: 10.1186/1471-2377-5-9
55. King AJ. The use of animal models in diabetes research. *Br J Pharmacol*. (2012) 166:877–894. doi: 10.1111/j.1476-5381.2012.01911.x
56. Acharjee S, Ghosh B, Al-Dhubiab BE, Nair AB. Understanding type 1 diabetes: Etiology and models. *Can J Diabetes*. (2013) 37:269–76. doi: 10.1016/j.jcjd.2013.05.001
57. Sasase T, Ohta T, Masuyama T, Yokoi N, Kakehashi A, Shinohara M. The spontaneously diabetic Torii rat: An animal model of nonobese Type 2 Diabetes with severe diabetic complications. *J Diabetes Res*. (2013) 2013:1–12. doi: 10.1155/2013/976209
58. Kuwabara WM, Panveloski-Costa AC, Yokota CN, Pereira JN, Filho JM, Torres RP, et al. Comparison of goto-kakizaki rats and high fat diet-induced obese rats: are they reliable models to study type 2 diabetes mellitus? *PLoS ONE*. (2017) 12:e0189622. doi: 10.1371/journal.pone.0189622
59. Gheibi S, Kashfi K, Ghasemi A. A practical guide for induction of type-2 diabetes in rat: Incorporating a high-fat diet and streptozotocin. *Biomed Pharmacother*. (2017) 95:605–13. doi: 10.1016/j.biopha.2017.08.098
60. Szkudelski T. Streptozotocin-nicotinamide-induced diabetes in the rat. Characteristics of the experimental model. *Exp Biol Med*. (2012) 237:481–90.doi: 10.1258/ebm.2012.011372
61. Lau LH, Lew J, Borschmann K, Thijs V, Ekinci EI. Prevalence of diabetes and its effects on stroke outcomes: a meta-analysis and literature review. *J Diabetes Investig*. (2018). doi: 10.1111/jdi.12932. [Epub ahead of print].
62. Toni D, De Michele M, Fiorelli M, Bastianello S, Camerlingo M, Sacchetti ML, et al. Influence of hyperglycaemia on infarct size and clinical outcome of acute ischemic stroke patients with intracranial arterial occlusion. *J Neurol Sci*. (1994) 123:129–33.doi: 10.1016/0022-510X(94)90214-3
63. Snarska KK, Bachórzewska-Gajewska H, Kapica-Topczewska K, Drozdowski W, Chorazy M, Kułakowska A, et al. Hyperglycemia and diabetes have different impacts on outcome of ischemic and hemorrhagic stroke. *Arch Med Sci*. (2017) 13:100–8. doi: 10.5114/aoms.2016.61009
64. Fuentes B, Castillo J, San José B, Leira R, Serena J, Vivancos J, et al. The prognostic value of capillary glucose levels in acute stroke:the glycemia in acute stroke (GLIAS) study. *Stroke*. (2009) 40:562–8. doi: 10.1161/STROKEAHA.108.519926
65. Saha JK, Xia J, Engle SK, Chen YF, Glaesner W, Jakubowski JA. A model of controlled acute hyperglycemia in rats: Effects of insulin and glucagon-like peptide-1 analog. *J Pharmacol Exp Ther*. (2006) 316:1159–64.doi: 10.1124/jpet.105.093534
66. Di Filippo C, Marfella R, Cuzzocrea S, Piegari E, Petronella P, Giugliano D, et al. Hyperglycemia in streptozotocin-induced diabetic rat increases infarct size associated with low levels of myocardial HO-1 during ischemia/reperfusion. *Diabetes*. (2005) 54:803–10. doi: 10.2337/diabetes.54.3.803
67. Suk SH, Sacco RL, Boden-Albala B, Cheun JF, Pittman JG, Elkind MS, et al. Abdominal obesity and risk of ischemic stroke: The Northern Manhattan Stroke Study. *Stroke*. (2003) 34:1586–92. doi: 10.1161/01.STR.0000075294.98582.2F
68. Strazzullo P, D'Elia L, Cairella G, Garbagnati F, Cappuccio FP, Scalfi L. Excess body weight and incidence of stroke: Meta-analysis of prospective studies with 2 million participants. *Stroke*. (2010) 41:418–26. doi: 10.1161/STROKEAHA.109.576967
69. Yatsuya H, Folsom AR, Yamagishi K, North KE, Brancati FL, Stevens J, et al. Race- and sex-specific associations of obesity measures with ischemic stroke incidence in the Atherosclerosis Risk in Communities (ARIC) study. *Stroke*. (2010) 41:417–25. doi: 10.1161/STROKEAHA.109.566299
70. Pezzini A, Grassi M, Paciaroni M, Zini A, Silvestrelli G, Iacoviello L, et al. Obesity and the risk of intracerebral hemorrhage: The multicenter study on cerebral hemorrhage in Italy. *Stroke*. (2013) 44:1584–9. doi: 10.1161/STROKEAHA.111.000069
71. Lutz TA, Woods SC. Overview of animal models of obesity. *Curr Protoc Pharmacol*. (2012) 5.61:1–22. doi: 10.1002/0471141755.ph0561s58
72. Chang KA, Lee JH, Suh YH. Therapeutic potential of human adiposederived stem cells in neurological disorders. *J Pharmacol Sci*. (2014) 126:293–301. doi: 10.1254/jphs.14R10CP
73. Parker AM, Katz AJ. Adipose-derived stem cells for the regeneration of damaged tissues. *Expert Opin Biol Ther*. (2006) 6:567–78. doi: 10.1517/14712598.6.6.567
74. Ra JC, Shin IS, Kim SH, Kang SK, Kang BC, Lee HY, et al. Safety of intravenous infusion of human adipose tissue-derived mesenchymal stem cells in animals and humans. *Stem Cells Dev*. (2011) 20:1297–308. doi: 10.1089/scd.2010.0466
75. Gutiérrez-Fernández M, Rodríguez-Frutos B, Otero-Ortega L, Ramos-Cejudo J, Fuentes B, Díez-Tejedor E. Adipose tissue-derived stem cells in stroke treatment: from bench to bedside. *Discov Med*. (2013) 16:37–43.
76. Manwani B, Liu F, Xu Y, Persky R, Li J, McCullough LD. Functional recovery in aging mice after experimental stroke. *Brain Behav Immun*. (2011) 25:1689–700. doi: 10.1016/j.bbi.2011.06.015

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.

Copyright © 2019 Laso-García, Diekhorst, Gómez-de Frutos, Otero-Ortega, Fuentes, Ruiz-Ares, Díez-Tejedor and Gutiérrez-Fernández. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Neurology

ORIGINAL RESEARCH
published: 10 April 2019
doi: 10.3389/fneur.2019.00357

Combining Human Umbilical Cord Blood Cells With Erythropoietin Enhances Angiogenesis/ Neurogenesis and Behavioral Recovery After Stroke

Sunyoung Hwang[1], *Jeeln Choi*[2] *and MinYoung Kim*[1,2*]

[1] *Rehabilitation and Regeneration Research Center, CHA University, Seongnam, South Korea,* [2] *Department of Rehabilitation Medicine, CHA Bundang Medical Center, College of Medicine, CHA University, Seongnam, South Korea*

OPEN ACCESS

Edited by:
Paulo Henrique Rosado-de-Castro, Instituto D'Or de Pesquisa e Ensino (IDOR), Brazil

Reviewed by:
Samuel Greggio, Institute of the Brain of Rio Grande do Sul (InsCer), Brazil
Bharath Chelluboina, University of Wisconsin-Madison, United States

***Correspondence:**
MinYoung Kim
kmin@cha.ac.kr

Specialty section:
This article was submitted to Stroke, a section of the journal Frontiers in Neurology

Received: *15 January 2019*
Accepted: *22 March 2019*
Published: *10 April 2019*

Citation:
Hwang S, Choi J and Kim M (2019) Combining Human Umbilical Cord Blood Cells With Erythropoietin Enhances Angiogenesis/Neurogenesis and Behavioral Recovery After Stroke. Front. Neurol. 10:357. doi: 10.3389/fneur.2019.00357

Disruption of blood flow in the brain induces stroke, the leading cause of death and disability worldwide. However, so far the therapeutic options are limited. Thus, the therapeutic efficacy of cell-based approaches has been investigated to develop a potential strategy to overcome stroke-induced disability. Human umbilical cord blood cells (hUCBCs) and erythropoietin (EPO) both have angiogenic and neurogenic properties in the injured brain, and their combined administration may exert synergistic effects during neurological recovery following stroke. We investigated the therapeutic potential of hUCBC and EPO combination treatment by comparing its efficacy to those of hUCBC and EPO alone. Adult male Sprague-Dawley rats underwent transient middle cerebral artery occlusion (MCAO). Experimental groups were as follows: saline (injected once with saline 7 d after MCAO); hUCBC (1.2×10^7 total nucleated cells, injected once via the tail vein 7 d after MCAO); EPO (500 IU/kg, injected intraperitoneally for five consecutive days from 7 d after MCAO); and combination of hUCBC and EPO (hUCBC+EPO). Behavioral measures (Modified Neurological Severity Score [mNSS] and cylinder test) were recorded to assess neurological outcomes. Four weeks after MCAO, brains were harvested to analyze the status of neurogenesis and angiogenesis. *In vitro* assays were also conducted using neural stem and endothelial cells in the oxygen-glucose deprivation condition. Performance on the mNSS and cylinder test showed the most improvement in the hUCBC+EPO group, while hUCBC- and EPO-alone treatments showed superior outcomes relative to the saline group. Neurogenesis and angiogenesis in the cortical region was the most enhanced in the hUCBC+EPO group, while the findings in the hUCBC and EPO treatment alone groups were better than those in the saline group. Astrogliosis in the brain tissue was reduced by hUCBC and EPO treatment. The reduction was largest in the hUCBC+EPO group. These results were consistent with *in vitro* assessments that showed the strongest neurogenic and angiogenic effect

with hUCBC+EPO treatment. This study demonstrates that combination therapy is more effective than single therapy with either hUCBC or EPO for neurological recovery from subacute stroke. The common pathway underlying hUCBC and EPO treatment requires further study.

Keywords: stroke, human umbilical cord blood cell, erythropoietin, functional recovery, neurogenesis, angiogenesis

INTRODUCTION

Stroke, caused by the disruption of cerebral blood flow, is a leading cause of death and major disability throughout the world (1). However, the therapeutic options to deal with stroke are limited. Despite efforts to develop new therapies for stroke, all treatments have thus far failed to show a clinical effect or are known to have potential toxic effects. Although intra-arterial thrombolysis and intravenous tissue plasminogen activator therapy have been developed and used for ischemic stroke as effective approaches, those are actually significant only in acute phase with risk of cerebral hemorrhage (1–3). After the onset of stroke, patients experience the greatest amount of neurological recovery during the 3 months post-stroke, which does not last afterwards (4). Before fixation of the impairment in the chronic stage, subacute stroke patients require an effective therapeutic measure, which has remained elusive to date.

While many drugs from successful preclinical experiments have failed in clinical trials for stroke (5), cell therapy has been introduced and expected to be effective by ameliorating neurological impairments due to stroke *in vivo* with relevant mechanisms identified *in vitro* (6–8). Although cell-based therapy has therapeutic potential, to date, the greatest limitation must be the lack of clear evidence related to efficacy (9, 10) and safety issues that limit active clinical trials (10).

Human umbilical cord blood cells (hUCBCs) are a rich source of various progenitor cells, including hematopoietic stem cells that can be used as a cell therapy agent and are known to be safe based on 30 years of clinical application (7, 11). The therapeutic efficacy of hUCBC was supported by significant neurological recovery based on modified Neurological Severity Scores (mNSS) for both acute and subacute brain injury (7). Furthermore, evidence on neurogenesis and angiogenesis, in addition to functional recovery, in a stroke model was observed following hUCBC transplantation (12). Our previous clinical study in children with cerebral palsy also revealed functional improvements following hUCBC administration (13).

As a potential approach to enhance the therapeutic potency of cell therapy, combination therapy with a growth factor can be used (14). In this study, erythropoietin (EPO) was selected among the candidate molecules because it has been used clinically as a safe drug. EPO is a member of the hematopoietic cytokine superfamily and has neuroprotective effects against ischemic brain insults (15, 16). Repeated pre-treatment with EPO produced a neuroprotective effect in both focal and global ischemia models (15, 17, 18). Furthermore, EPO enhances angiogenesis and neurogenesis after ischemic stroke, leading to accompanying functional recovery (19–21). Therefore, EPO may be a promising therapeutic agent to enhance hUCBC treatment in ischemic stroke to induce neurogenesis and angiogenesis.

In recovery from stroke, not only neurogenesis, but also coupled angiogenesis, play central roles (22). For example, treatment with human bone marrow stromal cells (hBMSCs) has been found to enhance angiogenesis in the ischemic boundary zone after stroke (23). Moreover, combination treatment with simvastatin, sodium ferulate, and n-butylidenephthalide following hBMSC administration induced neurological improvement with findings of neurogenesis, angiogenesis, and arteriogenesis after cerebral ischemia (24, 25).

Therefore, in this study, we used a well-established rat model of transient ischemia (26) to represent subacute stroke to analyze the therapeutic efficacy of either hUCBC or EPO, and the additive effects of concomitant treatment with both. In addition, we have systematically investigated the underlying mechanism of neurogenesis and angiogenesis not only in *in vivo* but also *in vitro* experiments.

MATERIALS AND METHODS

Preparation of Human Umbilical Cord Blood Cells

Human UCB containing citrate phosphate dextrose adenine as an anticoagulant was provided from the cord blood bank of CHA Medical Center. Informed by previous clinical trials (27), we injected a total of 3×10^7 kg nucleated cells. This experiment was approved by the Institutional Ethics Committee of CHA Bundang Medical Center, Korea.

Animals

Male Sprague-Dawley rats (260–300 g; Charles River Laboratories, South Korea) were acclimated to their environments for 2 days before use. The rats were housed in a temperature-controlled room (22 ± 2°C), kept at constant humidity (50 ± 10%), and maintained on a 12-h light/dark cycle with *ad libitum* access to food and water. The provision of food was stopped from the night before surgery until the day of surgery, but water was continuously supplied (26). Neural stem cells (NSCs) were isolated from 1-to 3-day-old ICR mice (Charles River Laboratories, South Korea). All experimental procedures involving animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and were approved by CHA University Institutional Animal Care & Use Committee (IACUC150018, IACUC180018, IACUC180181).

In vivo Ischemia Model: Transient Middle Cerebral Artery Occlusion (MCAO)

Rats were initially anesthetized with 3.0% isoflurane in 70% N_2O and 30% O_2 (v/v) mixture supplied via a facemask and maintained with 2.5% isoflurane. Briefly, to occlude the middle cerebral artery (MCA), a 4-0 monofilament nylon suture (Ethicon Johnson & Johnson, Brussels, Belgium) with a heat-blunted end was inserted into the right external carotid artery and advanced into the internal carotid artery.

After a midline incision was made, the right MCA was occluded for 90 min as previously described. It was then reperfused by gentle removal of the suture. Body temperature was maintained at 37°C with a homeothermic blanket and checked using a rectal temperature probe.

Treatment and Experimental Groups

The number of total nucleated cells of hUCBC used in treatment was based on our previous clinical trial which was performed in patients with traumatic brain injury (27). Furthermore, the dosage of EPO was selected based on a corresponding dose in a clinical trial for cerebral palsy (28) that is considered safe for humans.

A total of adult male Sprague–Dawley rats were randomly divided into four groups: (1) saline group (injected into the tail vein once at 7 d post-MCAO / intraperitoneal injection for five consecutive days from 7 d post-MCAO); (2) EPO group (EPO, 500 IU/kg, intraperitoneal injection for 5 consecutive days from 7 d post-MCAO); EPO set included saline ($n = 6$) and EPO ($n = 5$) groups; (3) hUCBC group (hUCBC, 1.2×10^7 total nucleated cells, tail vein injection once at 7 d post-MCAO; hUCBC set included saline ($n = 9$) and hUCBC ($n = 6$) groups; (4) hUCBC+EPO group (hUCBC+EPO treatment at the same dose and schedule as the other groups; hUCBC+EPO set included saline ($n = 6$) and hUCBC+EPO ($n = 5$) groups. At 7 d post-MCAO, behavioral studies were performed immediately before injections.

Behavioral Tests to Assess Neurological Function

The mNSS is a composite scale assessing motor function, sensory disturbance, reflex, and balance tests. **Table 1** shows mNSS scores obtained in this study (7, 29). Neurological function was graded on a scale of 0–18 (normal score: 0; maximal deficit score: 18). Only rats with 11–13 points on the mNSS 1 day post-reperfusion were used in this study. Overall exclusion rate due to score criteria at 1 d post-MCAO was 20%, and survival rate to the end of the experiment was 62.5% among the included subjects.

The cylinder test was performed to assess the degree of forepaw asymmetry. Rats were placed in a transparent Plexiglas cylinder (diameter: 20 cm, height: 30 cm), and the number of forepaw contacts with the cylinder wall was counted until a total of 20 contacts was reached (30). The study team has previously established the reliabilities (26) of the mNSS and cylinder tests, which were performed at 0, 1, 3, 7, 14, 21, and 28 days after MCAO.

TABLE 1 | Modified Neurological Severity Score (mNSS).

	Points
Motor Tests	
Raising rat by the tail 1 Flexion of forelimb 1 Flexion of hindlimb 1 Head moved > 10° to vertical axis within 30 s	3
Placing rat on the floor (normal = 0; maximum = 3) 1 Normal walk 1 Inability to walk straight 1 Circling toward the paretic side 1 Fall down to the paretic side	3
Sensory tests 1 Placing test (visual and tactile test) 1 Proprioceptive test (deep sensation, pushing the paw against the table edge to stimulate limb muscles)	2
Beam balance tests (normal = 0; maximum = 6) 1 Balances with steady posture 1 Grasps side of beam 1 Hugs the beam and one limb falls down from the beam 1 Hugs the beam and two limbs fall down from the beam, or spins on beam(>60 s) 1 Attempts to balance on the beam but falls off (>40 s) 1 Attempts to balance on the beam but falls off (>20 s) 1 Falls off: no attempt to balance or hang on to the beam (<20 s)	6
Reflexes absent and abnormal movements 1 Pinna reflex (head shake when touching the auditory meatus) 1 Corneal reflex (eye blink when lightly touching the cornea with cotton) 1 Startle reflex (motor response to a brief noise from snapping a clipboard paper) 1 Seizures, myoclonus, myodystony	4
Maximum points	18

One point is awarded for the inability to perform the tasks or for the lack of a tested reflex; 13–18 indicates severe injury; 7–12, moderate injury; 1–6, mild injury.

Gross Finding of Brain Section With Cresyl Violet Staining

Serial 8-μm coronal brain sections were prepared using a cryostat and stained with 0.5% cresyl violet. Image of each cresyl violet-stained brain slice was obtained using a light microscope (Nikon, USA).

Immunofluorescence Staining

At 28 d after MCAO (21 d post-therapy), animals were anesthetized with 3.0% isoflurane in 70% N_2O and 30% O_2 (v/v) and then perfused transcardially with a freshly prepared solution of 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were removed and post-fixed overnight in the

same fixative before being immersed in a solution of 30% sucrose in PBS. Serial 8-μm-thick coronal tissue sections were cut using a microtome and immunostained as free-floating sections. The sectioned brain tissues were washed in PBS and incubated for 1 h in blocking solution (2% normal goat serum, 0.5% Triton^{TM} X-100 in PBS). The fluorescent target was selected according to the desired purpose; Sox2 was used to assess neuronal stem cell, Ki67 as a proliferation marker, NeuN to assess neuronal survival, glial fibrillary acidic protein (GFAP) to reveal astrogliosis status, CD31, which is also known as PECAM-1, to assess endothelial expression, and VEGF as an angiogenic marker. The brain tissues were incubated overnight at 4°C with the following primary antibodies: anti-Sox2 (1:500, Santa Cruz, USA), anti-Ki67 (1:500, Abcam, UK), anti-NeuN (1:1000, Novus, USA), anti-GFAP (1:1000, Abcam, UK), anti-CD31 (1:100, Abcam, UK), and anti-VEGF (1:100, Novus, USA). After rinsing with primary antibodies, the tissues were incubated with secondary antibodies for 1 h at 22–25°C. The brain tissues with DAPI (Molecular Probes, Invitrogen, USA) mounting solution were imaged using a fluorescence microscope (Nikon, USA).

Primary Neural Stem Cell Culture

Neural stem cells (NSCs) were isolated from 1-to 3-day-old ICR mice (Charles River Laboratories, South Korea). The whole cortex of the mouse brain was dissected and cultured with basal medium, including Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Gibco, USA), 2% B-27 supplement (Gibco, USA), 1×GlutaMAX (Gibco, USA), basic fibroblast growth factor (bFGF, 20 ng/ml; Gibco, USA), and epidermal growth factor (EGF, 10 ng/ml; Gibco, USA). NSCs were maintained at 37°C in a 5% CO_2 incubator and were subcultured when 90% confluence was reached following a 3-day interval.

Mouse bEnd.3 Cell Culture

bEnd.3 mouse brain endothelial cells were purchased from the ATCC (Manassas, VA, USA) and cultured with medium, including DMEM (Gibco, USA), 10% fetal bovine serum (FBS; Gibco, USA), and 1% penicillin and streptomycin (Gibco, USA). bEnd.3 cells were subcultured when 90% confluence was reached using 0.25% trypsin EDTA and were maintained at 37°C in a 5% CO_2 incubator.

Oxygen-Glucose Deprivation and Cell Treatment

For hypoxic experiments in bEnd.3 cells and NSCs, cells were seeded in 6-well plates 5×10^5cells per well and underwent oxygen-glucose deprivation (OGD) conditioning under 5% CO_2 and 95% N_2 at 37°C in the chamber for 24 h for bEnd.3 cells and 5 h for NSCs (31, 32). After OGD, we treated cells with EPO (0.5 IU/ml), co-cultured with hUCBCs (2×10^4 total nucleated cells per well), or combined hUCBC co-culture and EPO treatment to confirm therapeutic effect in a transwell (0.4-μm pore size; SPL, South Korea), and analyzed 24 h after treatment.

Cell Viability

Cell concentration was measured by cell counting after staining with trypan blue (STEMCELL, USA) using a cell counter (Thermo Fisher, USA). Cell viability was evaluated using a Cellrix viability assay kit (Cellrix, USA). Cells were cultured overnight at 1×10^3 cells per well in 96-well plates. After adding 10 μl/per well of WST-8 into the cell media, we detected absorbance at 450 nm after 30 min incubation. In all viability assays, wells were assessed in triplicate for each group.

Conventional Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from bEnd.3 cells and NSCs using TRIZOL (Life Technologies, USA) and cDNA was synthesized using a reverse transcriptase kit (Toyobo, Japan). Using Taq PCR Master mix (Bioneer, South Korea), PCR was performed. The levels of neuronal markers, Sox2 and Nestin (33), and angiogenesis markers, VEGF, and bFGF (34), were measured after administration of EPO, hUCBC, and hUCBC+EPO in OGD-induced cells. The primers for neuronal markers were Sox2 forward, 5′-CAATCCCATCCAAATTAACGCA-3′, Sox2 reverse, 5′- AAGCTGCAGAATCAAAACCC-3′, Nestin forward, 5′-ACCTATGTCTGAGGCTCCCTATCCTA-3′, and Nestin reverse, 5′-GAGGTTGGATCATCAGGGAAGTG-3′; for angiogenic markers, VEGF forward, 5′- CACAGCAGATGTGAATGCAG-3′, VEGF reverse, 5′-TTTACACGTCTGCGGATCTT-3′, bFGF forward, 5′-CAACCGGTACCTTGCTATGA-3′, and bFGF reverse, 5′-TCCGTGACCGGTAAGTATTG-3′. The following cycling conditions were used: 5 min at 95°C for polymerase activation, followed by 95°C for 30 s and 60°C for 30 s. This was repeated for 35 cycles, and findings were normalized to β-actin.

Western Blotting

To confirm the difference in expressed proteins between the bEnd.3 cells and NSCs, proteins were extracted from ipsilesional brain tissue using a RIPA lysis buffer (Thermo Fisher, USA). For western blotting, equal amounts (30 μg) of protein extracts in a lysis buffer were used in a 12% SDS-PAGE analysis and transferred onto the polyvinylidene fluoride membranes. A 5% skimmed milk solution was used to block the membrane for 1 h at room temperature on a rocker. The housekeeping gene, β-actin, was employed as a loading control. Anti-Sox2 (1:1000, Santa Cruz, USA), anti-Nestin (1:1000, Abcam, UK), anti-VEGF (1:1000, Novus, USA), anti-bFGF (1:1000, Santa Cruz, USA), anti-CD31 (1:000, Abcam, UK), and anti-β-actin (1:1000, Santa Cruz, USA) antibodies were incubated with the membranes at 4°C overnight. After washing the membranes with TBST buffer, a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Santa Cruz, USA) at a dilution of 1:20,000 or an anti-mouse IgG antibody (KPL, Inc., Gaithersburg, MD, USA) at a dilution of 1:10,000 was added to the corresponding primary antibodies, followed by incubation for 1 h at room temperature. Bands were detected using ECL reagent (Millipore, USA).

Tube Formation

The tube formation assays were performed using 24-well plates coated with 200 μl of Matrigel (Gibco, USA) per

well, which was incubated at 37°C for 30 min to solidify. Next, 1×10^5 bEnd.3 cells were seeded per well in a coated plate. Tube formation was estimated after 8 h of cell culture. As a positive control, bFGF (20 ng/ml) was added to induce angiogenesis in endothelial cell (35). Three random areas in the cortex were imaged using a microscope (Nikon, USA), and the total number of tube structures was calculated.

Statistical Analyses

Data are presented as the mean ± standard error of mean (N = 3–9 per group). Each experiment was repeated at least three times. Statistical comparisons between groups was performed using one-way ANOVA and Prism6.0 software (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

RESULTS

EPO, hUCBC, and hUCBC+EPO Attenuated Ischemic Brain Damage

We investigated whether EPO, hUCBC, and hUCBC+EPO affect neuronal damage after brain ischemia at 21 d post-therapy. Animals that received hUCBC+EPO exhibited the highest reduction of brain tissue volume loss; the hUCBC-alone group also exhibited more reduction of brain tissue volume loss than the saline group (**Figure 1A**).

Brains were histologically examined with cresyl violet staining to determine the neuroprotective effects of EPO, hUCBC, and hUCBC+EPO. Treatment with hUCBC+EPO achieved the most significant alleviation of cell damage in rat brains as evinced by the number of intact neurons ($Ps < 0.01$ vs. saline, EPO, hUCBC); the hUCBC- and EPO-alone groups also exhibited significant reductions in the number of injured and

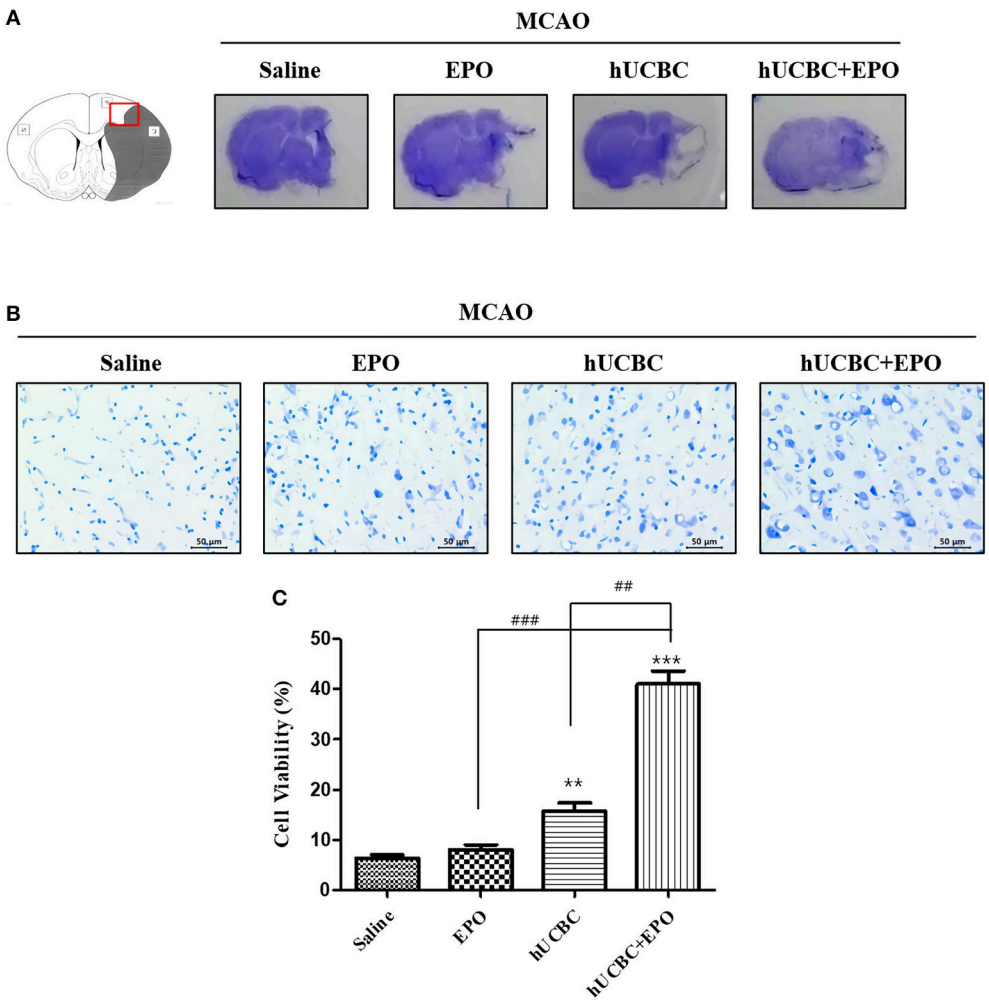


FIGURE 1 | EPO, hUCBC, and hUCBC+EPO attenuated brain damage in MCAO. **(A)** Gross finding of brain section 28 d after MCAO. **(B)** Representative images of cresyl violet staining in the cortex (**B**, scale bar 50 μm). **(C)** Quantification of the number of neurons in the cortex. Data represent mean ± SEM. $N = 3$ per group. $^{**}P < 0.01$, $^{***}P < 0.005$ vs. Saline group. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.005$ for inter-treatment group comparison.

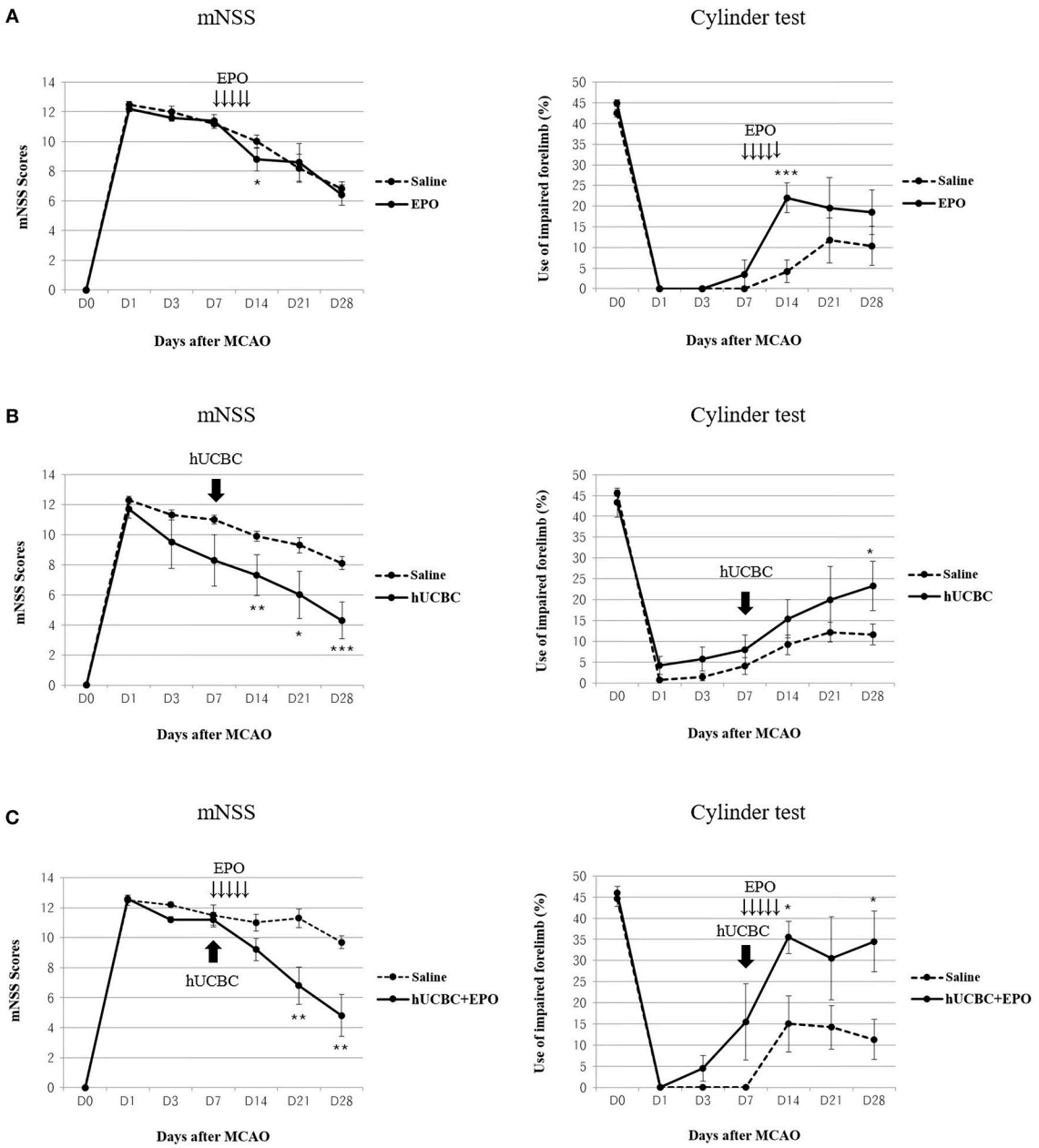


FIGURE 2 | Behavior changes after EPO, hUCBC and hUCBC+EPO administration in comparison with saline treatment for each set of experiments in a rat model of middle cerebral artery occlusion (MCAO). Saline (MCAO alone); EPO (500 IU/kg, injected intraperitoneally for 5 consecutive days from 7 d after MACO); hUCBC (1.2×10^7, injected via tail vein once at 7 d after MCAO); and combination of hUCBC and EPO (hUCBC+EPO). Data represent mean ± standard error of mean (SEM) of mNSS and Cylinder test scores **(A–C)**. $N = 5$–9 per group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ vs. Saline group.

shrunken neurons compared to the saline group ($Ps < 0.01$ vs. saline) (**Figures 1B,C**).

Therapeutic Efficacy of EPO, hUCBC, and hUCBC+EPO in the Subacute Stroke Model

Functional performance as assessed using the mNSS and cylinder test showed bigger improvement in the hUCBC+EPO group ($P < 0.01$ for mNSS at 14 d and 21 d post-therapy; $P < 0.05$ for cylinder test at 7 d and 21 d post-therapy) than the saline group (**Figure 2C**). Also, hUCBC-alone ($P < 0.05$ for mNSS at 7 d, 14 d, and 21 d post therapy; $P = 0.05$ for cylinder test at 21 d post-therapy) and EPO-alone ($P < 0.05$ for mNSS at 7 d post therapy; $P < 0.01$ for cylinder test at 7 d post-therapy) groups showed bigger improvements than the saline group (**Figures 2A,B**). Improvement following EPO

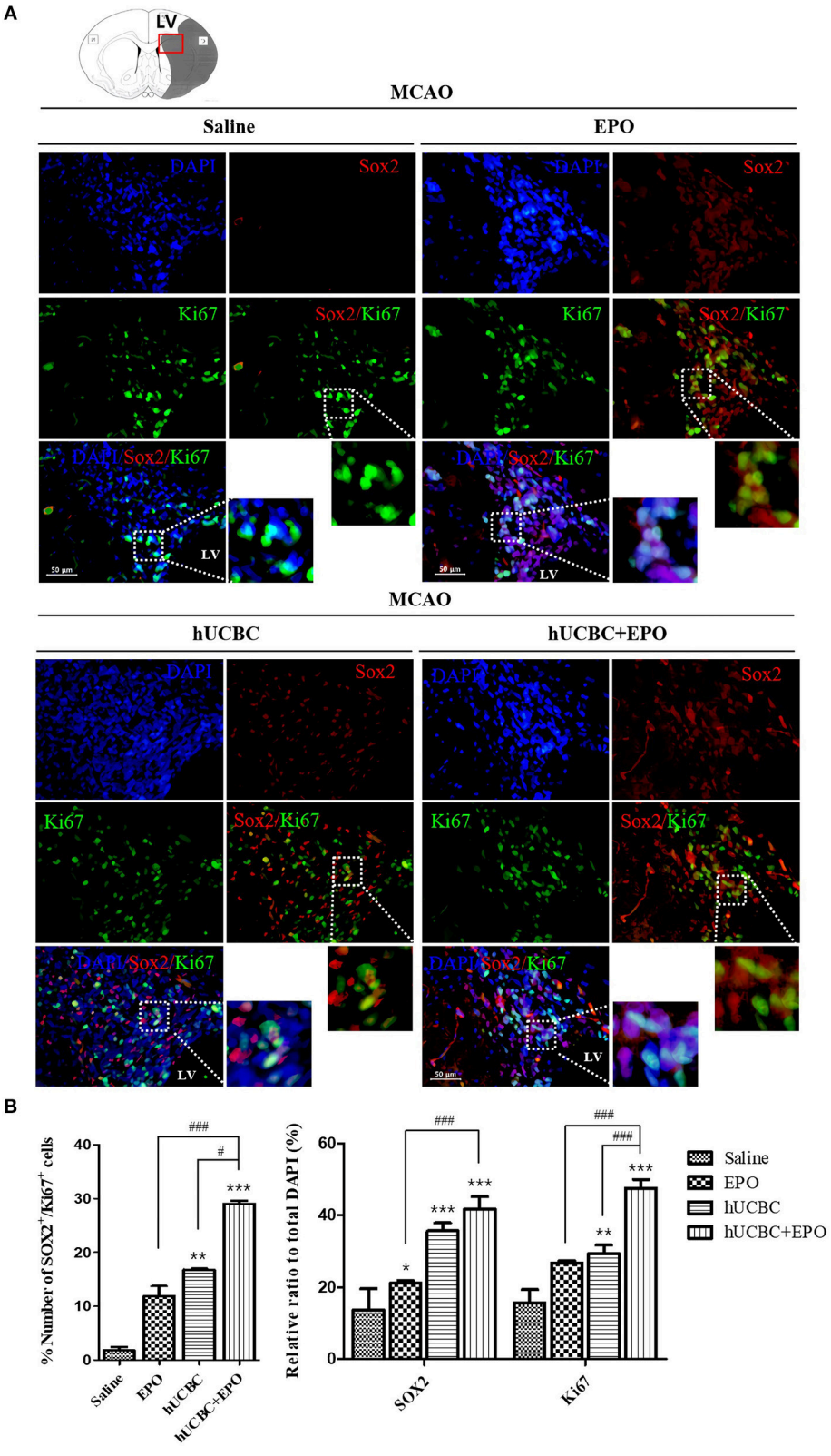


FIGURE 3 | Immunohistochemistry findings in subventricular zone of lateral ventricle following EPO, hUCBC and hUCBC+EPO treatments which administered at or from 7 d after MCAO. Coronal brain slices were stained with Ki67 or Sox2 antibodies at 28 d after MCAO. **(A)** The co-localization of Sox2 (Red) and Ki67 (Green)

(Continued)

FIGURE 3 | identifies proliferation of neuronal cells in the lateral ventricle. White boxes in images indicate the zone of co-localization for further zoomed images. Representative images (scale bar 50 μm). **(B)** Quantification of the number of Sox2(+) cells, Ki67(+) cells, and Sox(+)/Ki67(+) in the subventricular zone of lateral ventricle. Data represent mean ± SEM. $N = 3$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ vs. Saline group. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.005$ for inter-treatment group comparison.

treatment was significant only at 7 d post-therapy (**Figure 2A**). On the contrary, the efficacy of hUCBC administration was most prominent at 21 d post-therapy (**Figure 2B**). Only improvement in the hUCBC+EPO group was apparent from 7 d post-therapy and continued to 21 d post-therapy and the amount of improvement was biggest compared to the EPO-alone and hUCBC alone groups (**Figure 2C**) although the efficacy tests were conducted for each pair of the intervention with saline group.

EPO, hUCBC, and hUCBC+EPO Increased the Proliferation of Neuronal Cells in the Subacute Stroke Model

The quantitative analysis of fluorescent immunostaining images of Sox2(+) and Ki67(+) in subventricular zone of lateral ventricle was conducted. The numbers of Sox2(+) cells, Ki67(+) cells, and Ki67/Sox2 double(+) cells in the subventricular zone were higher in the EPO- and hUCBC-alone groups and hUCBC+EPO group than in the saline group (**Figures 3A,B**). Co-localization of Ki67 and Sox2 was greatest in hUCBC+EPO group. This result indicates increment of proliferation of neuronal cells in the lateral subventricular zone by treatment with EPO, hUCBC, and hUCBC+EPO.

EPO, hUCBC, and hUCBC+EPO Increased Neurogenesis and Reduced Inflammation in the Subacute Stroke Model

To examine whether the administration of EPO, hUCBC, and hUCBC+EPO affected neurogenesis, NeuN(+) cells were counted, and astrogliosis was assessed by counting GFAP(+) cells in the cortex at 28 days after MCAO. **Figures 4A,B** show the significantly more NeuN(+) neuronal cells in the hUCBC+EPO group than in saline ($P < 0.0001$) or EPO-alone groups ($P < 0.01$). The hUCBC group featured the next greatest number of NeuN(+) neuronal cells, which was also higher than those in the saline and in EPO groups ($P < 0.01$).

The number of GFAP(+) cells were markedly reduced in the hUCBC+EPO ($P < 0.001$) group in the cortical region, while the EPO- ($P < 0.01$) and hUCBC- ($P < 0.001$) alone treatment groups also showed reductions in the number of GFAP(+) cells, although to a lesser extent ($P < 0.05$ for EPO vs. hUCBC+EPO). In contrast, astrogliosis was obvious in the saline group with many GFAP(+) cells present (**Figures 4A,C**).

More microglia survived at ischemic lesions in the EPO, hUCBC, and hUCBC+EPO groups than in the saline group, as evinced by the decrease in the number of dying Iba-1 (+) cells with fragmented cellular processes (**Figure 4D**).

EPO, hUCBC, and hUCBC+EPO Enhanced Angiogenesis in the Subacute Stroke Model

The quantitative analysis of fluorescent immunostaining images showed that hUCBC+EPO brains exhibited the greatest cortical vessel density of VEGF(+); cortical vessel density of VEGF(+) cells was higher in the EPO- and hUCBC-alone groups than the saline group ($P < 0.05$) (**Figures 5A,B**).

Similarly, the greatest vessel density of cells positive for CD31, a vascular endothelial marker, was observed in the hUCBC+EPO group; the EPO- and hUCBC-alone groups exhibited more vessel density expression of CD31(+) cells than did the saline group ($P < 0.01$) (**Figures 5A,C**).

EPO, hUCBC, and hUCBC+EPO Enhanced Neurogenesis in OGD-Injured NSC

To evaluate the effect on neuronal proliferation in OGD-injured NSCs and to identify the appropriate concentration of culture media, NSCs were treated with 0, 0.5, 1, 5, or 10 IU/ml of EPO for 24 h after OGD (**Figure 6A**). A dose of 0.5 IU/ml of EPO was selected because it produced the best survival rate for NSCs. After OGD, WST assays were conducted; the results showed remarkable reduction in the viability of NSCs (**Figure 6B**; $P < 0.05$). The cell viability assay revealed the best survival rates in NSCs that received hUCBC and EPO combination treatment ($P < 0.001$), while hUCBC—($P = 0.05$) or EPO—($P < 0.01$) alone treatment also improved cell viability relative to OGD alone (**Figure 6B**). Next, we assessed Sox2, a neuronal proliferation marker, to evaluate the neurogenic effect of treatment with EPO, hUCBC, or hUCBC+EPO after OGD. Sox2 gene and protein expression were significantly upregulated in the hUCBC+EPO group ($P < 0.05$), while a similar response but to a lesser extent was seen for Nestin ($P = 0.06$) expression (**Figures 6C,D**).

EPO, hUCBC, and hUCBC+EPO Enhanced Angiogenesis in OGD-Injured Endothelial Cell

Next, we administered EPO at the same dosages of 0, 0.5, 1, 5, or 10 IU/ml to bEnd.3 cells after OGD, and analyzed the results after 24 h (**Figure 7A**). Again, 0.5 IU/ml of EPO ($P < 0.01$) was selected because it yielded the best survival rate for bEnd.3 cells. After exposure to OGD for 24 h, viability was attenuated by ∼50% as reported previously (**Figure 7B**). In OGD-injured bEnd.3 cells, treatment with hUCBC+EPO resulted in the best cell viability, while hUCBC-alone led to better viability than OGD injury without treatment (**Figure 7B**). According to an angiogenesis factor expression assay examining gene expressions of VEGF and bFGF, hUCBC+EPO (VEGF, $P < 0.05$; bFGF, $P < 0.01$) treatment resulted in highest levels of expression of both genes, while treatment with hUCBC—alone also increased

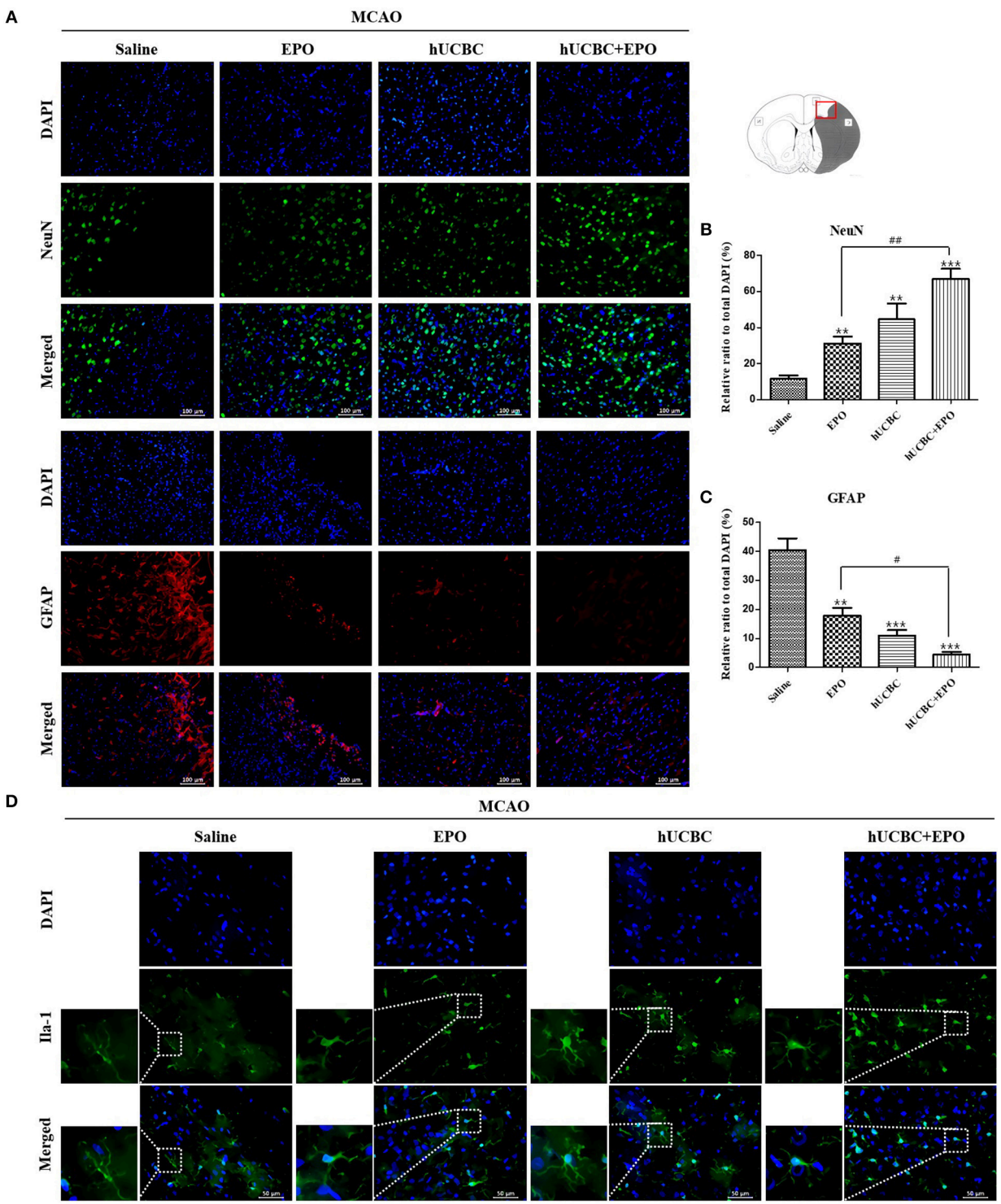


FIGURE 4 | Immunohistochemistry findings in the cerebral cortex following EPO, hUCBC and hUCBC+EPO treatments which were given at or from 7 d after MCAO. Coronal brain slices were stained with anti-NeuN-, anti-GFAP-, or Iba-1 antibodies 28 d after MCAO. **(A)** Representative images [**(A)**, scale bar 100 μm]. **(B,C)** Quantification of the cell density of NeuN(+) and GFAP(+) cells in the cortex of ipsilesional are provided. The treatments promoted neuron, reduced astrogliosis, and changed microglia morphology in the cortex; the strongest findings were observed in the hUCBC+EPO group. **(D)** Representative images (**D**, scale bar 50 μm). Data represent mean ± SEM. $N = 3$ per group, $^{**}P < 0.01$, $^{***}P < 0.005$ vs. saline-treatment group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ for inter-treatment group comparison.

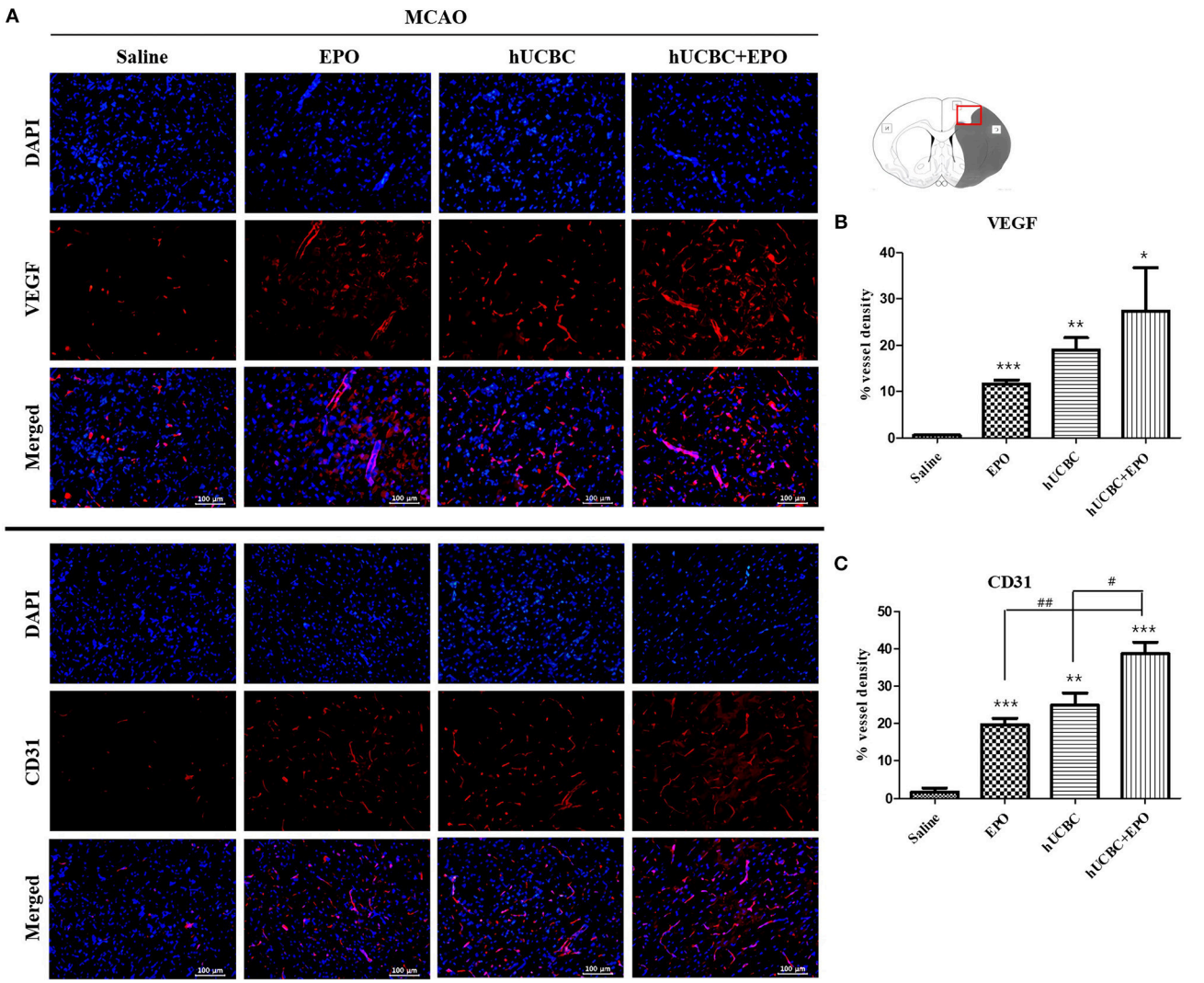


FIGURE 5 | Immunohistochemistry findings in the cerebral cortex following EPO, hUCBC and hUCBC+EPO treatments which were given at or from 7 d after MCAO. Coronal brain slices were stained with anti-VEGF or anti-CD31 antibodies 28 d after MCAO. **(A)** Representative images (**A**, scale bar 100 μm). **(B,C)** Quantification for the vessel density of VEGF(+) and CD31(+) cells in the cortex, and the highest densities of cells were observed in hUCBC+EPO group. Data represent mean ± SEM. $N = 3$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ vs. saline-treated group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ for inter-treatment group comparison.

expression of those genes after OGD (**Figure 7C**). Levels of the endothelial cell marker CD31 and the angiogenic protein markers VEGF and bFGF increased in the hUCBC+EPO group (CD31, $P < 0.005$; VEGF, $P < 0.05$; bFGF, $P < 0.0001$) relative to those following OGD injury without treatment (**Figure 7D**).

Tube Formation Effect of EPO, hUCBC, and hUCBC+EPO in OGD in Endothelial Cell

Because tubular structure formation is a critical process in angiogenesis, we conducted a tube formation assay after OGD. As a result, the hUCBC+EPO ($P < 0.0001$) treatment remarkably increased the number of tubes, while treatment with either hUCBC- or EPO-alone also increased the number of tubes relative to OGD injury without treatment (**Figure 8**).

DISCUSSION

In this study, the efficacy of cell-based therapy in *in vivo* ischemic conditions was investigated. Post-ischemic treatment with EPO, hUCBC, or hUCBC+EPO improved functional outcomes in a subacute stroke rat model. In particular, hUCBC+EPO administration promoted functional recovery as measured using the mNSS and cylinder test better than treatment with EPO- or hUCBC-alone. Most previous studies tested the efficacy of cell-based therapy at 24 h after ischemic injury which reflects the acute stage of stroke (36, 37). The study is significant as it shows remarkable improvement in the behavioral test results of adult rats given hUCBC intravenously at 7 d after MCAO, a model representative of subacute stroke that was not been frequently studied (7). The current study also

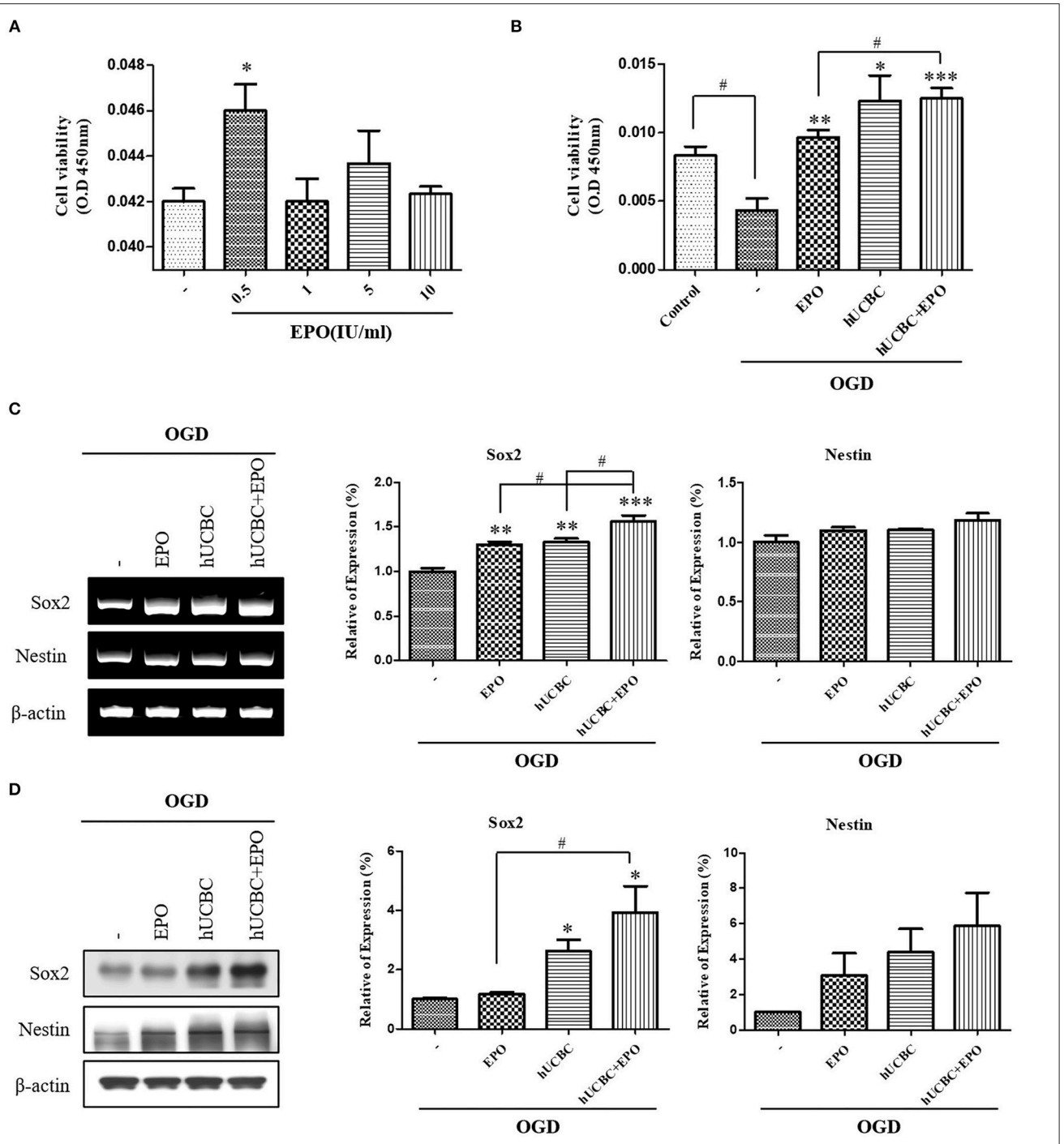


FIGURE 6 | *In vitro* assays for neurogenesis following EPO, hUCBC and hUCBC+EPO treatment using OGD-injured neural stem cells. **(A)** Cell viability of EPO concentration in OGD-injured neural stem cells **(B)** cell viability was decreased by OGD, and increased by EPO and hUCBC and the most by hUCBC+EPO. **(C)** Reverse transcription polymerase chain reaction (RT-PCR) for expression of Sox2 and Nestin. Sox2 gene expression was significantly increased by EPO or hUCBC treatment, and it was most increased by hUCBC+EPO. **(D)** Western blotting for expression of Sox2 and Nestin. Sox2 was upregulated by hUCBC and hUCBC+EPO treatment. Data represent mean ± SEM. $N = 3$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ vs. OGD-injured cells. $^{\#}P < 0.05$ for inter-treatment effect comparison.

demonstrated the therapeutic efficacy of delayed administration of EPO during the subacute stage of stroke, which is in accordance with previous research (30). However, the effect of EPO seems to last only for a few days in this study. Furthermore, the efficacy of hUCBC administration appeared later than EPO and persisted. The combination of hUCBC and

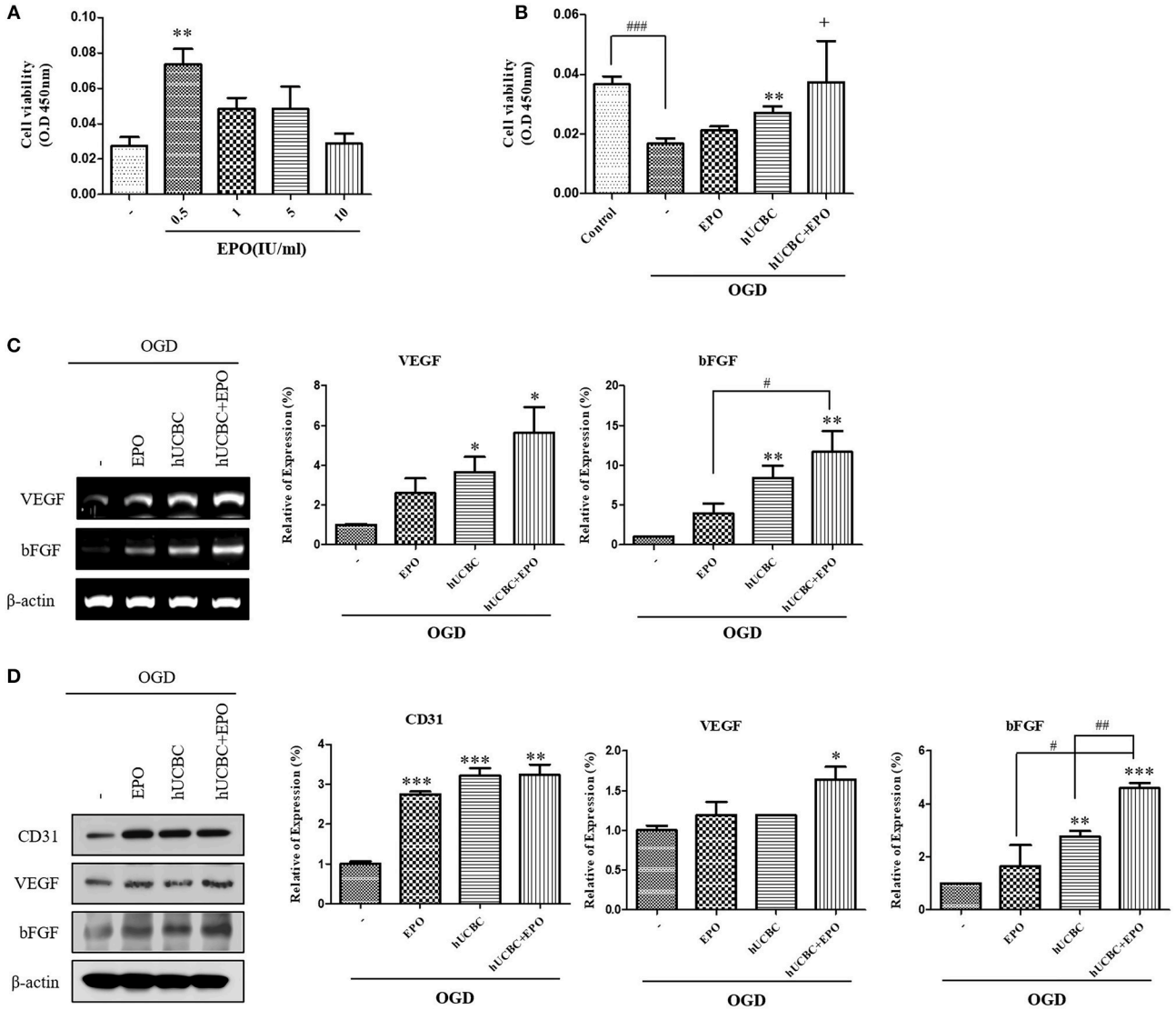


FIGURE 7 | *In vitro* assays for angiogenesis following EPO, hUCBC and hUCBC+EPO treatment using OGD-injured bEnd.3 cells. **(A)** Cell viability of EPO concentration in OGD-injured bEnd.3 cells **(B)** Survival and cell viability were decreased by OGD, and increased by hUCBC and the most by hUCBC+EPO with marginal significance (+P =0.057). **(C)** Reverse transcription polymerase chain reaction (RT-PCR) for expression of VEGF and bFGF. VEGF and bFGF gene expressions significantly increased by hUCBC treatment, and it was most increased by hUCBC+EPO. **(D)** Western blotting for expression of CD31, VEGF and bFGF. VEGF was upregulated by hUCBC+EPO. CD31 and bFGF was upregulated by hUCBC, and the most by hUCBC+EPO. Data represent mean ± SEM. $N = 3$ per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ vs. OGD-injured cells. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.005$ for inter-treatment effect comparison.

EPO showed a rapid initial response and long-lasting effect, from a week to 3 weeks after the therapy, and had the greatest efficacy of all the examined interventions. Therefore, these results indicate the possibility of overcoming issues regarding the weak efficacy of cell-based therapy in neurorestoration following stroke.

The therapeutic efficacy of hUCBC and EPO was also apparent in the brain tissue with manifestations of neurogenesis and angiogenesis in the present study. During the neurological recovery from stroke, both neurogenesis and angiogenesis occur (20, 22). Also in this study, neurogenesis and angiogenesis were enhanced together both *in vivo* and *in vitro* by treatment with EPO, hUCBC, or hUCBC+EPO. However, in terms of VEGF, its gene expression was elevated by hUCBC and hUCBC+EPO treatments (PCR), and its protein expression was elevated only by hUCBC+EPO treatments (western blotting) *in vitro*. The VEGF, a key angiogenic and permeability factor (24, 25), increases cerebral microvascular perfusion and induces neurological recovery when administered 48 h after ischemic stroke (38). VEGF is also likely to mediate the coupling of angiogenesis and neurogenesis after stroke (39). The results therefore indicate that only hUCBC+EPO treatment may induce

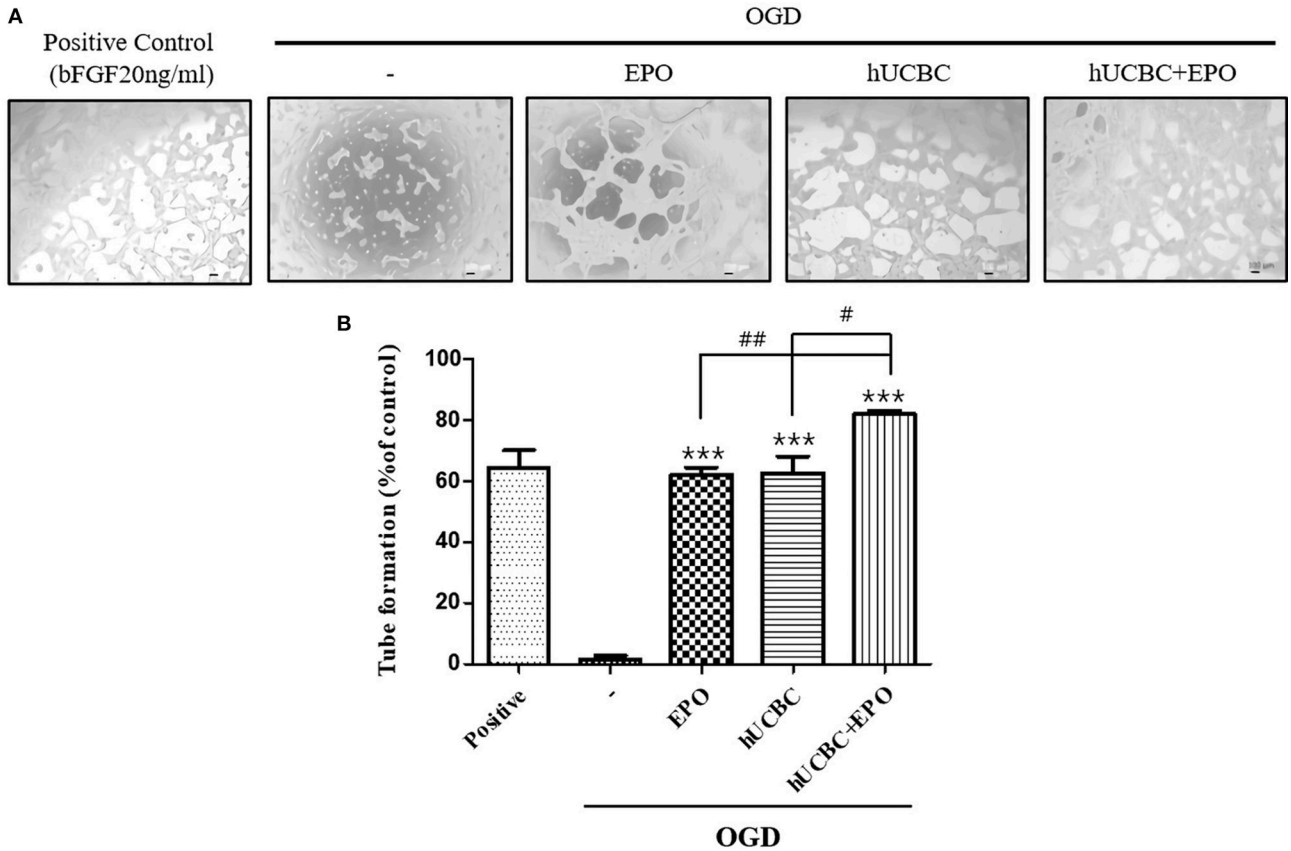


FIGURE 8 | Tube formation assay by EPO, hUCBC and hUCBC+EPO treatment in OGD-induced bEnd.3 cells. **(A)** Images of tube formation in each groups: positive control was treated bFGF (20 ng/ml); control was not; co-cultured with EPO, hUCBC and hUCBC+EPO in OGD-induced cells. **(B)** Quantitative analysis of total number of tube formation. Total number of tubes were increased by EPO or hUCBC treatment, and the most by hUCBC+EPO. Data represent mean ± SEM. $N = 3$ per group. $^{***}P < 0.005$ vs. OGD-injured cells. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ for inter-treatment effect comparison.

the enhancement of VEGF expression, the most significant factor in neurogenesis and angiogenesis, even in later stage post-stroke.

The highest levels of neurogenesis and angiogenesis in the cortex occurred following hUCBC+EPO treatment; similar responses were seen for NSC and endothelial cells. Treatment with either hUCBC- or EPO-alone resulted in similar findings regarding neurogenesis and angiogenesis *in vivo* and *in vitro*, but to a lesser extent than that following hUCBC+EPO treatment. It was interesting that the number of NeuN(+) neuronal cells was increased by treatment with either hUCBC or EPO, but increased most following treatment with hUCBC+EPO. On the contrary, the number of GFAP(+) cells were decreased inversely by the treatments and hUCBC+EPO induced the greatest reduction. GFAP can be expressed by many cell types although astrocytes are the most representative and the role of astrocytes in maladaptive plasticity in the ischemic brain has been reported (40). Although astrocytes have two roles in the pathological mechanisms of neurological diseases (41), our finding of a reduction in the number of GFAP(+) cells can be interpreted as a reduction of astrogliosis. In relation to this reaction, it is notable that another glial maker, Iba-1(+) cells showed reversed reaction. Since this finding indicates more survival or activation of microglia by hUCBC or EPO treatment, role of the cells in neurogenesis can be inferred.

This study is not the first to illustrate the potential of cell-based therapy via combination with a growth factor. Because a study where brain-derived neurotrophic factor was administered alongside hBMSC in a stroke model showed positive results (42), variable approaches have been reported to enhance the neurorestorative effect of cell-based therapy (43). To present, reports have also revealed the therapeutic advantages of concomitant administration of other factors in cell-based therapy (44, 45). However, not all trials were successful. A recent study revealed potential side effects that are harmful in ischemic stroke when hBMSC were transplanted with granulocyte colony-stimulating factor, leading to findings of increased hemorrhagic transformation and astrogliosis with alterations in the blood-brain barrier (46). In the present study, the results seem to show an additive or synergistic effect of combined treatment with hUCBC and EPO, without a diluting effect, both for neurogenesis and angiogenesis. Furthermore, the finding of a reduction in astrogliosis was inversely correlated with the

increase in neurogenesis and angiogenesis for all treatments among the groups, both *in vivo* and *in vitro*. Taken together, hUCBC and EPO appear to share the same signaling pathways in their therapeutic mechanisms for recovery after stroke. This therapeutic combination did not result in harmful adverse events, which is the most important point in clinical applications.

This study has certain limitations. First, the perfectly uniform allocation of the rats into four groups was not possible for all experimental subsets. However, this study was conducted following the principles of randomization. Second, the increase in the number of NeuN(+) cells in the cortex following treatment that was taken as reflective of neurogenesis can be the result of neuroprotection with increased survival of neuronal cells. We did not observe neuroregeneration *per se* after loss of neurons in the damaged area. Third, although the results in behavior, histological findings, and *in vitro* assays represent manifestations of neurogenesis and angiogenesis, more direct mechanisms that may explain the response of the host induced by hUCBC and EPO administration should be clarified. Also, this study did not reveal engraftment status of the intravenously administered cells. The authors presume that the treatments were given systemically, the response must be systemic and must have affected the brain. Considering their availability in clinical usage, it is difficult to give therapeutic cells directly to the brain, and because peripherally introduced cells demonstrated efficacy, the mechanism of action would be important to identify. In relation, further research on the blood-brain barrier might enlighten the therapeutic mechanism. Lastly, the common signaling pathway of hUCBC and EPO treatment for stroke injury should be elucidated.

In conclusion, our results suggest that hUCBC infusion in combination with EPO administration demonstrates therapeutic efficacy in the treatment of stroke-induced injury by promoting neurogenesis and angiogenesis. Further research that delineates the therapeutic mechanism of systemically administered hUCBC and EPO is required.

ETHICS STATEMENT

All experimental procedures involving animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U. S. National Institutes of Health and were approved by CHA University Institutional Animal Care & Use Committee (IACUC150018, IACUC180018, IACUC180181).

AUTHOR CONTRIBUTIONS

SH conducted animal experiments, analyzed the data, and drafted the manuscript. JC performed *in vitro* assays and analyzed the data. MK designed and conceptualized this study, interpreted the results, and revised the manuscript.

FUNDING

This work was supported by Industrial Strategic Technology Development Program (10051152, Development of advanced cell therapy technology using growth factor for stroke treatment) funded by the Ministry of Trade, Industry, and Energy (MOTIE, Korea).

ACKNOWLEDGMENTS

The hUCBCs were supplied from CHA Medical Center Cord Blood Bank. We thank to the Enditage for English editing, and to Dr. Kye Hee Cho for discussion.

REFERENCES

1. Murakami K, Suzuki M, Suzuki N, Hamajo K, Tsukamoto T, Shimojo M. Cerebroprotective effects of TAK-937, a novel cannabinoid receptor agonist, in permanent and thrombotic focal cerebral ischemia in rats: therapeutic time window, combination with t-PA and efficacy in aged rats. *Brain Res.* (2013) 1526:84–93. doi: 10.1016/j.brainres.2013.06.014
2. Xu SY, Pan SY. The failure of animal models of neuroprotection in acute ischemic stroke to translate to clinical efficacy. *Med Sci Monit Basic Res.* (2013) 19:37–45. doi: 10.12659/MSMBR.883750
3. Chen SY, Zhang XR, Chen J, Ge WQ, Wang WW, Wang XS, et al. An overview of meta-analyses of endovascular bridging therapies for acute ischemic stroke. *Biomed Res Int.* (2018) 2018:9831210. doi: 10.1155/2018/9831210
4. Skilbeck CE, Wade DT, Hewer RL, Wood VA. Recovery after stroke. *J Neurol Neurosurg Psychiatr.* (1983) 46:5–8. doi: 10.1136/jnnp.46.1.5
5. Chen X, Wang K. The fate of medications evaluated for ischemic stroke pharmacotherapy over the period 1995-2015. *Acta Pharm Sin B.* (2016) 6:522–30. doi: 10.1016/j.apsb.2016.06.013
6. Chen J, Li Y, Wang L, Zhang Z, Lu D, Lu M, et al. Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke.* (2001) 32:1005–11. doi: 10.1161/01.STR.32.4.1005
7. Chen J, Sanberg PR, Li Y, Wang L, Lu M, Willing AE, et al. Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. *Stroke.* (2001) 32:2682–8. doi: 10.1161/hs1101.098367
8. Nam HS, Kwon I, Lee BH, Kim H, Kim J, An S, et al. Effects of mesenchymal stem cell treatment on the expression of matrix metalloproteinases and angiogenesis during ischemic stroke recovery. *PLoS ONE.* (2015) 10:e0144218. doi: 10.1371/journal.pone.0144218
9. Liu X, Ye R, Yan T, Yu SP, Wei L, Xu G, et al. Cell based therapies for ischemic stroke: from basic science to bedside. *Prog Neurobiol.* (2014) 115:92–115. doi: 10.1016/j.pneurobio.2013.11.007
10. Marei HE, Hasan A, Rizzi R, Althani A, Afifi N, Cenciarelli C, et al. Potential of stem cell-based therapy for ischemic stroke. *Front Neurol.* (2018) 9:34. doi: 10.3389/fneur.2018.00034
11. Gluckman E. History of cord blood transplantation. *Bone Marrow Transplant.* (2009) 44:621–6. doi: 10.1038/bmt.2009.280
12. Cui X, Chopp M, Shehadah A, Zacharek A, Kuzmin-Nichols N, Sanberg CD, et al. Therapeutic benefit of treatment of stroke with simvastatin and human umbilical cord blood cells: neurogenesis, synaptic plasticity, and axon growth. *Cell Transplant.* (2012) 21:845–56. doi: 10.3727/096368911X627417
13. Kang M, Min K, Jang J, Kim SC, Kang MS, Jang SJ, et al. Involvement of immune responses in the efficacy of cord blood cell therapy for cerebral palsy. *Stem Cells Dev.* (2015) 24:2259–68. doi: 10.1089/scd.2015.0074
14. Yu JH, Seo JH, Lee JE, Heo JH, Cho SR. Time-dependent effect of combination therapy with erythropoietin and granulocyte colony-stimulating factor in a mouse model of hypoxic-ischemic brain injury. *Neurosci Bull.* (2014) 30:107–17. doi: 10.1007/s12264-013-1397-9
15. Li Y, Lu Z, Keogh CL, Yu SP, Wei L. Erythropoietin-induced neurovascular protection, angiogenesis, and cerebral blood flow restoration after

focal ischemia in mice. *J Cereb Blood Flow Metab.* (2007) 27:1043–54. doi: 10.1038/sj.jcbfm.9600417
16. Zhou Z, Wei X, Xiang J, Gao J, Wang L, You J, et al. Protection of erythropoietin against ischemic neurovascular unit injuries through the effects of connexin43. *Biochem Biophys Res Commun.* (2015) 458:656–62. doi: 10.1016/j.bbrc.2015.02.020
17. Unden J, Sjolund C, Lansberg JK, Wieloch T, Ruscher K, Romner B. Post-ischemic continuous infusion of erythropoeitin enhances recovery of lost memory function after global cerebral ischemia in the rat. *BMC Neurosci.* (2013) 14:27. doi: 10.1186/1471-2202-14-27
18. Wang R, Li J, Duan Y, Tao Z, Zhao H, Luo Y. Effects of erythropoietin on gliogenesis during cerebral ischemic/reperfusion recovery in adult mice. *Aging Dis.* (2017) 8:410–9. doi: 10.14336/AD.2016.1209
19. Sakanaka M, Wen TC, Matsuda S, Masuda S, Morishita E, Nagao M, et al. *In vivo* evidence that erythropoietin protects neurons from ischemic damage. *Proc Natl Acad Sci USA.* (1998) 95:4635–40. doi: 10.1073/pnas.95.8.4635
20. Wang L, Zhang Z, Wang Y, Zhang R, Chopp M. Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. *Stroke.* (2004) 35:1732–7. doi: 10.1161/01.STR.0000132196.49028.a4
21. Gandin C, Widmann C, Lazdunski M, Heurteaux C. MLC901 Favors angiogenesis and associated recovery after ischemic stroke in mice. *Cerebrovasc Dis.* (2016) 42:139–54. doi: 10.1159/000444810
22. Ruan L, Wang B, Zhuge Q, Jin K. Coupling of neurogenesis and angiogenesis after ischemic stroke. *Brain Res.* (2015) 1623:166–73. doi: 10.1016/j.brainres.2015.02.042
23. Chen J, Zhang ZG, Li Y, Wang L, Xu YX, Gautam SC, et al. Intravenous administration of human bone marrow stromal cells induces angiogenesis in the ischemic boundary zone after stroke in rats. *Circ Res.* (2003) 92:692–9. doi: 10.1161/01.RES.0000063425.51108.8D
24. Cui X, Chopp M, Zacharek A, Roberts C, Lu M, Savant-Bhonsale S, et al. Chemokine, vascular and therapeutic effects of combination Simvastatin and BMSC treatment of stroke. *Neurobiol Dis.* (2009) 36:35–41. doi: 10.1016/j.nbd.2009.06.012
25. Zhang Q, Zhao Y, Xu Y, Chen Z, Liu N, Ke C, et al. Sodium ferulate and n-butylidenephthalate combined with bone marrow stromal cells (BMSCs) improve the therapeutic effects of angiogenesis and neurogenesis after rat focal cerebral ischemia. *J Transl Med.* (2016) 14:223. doi: 10.1186/s12967-016-0979-5
26. Yu J, Moon J, Jang J, Choi JI, Jung J, Hwang S, et al. Reliability of behavioral tests in the middle cerebral artery occlusion model of rat. *Lab Anim.* (2018). doi: 10.1177/0023677218815210. [Epub ahead of print].
27. Min K, Song J, Lee JH, Kang MS, Jang SJ, Kim SH, et al. Allogenic umbilical cord blood therapy combined with erythropoietin for patients with severe traumatic brain injury: three case reports. *Restor Neurol Neurosci.* (2013) 31:397–410. doi: 10.3233/RNN-120289
28. Kim M, Cho K, Min K, Lee S. Safety and efficacy of allogeneic umbilical cord blood therapy combined with erythropoietin in children with cerebral palsy: study protocol for a double-blind, randomized, placebo-controlled trial. *Asia Pac J Clin Trials.* (2017) 2:129–39. doi: 10.4103/2542-3932.217491
29. Morimoto J, Yasuhara T, Kameda M, Umakoshi M, Kin I, Kuwahara K, et al. Electrical stimulation enhances migratory ability of transplanted bone marrow stromal cells in a rodent ischemic stroke model. *Cell Physiol Biochem.* (2018) 46:57–68. doi: 10.1159/000488409
30. Larpthaveesarp A, Georgevits M, Ferriero DM, Gonzalez FF. Delayed erythropoietin therapy improves histological and behavioral outcomes after transient neonatal stroke. *Neurobiol Dis.* (2016) 93:57–63. doi: 10.1016/j.nbd.2016.04.006
31. Ku JM, Taher M, Chin KY, Grace M, Mcintyre P, Miller AA. Characterisation of a mouse cerebral microvascular endothelial cell line (bEnd.3) after oxygen glucose deprivation and reoxygenation. *Clin Exp Pharmacol Physiol.* (2016) 43:777–86. doi: 10.1111/1440-1681.12587
32. Yu X, Wang X, Zeng S, Tuo X. Protective effects of primary neural stem cell treatment in ischemic stroke models. *Exp Ther Med.* (2018) 16:2219–28. doi: 10.3892/etm.2018.6466
33. Wang Q, Yang L, Wang Y. Enhanced differentiation of neural stem cells to neurons and promotion of neurite outgrowth by oxygen-glucose deprivation. *Int J Dev Neurosci.* (2015) 43:50–7. doi: 10.1016/j.ijdevneu.2015.04.009
34. Yoo SY, Kwon SM. Angiogenesis and its therapeutic opportunities. *Mediators Inflamm.* (2013) 2013:127170. doi: 10.1155/2013/127170
35. Makarevich P, Tsokolaeva Z, Shevelev A, Rybalkin I, Shevchenko E, Beloglazova I, et al. Combined transfer of human VEGF165 and HGF genes renders potent angiogenic effect in ischemic skeletal muscle. *PLoS ONE.* (2012) 7:e38776. doi: 10.1371/journal.pone.0038776
36. Chen J, Zhang C, Jiang H, Li Y, Zhang L, Robin A, et al. Atorvastatin induction of VEGF and BDNF promotes brain plasticity after stroke in mice. *J Cereb Blood Flow Metab.* (2005) 25:281–90. doi: 10.1038/sj.jcbfm.9600034
37. Zhao Q, Hu J, Xiang J, Gu Y, Jin P, Hua F, et al. Intranasal administration of human umbilical cord mesenchymal stem cells-conditioned medium enhances vascular remodeling after stroke. *Brain Res.* (2015) 1624:489–96. doi: 10.1016/j.brainres.2015.08.003
38. Zhang ZG, Zhang L, Jiang Q, Zhang R, Davies K, Powers C, et al. VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. *J Clin Invest.* (2000) 106:829–38. doi: 10.1172/JCI9369
39. Teng H, Zhang ZG, Wang L, Zhang RL, Zhang L, Morris D, et al. Coupling of angiogenesis and neurogenesis in cultured endothelial cells and neural progenitor cells after stroke. *J Cereb Blood Flow Metab.* (2008) 28:764–71. doi: 10.1038/sj.jcbfm.9600573
40. Wang YF, Parpura V. Central role of maladapted astrocytic plasticity in ischemic brain edema formation. *Front Cell Neurosci.* (2016) 10:129. doi: 10.3389/fncel.2016.00129
41. Pekny M, Pekna M, Messing A, Steinhauser C, Lee JM, Parpura V, et al. Astrocytes: a central element in neurological diseases. *Acta Neuropathol.* (2016) 131:323–45. doi: 10.1007/s00401-015-1513-1
42. Chen J, Li Y, Chopp M. Intracerebral transplantation of bone marrow with BDNF after MCAo in rat. *Neuropharmacology.* (2000) 39:711–6. doi: 10.1016/S0028-3908(00)00006-X
43. Barkho BZ, Zhao X. Adult neural stem cells: response to stroke injury and potential for therapeutic applications. *Curr Stem Cell Res Ther.* (2011) 6:327–38. doi: 10.2174/157488811797904362
44. Pirzad Jahromi G, Seidi S, Sadr SS, Shabanzadeh AP, Keshavarz M, Kaka GR, et al. Therapeutic effects of a combinatorial treatment of simvastatin and bone marrow stromal cells on experimental embolic stroke. *Basic Clin Pharmacol Toxicol.* (2012) 110:487–93. doi: 10.1111/j.1742-7843.2011.00848.x
45. Cho DY, Jeun SS. Combination therapy of human bone marrow-derived mesenchymal stem cells and minocycline improves neuronal function in a rat middle cerebral artery occlusion model. *Stem Cell Res Ther.* (2018) 9:309. doi: 10.1186/s13287-018-1011-1
46. Strecker JK, Olk J, Hoppen M, Gess B, Diederich K, Schmidt A, et al. Combining growth factor and bone marrow cell therapy induces bleeding and alters immune response after stroke in mice. *Stroke.* (2016) 47:852–62. doi: 10.1161/STROKEAHA.115.011230

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.

Copyright © 2019 Hwang, Choi and Kim. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Neurology

SYSTEMATIC REVIEW
published: 24 April 2019
doi: 10.3389/fneur.2019.00405

World-Wide Efficacy of Bone Marrow Derived Mesenchymal Stromal Cells in Preclinical Ischemic Stroke Models: Systematic Review and Meta-Analysis

Nikunj Satani[1], Chunyan Cai[1,2,3], Kaavya Giridhar[1], Daryl McGhiey[1], Sarah George[1], Kaushik Parsha[1,4], Duyen M. Nghiem[1], Krystal S. Valenzuela[5], Jenny Riecke[6], Farhaan S. Vahidy[1] and Sean I. Savitz[1]*

[1] McGovern Medical School, Institute for Stroke and Cerebrovascular Diseases, The University of Texas Health Science Center at Houston, Houston, TX, United States, [2] McGovern Medical School, Department of Internal Medicine, The University of Texas Health Science Center at Houston, Houston, TX, United States, [3] Center for Clinical and Translational Sciences, McGovern Medical School, The University of Texas Health Science Center at Houston, Houston, TX, United States, [4] Department of Neurology, McGovern Medical School, The University of Texas Health Science Center at Houston, Houston, TX, United States, [5] Department of Psychology, The University of Texas Health Science Center at Austin, Austin, TX, United States, [6] Department of Neurology, The University of Texas Southwestern Medical Center, Dallas, TX, United States

OPEN ACCESS

Edited by:
Paulo Henrique Rosado-de-Castro, D'Or Institute for Research and Education (IDOR), Brazil

Reviewed by:
Simon Koblar, University of Adelaide, Australia
Aurel Popa-Wagner, University Hospital Essen, Germany

***Correspondence:**
Nikunj Satani
nikunj.b.satani@uth.tmc.edu

Specialty section:
This article was submitted to Stroke, a section of the journal Frontiers in Neurology

Received: *15 January 2019*
Accepted: *04 April 2019*
Published: *24 April 2019*

Citation:
Satani N, Cai C, Giridhar K, McGhiey D, George S, Parsha K, Nghiem DM, Valenzuela KS, Riecke J, Vahidy FS and Savitz SI (2019) World-Wide Efficacy of Bone Marrow Derived Mesenchymal Stromal Cells in Preclinical Ischemic Stroke Models: Systematic Review and Meta-Analysis. Front. Neurol. 10:405. doi: 10.3389/fneur.2019.00405

Background: Following extensive, positive results in pre-clinical experiments, Bone Marrow Derived-Mesenchymal Stromal Cells (BM-MSCs) are now being tested as a novel therapy for ischemic stroke in ongoing clinical trials. However, multiple critical questions relating to their translational application remain to be clarified. We performed a comprehensive, systematic review and meta-analysis of pre-clinical studies to evaluate the efficacy of BM-MSCs on functional outcomes after ischemic stroke, as well as the independent role of translational factors on their effect size.

Methods: We systematically reviewed the literature and identified articles using BM-MSCs in animal models of focal ischemic stroke. After abstraction of all relevant data, we performed a meta-analysis to estimate the combined effect size of behavioral endpoints after BM-MSC administration. To describe the effect size across many behavioral outcomes, we divided these outcomes into four categories: (1) Composite scores, (2) Motor Tests, (3) Sensorimotor Tests, and (4) Cognitive Tests. We also performed a meta-regression analysis for measuring the effect of individual characteristics of BM-MSC administration on the effect size.

Results: Our results from 141 articles indicate a significant beneficial effect on composite, motor, and sensorimotor outcomes after treatment with BM-MSCs compared to control groups. We found no major differences in treatment effect based on delivery route, dose, fresh vs. frozen preparation, or passage number. There were no consistent findings supporting a difference in treatment effect based on time windows from acute periods (0–6 h) vs. later windows (2–7 days). Furthermore, these positive treatment effects on functional outcome were consistent across different labs in different parts of the world as well as over the last 18 years. There was a negative correlation between publication year and impact factor.

Conclusions: Our results show worldwide efficacy of BM-MSCs in improving functional outcomes in pre-clinical animal models of stroke and support testing these cells in clinical trials in various ranges of time windows using different delivery routes. The continued growing number of publications showing functional benefit of BM-MSCs are now adding limited value to an oversaturated literature spanning 18 years. Researchers should focus on identifying definitive mechanisms on how BM-MSCs lead to benefit in stroke models.

Keywords: ischemic stroke, meta-analysis, functional outcome, mesenchymal stromal cells, treatment effect, timing of administration, co-morbidities, gender differences

INTRODUCTION

Ischemic stroke is the 5th leading cause of death and the leading cause of long term disability in the United States (1, 2). Currently, tPA is the only FDA approved medical, non-invasive treatment for ischemic stroke, its use restricted by a narrow time window of 3 h (4.5 h in certain eligible patients) after symptom onset. This has limited the use of tPA to only a fraction of ischemic stroke patients.

Ischemic stroke results in a complex cascade of events leading to the loss of neural tissue, including neurons and their supporting structures (3, 4). Concurrently, there is an initiation of local and systemic inflammatory responses that evolve over a period of days, and the extent of which determines the eventual degree of damage (4). Considering the involvement of multiple parallel processes contributing to recovery, it is not surprising that stroke researchers have tested a multitude of investigational therapies for ischemic stroke with no significant advancement in stroke treatment.

Over the last two decades, Bone Marrow derived Mesenchymal Stromal Cells (BM-MSCs) have been investigated extensively as potential novel approach to promote recovery after ischemic stroke (5–7), and have advanced to clinical trials (8–10). BM-MSCs have demonstrated their beneficial effects by immunomodulation via multiple processes such as release of trophic factors, increasing angiogenesis, recruitment of neural precursors, synaptogenesis, as well as modulating immune responses from peripheral organs such as lungs and spleen (11, 12). Multiple preclinical animal studies have yielded encouraging results favoring the use of BM-MSCs for the treatment of ischemic stroke. These preclinical animal studies differ in various factors such as cell dose, timing, and route of administration, use of fresh or cultured cells, cell passage and the species from which the cells were isolated. In addition, the methodology of how and which outcomes are measured varies between different laboratories and researchers across the world.

As the use of BM-MSCs for ischemic stroke is translated to human clinical trials, there is a need to better understand the impact of individual, clinically relevant factors, as they most likely play a critical role in the potential treatment effects of BM-MSCs, and the design of clinical trials. Previously published meta-analyses have studied the results of preclinical studies using mesenchymal cells to evaluate their functional effect; however none of them extensively studied whether methodological variability between these preclinical trials affects functional outcome (13–15). Any reasonable interpretation of effect size, particularly with respect to timing of administration and delivery route was further limited by smaller sample size of studies included and behavioral tests they characterized. In addition, previous meta-analyses did not examine important clinically relevant variables such as gender of stroke animals, age of stroke animals, species from which MSCs were harvested, as well as the effect of stroke co-morbidities.

To address these issue, we conducted a comprehensive, updated, systematic review, and meta-analysis of all the preclinical publications between the years 2000 to 2018, which investigated the use of BM-MSCs to evaluate the efficacy of BM-MSCs in animal models of focal ischemic stroke. The primary aim of our analysis was to compare the effect size of behavioral improvement after BM-MSC administration vs. vehicle administration. To simplify the results from many behavioral tests in these articles, we divided these outcomes into four categories as previously described by our group (16): (1) Composite scores, (2) Motor Tests, (3) Sensorimotor Tests, and (4) Cognitive Tests. We also performed a meta-regression analysis to study whether heterogeneity among results of multiple studies is related to any specific characteristics of the treatment factors such as cell dose, labeling, timing and route of administration, time when outcome was measured, use of fresh or cultured cells, cell passage, species, and gender of animals from which cells were isolated, species and gender of stroke animals, co-morbidities as well as the laboratory where study was conducted.

MATERIALS AND METHODS

Search Strategy

Previous reports investigating the use of BM-MSCs in ischemic stroke were identified by an electronic search of PUBMED and Google Scholar using the following search terms: "marrow OR bone marrow" AND "mesenchymal stem cell OR stromal cell OR mesenchymal cell" AND "stroke OR cerebrovascular OR cerebral ischemia OR middle cerebral artery OR MCA OR anterior cerebral artery OR ACA." We excluded all reports which did not study functional outcome. In order to be maximally relevant to human clinical trials, studies investigating the use of gene modified BM-MSCs were also excluded. Studies in languages other than English were excluded. All other exclusion criteria are shown in **Figure 1**. A total of 141 articles were selected using the search strategy (**Figure 1**) and

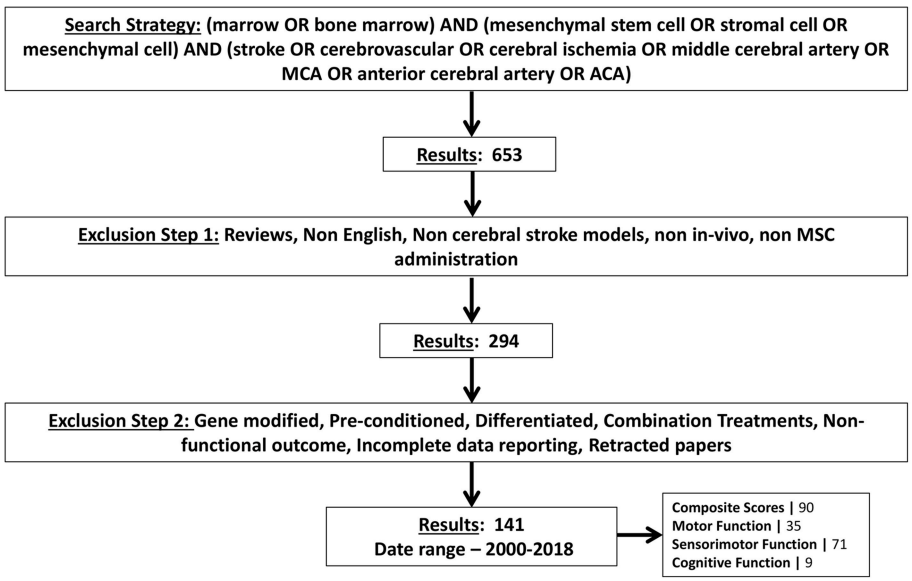


FIGURE 1 | Schematic showing the search strategy for selecting articles for meta-analysis. Exclusion criteria are also shown. Number of articles found by this search strategy are shown and further stratified by type of functional tests they measured.

data were subsequently extracted. The final search was carried out on December 12, 2018.

Inclusion Criteria

Published studies were included if they fulfilled all of the following criteria: (1) assessed bone marrow derived mesenchymal stromal cells in animal models of focal ischemic stroke; and (2) reported functional outcomes.

Exclusion Criteria

We excluded review articles, editorials, commentaries, letters which reported no new data, meta-analyses as well as abstracts. In addition, we excluded the following articles that: (1) were not written in English; (2) studied non-focal stroke models; (3) had no *in-vivo* experiments; (4) did not use bone marrow derived mesenchymal stromal cells; (5) used gene-modified BM-MSCs; (6) used preconditioned BM-MSCs; (7) used differentiated BM-MSCs; (8) used BM-MSCs with other drugs or cells as combination treatments; (9) did not assess functional outcomes; (10) did not report SD or SE; (11) had SD or SE as 0 (zero); and (12) did not report sample size.

Data Extraction

After running the search strategy and applying inclusion and exclusion criteria, we found 141 articles. We extracted data of all functional outcomes measured in these studies. We collected functional endpoints from all tables and the results. For line graphs, the data were extracted from the graphics using Web Plot Digitizer Version 4.1 - (https://automeris.io/WebPlotDigitizer/index.html). Two independent abstractors collected each outcome. Average of the data collected from the two users was used to run statistical analysis.

We collected data on behavioral tests conducted in these articles and divided them into four categories: (1) Composite Scores—which included Neurological severity score, Garcia Score, Roger Scale, Bederson Score, and Longa Score; (2) Motor Tests—which included cylinder test, foot fault test, limb placement, beam balance test, tightrope test and grid walking test; (3) Sensorimotor tests—which included adhesive removal test, treadmill stress test, rotarod test, corner test, elevated body swing test and limb stride length measurement; and (4) Cognitive tests—which included water maze test, eight arm radial maze test and novel object recognition test. We used this classification of functional outcomes as per our previously published paper (16). Considering the comprehensive nature of our meta-analysis, this classification allowed us to analyze and organize meta-analysis and meta-regression data from all functional tests and provided a guide for future pre-clinical and clinical trials assessing functional data.

In addition to collecting the functional outcomes, we also collected information about the BM-MSCs used to administer in these articles. We collected information on variables such as cell passage, cell labeling, time when functional outcome was measured, time when cells were administered, dosage of cells used, whether the cells were used fresh or cryopreserved before use, donor and species of BM-MSCs, the laboratory where the experiments were conducted. These variables were categorized as per **Table 1**. Only the last time point measurements were examined for behavioral testing if outcomes were reported at multiple time points within the same experiment. When there were multiple independent experiments in one article, we included all of these experiments into the analysis. For calculating dose of BM-MSCs, we took the average reported weight of stroke animals and converted all doses to cells per kilogram. We also

TABLE 1 | List of all variables collected in this meta-analysis.

Variables collected from 141 articles
Cell labeling
Yes | No
Route of administration
Intra-arterial | Intravenous | Intracranial
Time when outcome was measured
<2 weeks | 2–4 weeks | 4–12 weeks | >12 weeks
Dose of bm-mscs administered
>1*10E6 | <=1*10E6
Time of bm-msc Administration
0–6 h vs. > 7 Days
12–24 h vs. >7 Days
2–7 Days vs. >7 Days
0–6 h vs. 2–7 Days
12–24 h vs. 2–Days
0–6 h vs. 12–24 h
Were bm-mscs cryopreserved or not?
Fresh | Frozen
Passage of administered bm-mscs
2–4 vs. >4
Species of bm-msc donor
Rat | Mouse | Human
Gender oF bm-msc donor
Female|Male | Unknown
Species of stroke animal
Rat | Mouse | Rabbit | Monkey | Dog
Gender of stroke animal
Female | Male | Unknown
Age of stroke animal
Adult | Retired Breeder
Stroke animal co-morbidities
Normal | T1DM | T2DM | SCID
Continent
Asia | North America | Europe | South America
Year published
2000–2008 | 2009–2012 | 2013–2015 | 2016–2018
Impact factor of journal where article was published

analyzed effect sizes based on year of article publication. We calculated 25, 50, and 75 th quartiles of the publication year and categorized experiments into four groups based on publication year: 2000–2008, 2009–2012, 2013–2015, and 2016–2018.

Statistical Analysis

The effect size of BM-MSCs therapy was calculated as the standardized mean difference (SMD) of these interested outcomes between MSC and Vehicle-treated groups based on Hedges' method. The overall heterogeneity was examined by I^2 and Cochran's Q-statistic test (17). A $p < 0.1$ was considered statistically significant for the Cochran's Q-statistic test (18). Since heterogeneity exists for all four types of outcomes, random effects model using DerSimonian and Laird method was applied to obtain the pooled effect size. We multiplied the outcomes by-−1 for larger values, indicating superior outcome if needed. For each type of outcome, we generated forest plots to depict the SMD along with its 95% confidence interval (CI) for each individual experiment as well as the pooled SMD of all studies. The statistical significance of the pooled effect size was performed by z-test. To confirm whether our findings were driven by any single study, a leave-one-out sensitivity analysis was performed by iteratively removing one study at a time. Excessive influence was suspected if the main estimate of omitting an individual study lied outside the 95% CI of the combined analysis.

Meta regression analysis was performed to assess whether heterogeneity among results of multiple studies is related to any specific characteristics of the studies, if the significance of heterogeneity was found. Univariable meta-regression analyses based on a random effects model with restricted maximum likelihood estimation were performed for four types of outcomes. Potential publication bias was evaluated using funnel plots and Egger test was performed to evaluate the symmetry of the funnel plots (19). If asymmetry was observed, a Trim and Fill procedure (20) was applied to identify the existence of unpublished hidden studies. By imputing the presence of these potential missing studies, an adjusted pooled estimate was provided. All analyses were performed with StataMP 11 (StataCorp LP, College Station, TX).

RESULTS

Study Characteristics

The flow diagram shows that the initial search yielded 653 pubmed articles. After applying all exclusion criteria, we found 141 articles and relevant data was subsequently extracted. The number of articles identified and excluded at each step are depicted in **Figure 1**. Among all articles measuring functional outcomes, 90 articles reported composite scores, 35 articles reported motor outcomes, 71 articles reported sensorimotor outcomes, and 9 articles reported cognitive outcomes. Some articles conducted multiple independent, behavioral tests to measure outcome in the same category. Hence, a total of 105 experiments reported composite scores, 53 experiments reported motor function, 101 experiments reported sensorimotor function and 10 experiments reported cognitive function. Frequency and percentage of all interested variables by test categories was calculated in SAS (**Table S1**).

Treatment Effect Size

Composite Score

We performed a meta-analysis to evaluate the effect of BM-MSCs on overall neurological function, by assessing composite scores from 90 articles (105 experiments) based on tests of mNSS, Bederson score, Longa Score, and Roger scale. These tests are routinely used by different researchers around the world to assess severity of neurological damage after stroke. For the composite score, the pooled effect size of BM-MSC therapy was substantial and significant (1.26, 95% CI: 1.10–1.42), which demonstrates a significant decrease in the composite score in the BM-MSC group compared with vehicle treatment (**Figure 2**). Random

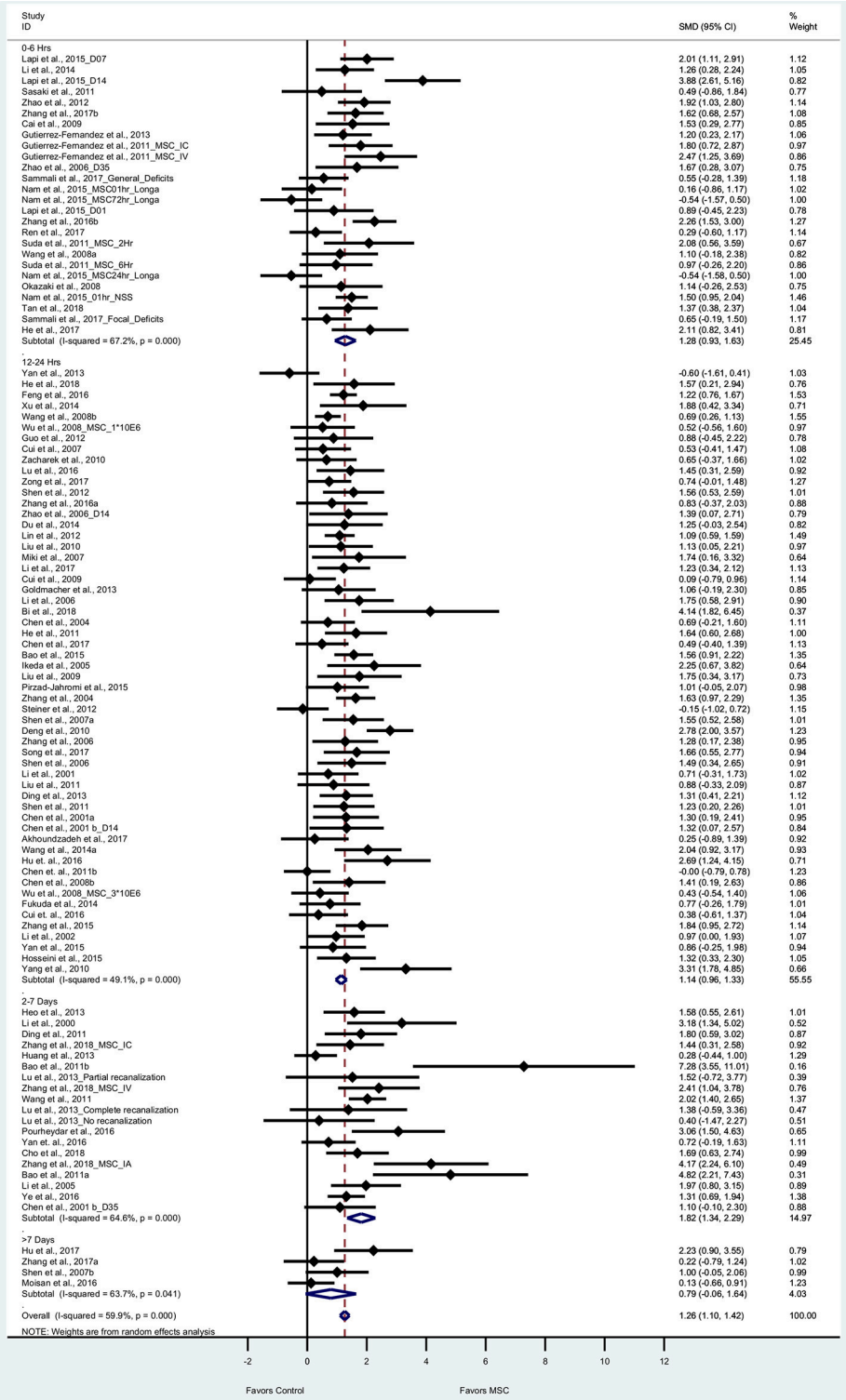


FIGURE 2 | Forest plot showing standardized mean difference of composite score between BM-MSC therapy and control groups, stratified by timing of administration of BM-MSCs. 95% confidence intervals are shown for all studies measuring composite scores. 95% confidence intervals are further stratified by timing of administration of BM-MSCs into 0–6 h, 12–24 h, 2–7 days, and >7 days. *p*-values are for heterogeneity measured by Cochran's Q-statistic test, values <0.1 are significant.

effects model was applied since significant heterogeneity was observed by Cochran's Q-statistic test ($p < 0.0001$). Furthermore, we stratified these experiments by timing of BM-MSC therapy: 0–6 h, 12–24 h, 1–7days and >7days (**Figure 2**). We found that the pooled effect size in each of these groups remained significant ($p < 0.0001$) except for >7days ($p = 0.07$).

Motor Function

Thirty five articles (53 experiments) reported motor outcomes based on tests that evaluate motor deficits such as cylinder test, beam walking test, elevated body swing test, foot fault, ladder-rung walking test, and grid walking test. For motor outcomes, the pooled effect size of BM-MSC therapy was substantial and significant as compared to vehicle group (0.98, 95% CI: 0.73–1.22), which demonstrates that BM-MSCs significantly improved motor outcomes (**Figure 3**). Random effects model was applied since significant heterogeneity was observed by Cochran's Q-statistic test ($p < 0.0001$). When we stratified motor outcomes by timing of treatment, we found that BM-MSCs significantly improved outcomes for treatment at 0–6 h, 12–24 h, and 2–7days ($p < 0.0001$ for all three groups, **Figure 3**). BM-MSC treatment at >7 days also improved motor outcomes ($p = 0.02$).

Sensorimotor Function

Seventy one articles (101 experiments) reported sensorimotor outcomes as measured by tests such as adhesive removal, rotarod, corner, and treadmill test. The pooled effect size of BM-MSC therapy based on sensorimotor outcomes was substantial and significant (1.36, 95% CI: 1.15–1.56), which demonstrates the significant increase of sensorimotor outcome in the BM-MSC group compared with vehicle treatment (**Figure 4**). Random effects models were applied due to significant heterogeneity assessed by Cochran's Q-statistic test. When we stratified sensorimotor outcomes based on timing of BM-MSC therapy, we found that BM-MSCs significantly improved outcomes for all treatment time-groups ($p < 0.0001$ for 0–6 h, 12–24 h, and 2–7days; $p = 0.001$ for >7day BM-MSC treatment, **Figure 4**).

Cognitive Function

Nine articles (10 experiments) reported cognitive outcomes from tests such as water maze, radial maze and novel object recognition tests. The pooled effect size of BM-MSC therapy based on cognitive function was substantial and significant (1.88, 95% CI: 0.73–3.02, $p = 0.001$), which demonstrated significant increase of cognitive function in the BM-MSC group compared with vehicle treatment (**Figure 5**). Random effects models were applied due to significant heterogeneity assessed by Cochran's Q-statistic test ($p < 0.0001$).

Meta-Regression

Cochran's Q-statistic test for heterogeneity suggested a significant effect of BM-MSCs for all four outcomes ($p < 0.0001$). We then performed univariable meta-regression analysis for composite score, motor function, and sensorimotor function to assess whether heterogeneity among results of multiple studies is related to any specific characteristics in these studies. Frequency and percentage of all interested variables by test categories are reported in **Table S1**. We did not perform meta-regression analysis for cognitive function due to the limited number of studies. The regression coefficients in **Tables 2–4** estimate how the effect of BM-MSCs on these outcomes in each subgroup differs from the reference group.

Route of Administration

There was no difference in treatment effect on improving composite scores and sensorimotor function regardless of route of BM-MSC administration (**Tables 2**, **4**). However, intravenously administered BM-MSCs showed significantly more treatment effect in improving motor outcomes as compared with intra-cranial injections ($p = 0.04$, **Table 3**).

Dose of BM-MSCs

There was no difference in treatment effect on all functional outcomes regardless of the dose of BM-MSCs used (**Tables 2–4** and **Figure S1**). There was a trend of an inverse correlation between dose (cells/kg) and treatment effect on composite scores. There was also a trend of direct correlation between dose (cells/kg) and treatment effect on sensorimotor function. However, none of these trends reached statistical significance. BM-MSCs produced significant improvement in all functional outcomes regardless of dose.

Timing of Administration

When BM-MSC were administered between 2 and 7 days, they significantly improved composite scores as compared to 12–24 h and >7days ($p = 0.02$ and 0.04, respectively). There was marginal significance on improvement of composite score for 2–7 days administration as compared with 0–6 h ($p = 0.09$, **Table 2**). BM-MSCs showed similar treatment effect in improving motor function regardless of timing of administration (**Table 3**). In addition, a significant difference in treatment effect size on sensorimotor function was found when BM-MSC were administered between 0 and 6 h as compared with 12–24 h ($p = 0.01$), 2–7days ($p = 0.03$), or >7 days ($p = 0.01$) (**Table 4**). The pooled effect size when BM-MSCs were administered after 7 days of ischemic stroke showed smaller significance as compared to when they were administered before 7 days ($p = 0.07$, 0.02, and 0.001 for composite score, motor function and sensorimotor function, respectively). Furthermore, when BM-MSCs were administered after 4 weeks, the forest plots do show benefit. However, the effect size was significantly less and inconsistent across studies (**Figure S2**).

Gender and Age Differences

BM-MSCs improved functional endpoints on all outcomes for both male and female stroke animals. However, female animals showed significantly more effect in improving sensorimotor function after BM-MSC treatment as compared to male animals ($p = 0.03$, **Table 4**). There was no such difference in treatment effect on improving composite scores or motor function. The pooled effect size for both composite score and sensorimotor function was significant in young as well as old stroke animals ($p < 0.0001$ for all groups, **Table S2**). However, the 95% confidence interval in old animals was wider due to limited sample size ($N = 5$).

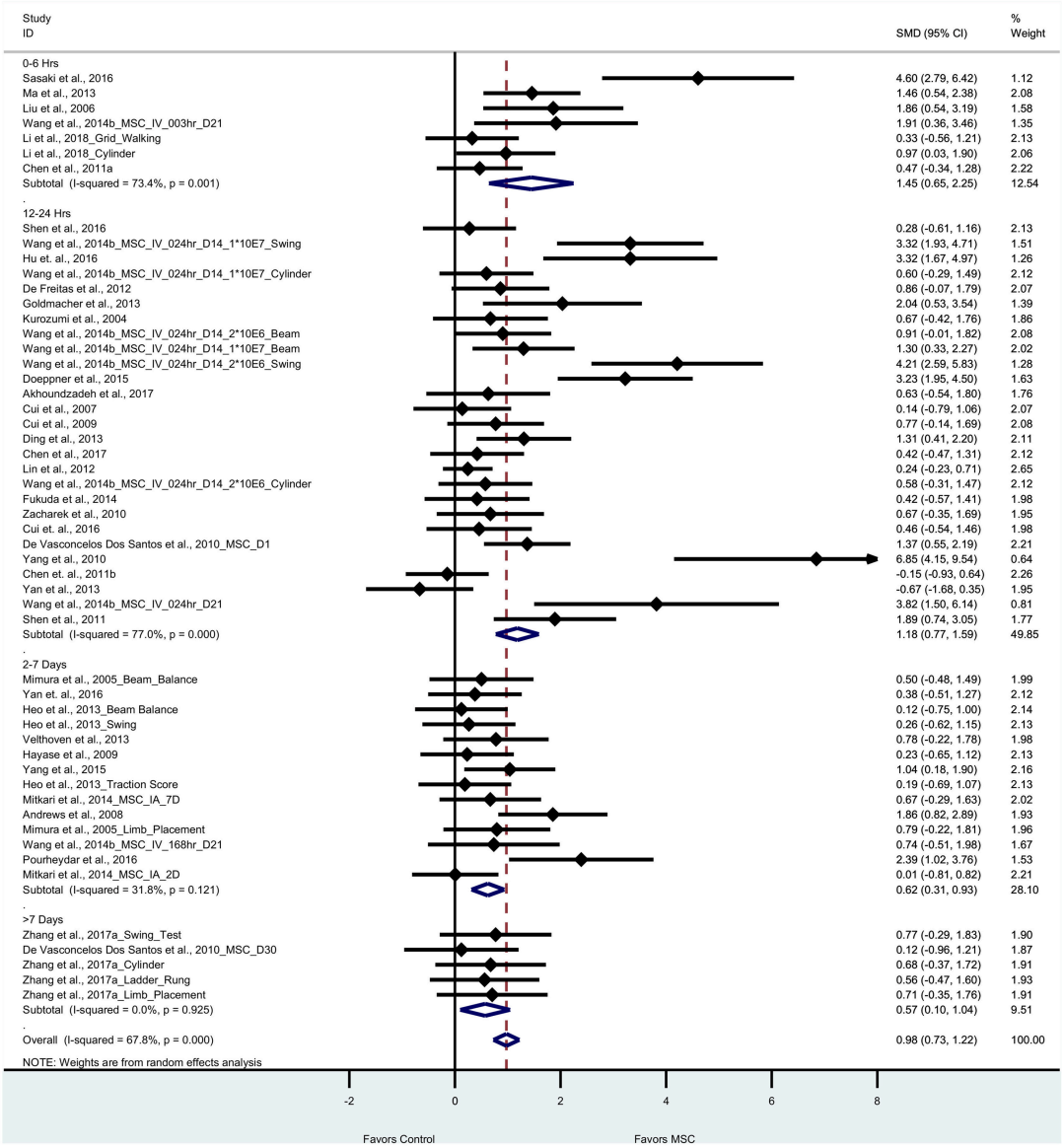


FIGURE 3 | Forest plot showing standardized mean difference of motor function between BM-MSC therapy and control groups, stratified by timing of administration of BM-MSCs. 95% confidence intervals are shown for all studies measuring motor function. 95% confidence intervals are further stratified by timing of administration of BM-MSCs into 0–6 h, 12–24 h, 2–7days, and >7 days. *p*-values are for heterogeneity measured by Cochran's Q-statistic test, values <0.1 are significant.

Co-morbidities

Although BM-MSCs improved outcomes in animals with and without comorbidities. Stroke animals without comorbidities showed significantly more improvement in composite scores after MSC treatment as compared to stroke animals with co-morbidities such as Type 1 Diabetes Mellitus (T1DM) or Type 2 Diabetes Mellitus (T2DM) ($p = 0.04$, **Table 2**).

BM-MSC Characteristics (Labeling, Donor Species, and Gender)

Labeling BM-MSCs did not change the efficacy of BM-MSCs on improving composite score, motor function and sensorimotor function. Furthermore, regardless of the species and gender of the donor of BM-MSCs, there was similar improvement in functional outcomes across all three categories. (**Tables 2**–**4**)

Fresh vs. Frozen BM-MSCs: Both fresh and frozen BM-MSCs had similar treatment effect in improving composite score and motor function (**Tables 2**, **3**). However, frozen BM-MSCs produced significantly improved sensorimotor outcome as compared with fresh BM-MSCs ($p = 0.02$, **Table 4**).

Passage of BM-MSCs

BM-MSCs improved functional outcomes regardless of the passage of cells used. There was no significant difference in

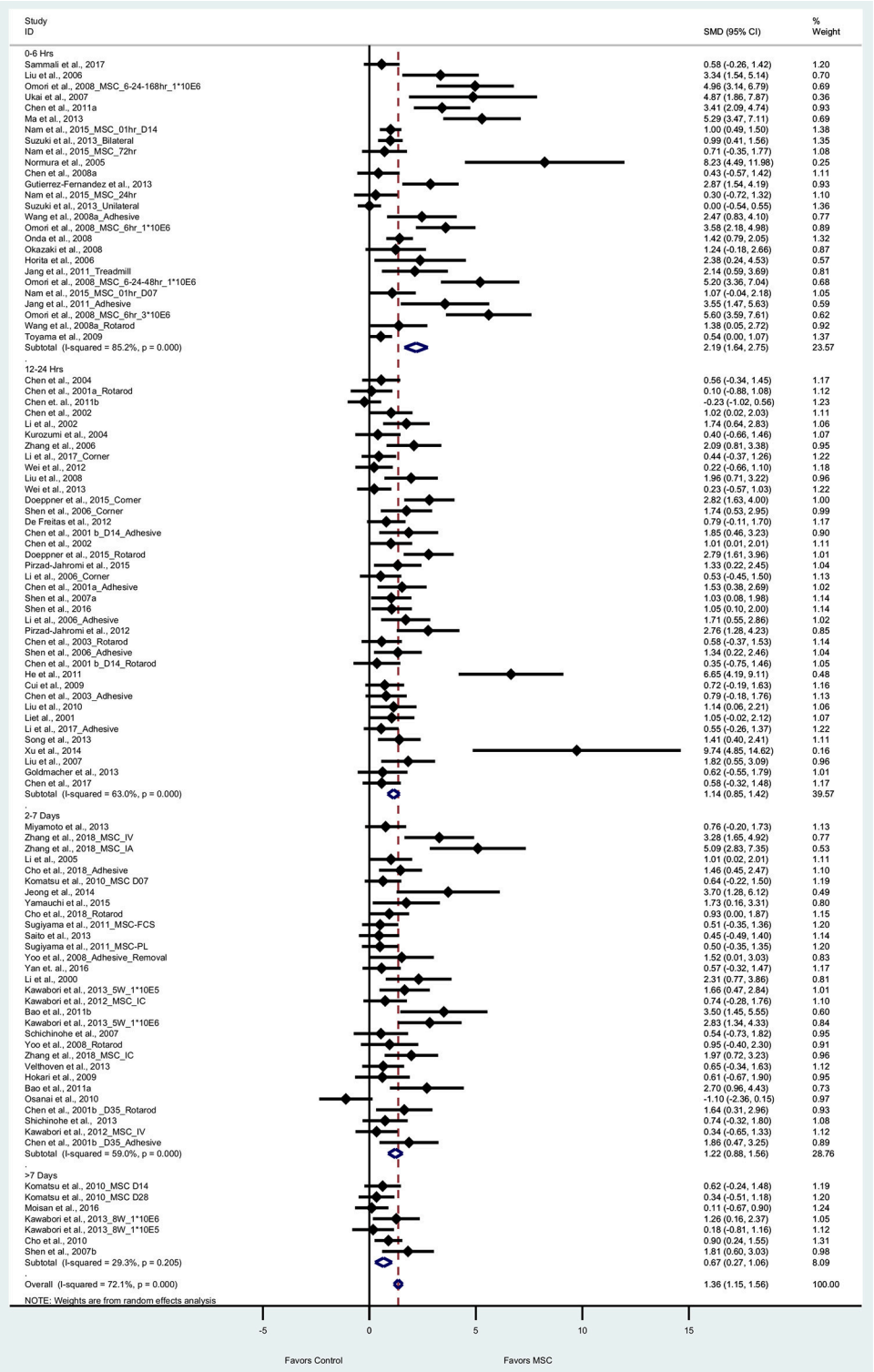


FIGURE 4 | Forest plot showing standardized mean difference of sensorimotor function between BM-MSC therapy and control groups, stratified by timing of administration of BM-MSCs. 95% confidence intervals are shown for all studies measuring sensorimotor function. 95% confidence intervals are further stratified by timing of administration of BM-MSCs into 0–6 h, 12–24 h, 2–7 days, and >7 days. *p*-values are for heterogeneity measured by Cochran's Q-statistic test, values <0.1 are significant.

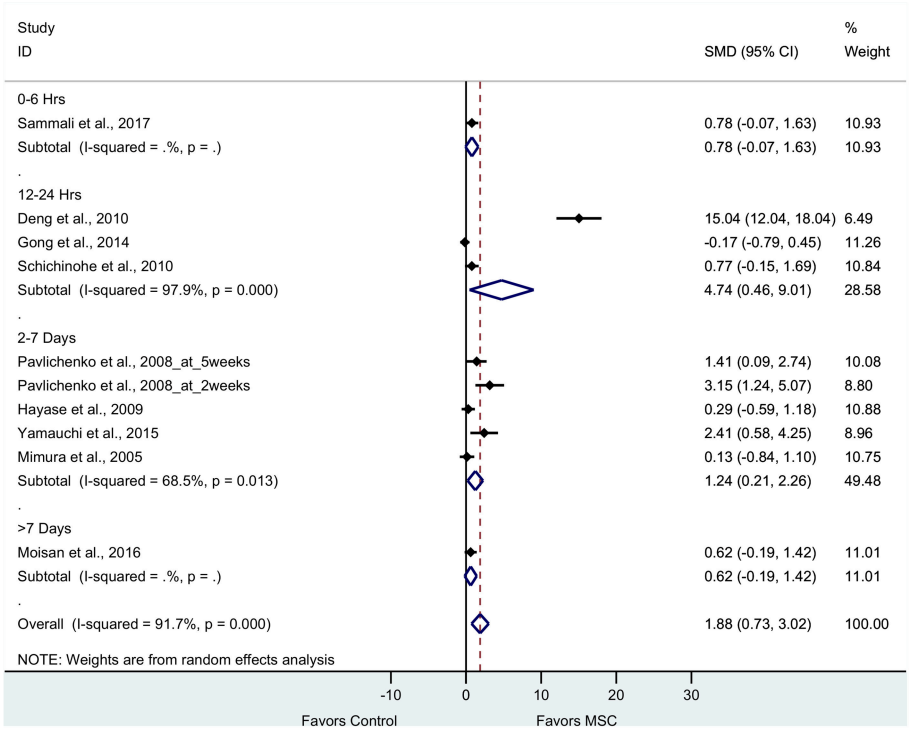


FIGURE 5 | Forest plot showing standardized mean difference of cognitive function between BM-MSC therapy and control groups, stratified by timing of administration of BM-MSCs. 95% confidence intervals are shown for all studies measuring cognitive function. 95% confidence intervals are further stratified by timing of administration of BM-MSCs into 0–6 h, 12–24 h, 2–7 days, and >7 days. *p*-values are for heterogeneity measured by Cochran's Q-statistic test, values <0.1 are significant.

treatment effect on improvement of composite scores whether cell passage was <4 or >4 (**Table 2**). BM-MSCs with >4 passages produced significant improvement in motor outcomes compared with passages <4 ($p = 0.006$, **Table 3**). BM-MSC with >4 passages also produced significant improvement in sensorimotor outcomes as compared to BM-MSC at 2–4 passages ($p = 0.03$, **Table 4**).

Laboratories

Laboratories from around the world showed significant improvement in all functional outcomes after BM-MSC treatment for ischemic stroke. On the composite outcome score, the effect size was more pronounced in studies from Asia as compared with North America ($p = 0.04$, **Table 2**).

Year of Publication

We calculated 25, 50, and 75 th quartiles of the publication year and categorized experiments into four groups based on publication year: 2000–2008, 2009–2012, 2013–2015, and 2016–2018. Publications from all four groups showed improvement in functional outcomes after BM-MSC administration with no significant difference between the groups.

Journal Impact Factor

We performed an analysis of impact factor since 2000 for all articles reporting functional outcomes after BM-MSC treatment. We observed a negative correlation between year and impact factor (Spearman correlation coefficient: −0.23, $p = 0.005$, **Figure 6**).

Sensitivity Analysis

To evaluate the robustness of the calculated pooled effect size, we performed a leave-one-out sensitivity analysis by iteratively removing one study at a time and recalculating the pooled effect size of the remaining studies for each test category. For composite scores, motor function, and sensorimotor function, the pooled effect size after removing each one of the studies was stable indicating that our results were not driven by any single study. However, for cognitive function, the pooled effect size after removing the experiment in Deng et al. (21) reduced from 1.88 (95% CI: 0.73–3.02) to 0.74 (95% CI: 0.22–1.26) and statistically significant ($p = 0.005$).

Publication Bias

Based on the funnel plots (**Figure 7**), we observed significant publication bias for the outcomes for composite scores, cognitive function, motor function, and sensorimotor function ($p = 0.002$, 0.002, <0.0001, and <0.0001, respectively). By trim and fill approach, for composite score, we added 25 unpublished hidden experiments and obtained the adjusted pooled effect size at 0.96 (95% CI: 0.78–1.13) after accounting for publication bias, which is still statistically significant ($p < 0.0001$). Similarly, for

TABLE 2 | Univariable meta-regression analysis to assess the impact of study variables related to MSC therapy on composite score.

Variables collected	Comparison	Coefficients	Standard error	*p*-value
Cell labeling	Yes vs. No	0.057	0.182	0.76
Route of administration	IA vs. IC	0.309	0.326	0.35
	IV vs. IC	−0.162	0.212	0.45
	IV vs. IA	−0.471	0.288	0.11
Time when outcome was measured	<2 weeks vs. >12 weeks	−0.262	0.540	0.63
	2–4 weeks vs. >12 weeks	0.049	0.554	0.93
	4–12 weeks vs. >12 weeks	0.097	0.576	0.87
	<2 weeks vs. 4–12 weeks	−0.359	0.256	0.16
	2–4 weeks vs. 4–12 weeks	−0.049	0.285	0.97
	<2 weeks vs. 2–4 weeks	−0.311	0.204	0.13
Cell dose (Total cells)	>1*10E6 vs. <=1*10E6	−0.109	0.180	0.55
Cell dose (per kilogram)	Cells/kg weight of stroke animal	$-8.69*10^{-9}$	$8.62*10^{-9}$	0.32
Timing of BM-MSC administration	0–6 h vs. > 7 Days	0.499	0.454	0.28
	12–24 h vs. >7 Days	0.378	0.437	0.39
	2–7 Days vs. >7 Days	0.973	0.476	**0.04**
	0–6 h vs. 2–7 Days	−0.474	0.276	**0.09**
	12–24 h vs. 2–7 Days	−0.595	0.247	**0.02**
	0–6 h vs. 12–24 h	0.121	0.203	0.55
Fresh/Frozen BM-MSCs	Fresh vs. Frozen	0.349	0.206	**0.09**
Passage of BM-MSCs	2–4 vs. >4	0.051	0.257	0.84
	Unknown vs. >4	0.015	0.298	0.96
	Unknown vs. 2–4	−0.036	0.217	0.87
Species of BM-MSC donor	Mouse vs. Human	0.276	0.451	0.54
	Rat vs. Human	0.395	0.205	**0.06**
	Dog vs. Human	0.086	0.762	0.91
	Mouse vs. Rat	−0.119	0.428	0.78
	Dog vs. Rat	−0.309	0.748	0.68
Donor gender	Female vs. Male	−0.364	0.357	0.31
	Unknown vs. Male	−0.069	0.193	0.72
	Unknown vs. Female	0.295	0.339	0.39
Gender of stroke animal	Female vs. Male	0.275	0.329	0.41
	Unknown vs. Male	0.140	0.310	0.65
	Unknown vs. Female	−0.134	0.431	0.76
Species of stroke animal	Mice vs. Rat	−0.525	0.320	0.10
Age of stroke animal	Adult vs. Retired Breeder	−0.211	0.402	0.60
Co–morbidities	Normal vs. Non-normal	0.820	0.384	**0.04**
Continent	Asia vs. North America	0.394	0.190	**0.04**
	Europe vs. North America	0.412	0.321	0.20
	Europe vs. Asia	0.018	0.302	0.95
Year	2009–2012 vs. 2000–2008	0.202	0.256	0.43
	2013–2015 vs. 2000–2008	−0.123	0.252	0.630
	2016–2018 vs. 2000–2008	0.056	0.244	0.82
	2013–2015 vs. 2009–2012	−0.325	0.252	0.2
	2016–2018 vs. 2009–2012	−0.146	0.244	0.55
	2016–2018 vs. 2013–2015	0.179	0.239	0.46

Positive coefficients indicate a larger MSC effect. Bold values indicate significance ($p < 0.05$) or showing a trend toward significance ($p < 0.1$).

motor function and sensorimotor function, we added 10 and 30 unpublished hidden experiments and obtained the adjusted pooled effect size at 0.63 (95% CI: 0.34–0.92) and 0.80 (95% CI: 0.56–1.03) after accounting for publication bias. For cognitive function, after trim and fill approach, we added 4 unpublished hidden experiments and obtained the adjusted pooled effect size at 0.37 (95% CI: −0.92 to 1.65), which led to statistically non-significant results on cognitive function ($p = 0.58$).

TABLE 3 | Univariable meta-regression analysis to assess the impact of study variables related to MSC therapy on motor function.

Variables collected	Comparison	Coefficients	Standard error	*p*-value
Cell labeling	Yes vs. No	−0.208	0.350	0.55
Route of administration	IA vs. IC	−0.203	0.659	0.76
	IV vs. IC	0.710	0.331	**0.04**
	IV vs. IA	0.913	0.630	0.15
Time when outcome was measured	<2 weeks vs. 4–12 weeks	0.187	0.468	0.69
	2–4 weeks vs. 4–12 weeks	0.171	0.497	0.73
	<2 weeks vs. 2–4 weeks	0.016	0.362	0.97
Cell dose (Total cells)	>1*10E6 vs. <=1*10E6	−0.128	0.313	0.69
Cell dose (per kilogram)	Cells/kg weight of stroke animal	$1.94*10^{-9}$	$8.84*10^{-9}$	0.83
Timing of BM-MSC administration	0–6 h vs. > 7 Days	0.852	0.662	0.20
	12–24 h vs. >7 Days	0.583	0.545	0.29
	2–7 Days vs. >7 Days	0.103	0.578	0.86
	0–6 h vs. 2–7 Days	0.749	0.523	0.16
	12–24 h vs. 2–7 Days	0.480	0.364	0.19
	0–6 h vs. 12–24 h	0.269	0.486	0.58
Fresh/Frozen BM-MSCs	Fresh vs. Frozen	0.147	0.365	0.69
Passage of BM-MSCs	2–4 vs. >4	−1.030	0.357	**0.006**
	Unknown vs. >4	−0.452	0.432	0.30
	Unknown vs. 2–4	0.575	0.357	0.11
Species of BM-MSC donor	Mouse vs. Human	0.165	0.657	0.80
	Rat vs. Human	0.199	0.377	0.60
	Mouse vs. Rat	−0.034	0.602	0.96
Donor gender	Unknown vs. Male	0.067	0.322	0.84
Gender of stroke animal	Unknown vs. Male	−0.175	0.601	0.77
Species of stroke animal	Mice vs. Rat	0.452	0.484	0.36
Co-morbidities	Normal vs. Non-normal	0.583	0.521	0.27
Continent	Asia vs. North America	−0.278	0.345	0.42
	Europe vs. North America	−0.079	0.618	0.90
	South America vs. North America	−0.330	0.689	0.63
	Asia vs. Europe	−0.199	0.623	0.75
	South America vs. Europe	−0.251	0.863	0.77
	Asia vs. South America	0.052	0.693	0.94
Year	2009–2012 vs. 2000–2008	−0.100	0.585	0.87
	2013–2015 vs. 2000–2008	0.168	0.535	0.76
	2016–2018 vs. 2000–2008	0.064	0.568	0.91
	2013–2015 vs. 2009–2012	0.268	0.426	0.53
	2016–2018 vs. 2009–2012	0.163	0.466	0.73
	2016–2018 vs. 2013–2015	−0.104	0.401	0.80

Positive coefficients indicate a larger MSC effect. Bold values indicate significance ($p < 0.05$) or showing a trend toward significance ($p < 0.1$).

DISCUSSION

Robust Efficacy Across Outcomes and Laboratories

We have performed a comprehensive assessment of preclinical studies testing the efficacy of BM-MSCs in preclinical stroke models. BM-MSCs were exclusively chosen because they are the most widely studied cell therapy in animal stroke models and BM-MSCs differ in their characteristics compared with MSCs derived from other tissue sources (22–24). Based on our meta-analysis of 141 articles, we found that BM-MSCs had broad treatment effects on a number of different functional outcomes that included composite scores, motor function, sensorimotor function, and cognitive function. Many prior meta-analyses have examined functional outcomes after BM-MSC therapy (13–15). We conducted a comprehensive meta-analysis to analyze all published articles in English from 2000 to 2018 and provided updated data on various clinically relevant factors. To provide more in-depth analyses, we included all behavioral tests from qualifying studies and grouped them into four categories based on our prior published work (16). We found significant improvement with a high mean effect size in all four categories of functional outcome, suggesting a robust effect across all outcomes. These effect sizes remained significant after adjusting for publication bias for all outcomes except cognitive outcome. Positive effects

TABLE 4 | Univariable meta-regression analysis to assess the impact of study variables related to MSC therapy on sensorimotor function.

Variables collected	Comparison	Coefficients	Standard error	*p*-value
Cell labeling	Yes vs. No	−0.426	0.284	0.14
Route of administration	IA vs. IC	0.940	0.558	0.10
	IV vs. IC	0.473	0.302	0.12
	IV vs. IA	−0.467	0.530	0.38
Time when outcome was measured	<2 weeks vs. >12 weeks	0.312	0.758	0.68
	2–4 weeks vs. >12 weeks	1.008	0.776	0.20
	4–12 weeks vs. >12 weeks	0.261	0.760	0.73
	<2 weeks vs. 4–12 weeks	0.051	0.312	0.87
	2–4 weeks vs. 4–12 weeks	0.748	0.353	**0.04**
	<2 weeks vs. 2– weeks	−0.696	0.349	**0.049**
Cell dose (Total cells)	>1*10E6 vs. <=1*10E6	0.268	0.273	0.33
Cell dose (per kilogram)	Cells/kg weight of stroke animal	$3.35*10^{-8}$	$1.98*10^{-8}$	**0.09**
Timing of BM-MSC administration	0–6 h vs. > 7 Days	1.361	0.536	**0.01**
	12–24 h vs. >7 Days	0.476	0.508	0.35
	2–7 Days vs. >7 Days	0.567	0.523	0.28
	0–6 h vs. 2–7 Days	0.794	0.362	**0.03**
	12–24 h vs. 2–7 Days	−0.091	0.320	0.78
	0–6 h vs. 12–24 h	0.885	0.339	**0.01**
Fresh/Frozen BM-MSCs	Fresh vs. Frozen	−0.700	0.302	**0.02**
	Unknown vs. Frozen	−1.304	0.533	**0.02**
	Unknown vs. Fresh	−0.604	0.495	0.23
Passage of BM-MSCs	2–4 vs. >4	−0.832	0.365	**0.03**
	Unknown vs. >4	−0.328	0.403	0.42
	Unknown vs. 2–4	0.504	0.306	0.10
Species of BM-MSC donor	Mouse vs. Human	−0.417	0.683	0.54
	Rat vs. Human	−0.486	0.296	0.10
	Mouse vs. Rat	0.069	0.660	0.92
Donor gender	Female vs. Male	−0.211	0.820	0.8
	Unknown vs. Male	−0.003	0.325	0.99
	Unknown vs. Female	0.208	0.787	0.79
Gender of stroke animal	Female vs. Male	0.897	0.406	**0.03**
	Unknown vs. Male	−0.147	0.481	0.76
	Unknown vs. Female	−1.044	0.593	**0.08**
Species of stroke animal	Mice vs. Rat	0.039	0.441	0.93
Age of stroke animal	Adult vs. Retired Breeder	−0.076	0.633	0.91
Continent	Asia vs. North America	0.453	0.291	0.12
	Europe vs. North America	0.413	0.586	0.48
	Asia vs. Europe	0.040	0.571	0.94
Year	2009–2012 vs. 2000–2008	−0.544	0.355	0.13
	2013–2015 vs. 2000–2008	−0.276	0.348	0.43
	2016–2018 vs. 2000–2008	−0.500	0.439	0.26
	2013–2015 vs. 2009–2012	0.268	0.390	0.49
	2016–2018 vs. 2009–2012	0.043	0.473	0.93
	2016–2018 vs. 2013–2015	−0.225	0.467	0.63

Positive coefficients indicate a larger MSC effect. Bold values indicate significance ($p < 0.05$) or showing a trend toward significance ($p < 0.1$).

on cognitive outcomes appeared driven by one study. It is possible that BM-MSCs do not improve cognitive outcome or the nature of the cognitive studies performed limit the ability of accurately measuring such outcomes in rodents. More studies might be required to assess if BM-MSCs can improve cognitive function.

Based on the results of our work and prior meta-analyses (13–15), we believe that an overwhelming body of literature now supports the robust efficacy of BM-MSCs to improve functional outcomes. In fact, when we examined different time windows when MSC studies were completed over the past 18 years broken out into 2000–2008, 2009–2012, 2013–2015, and 2016–2018, we

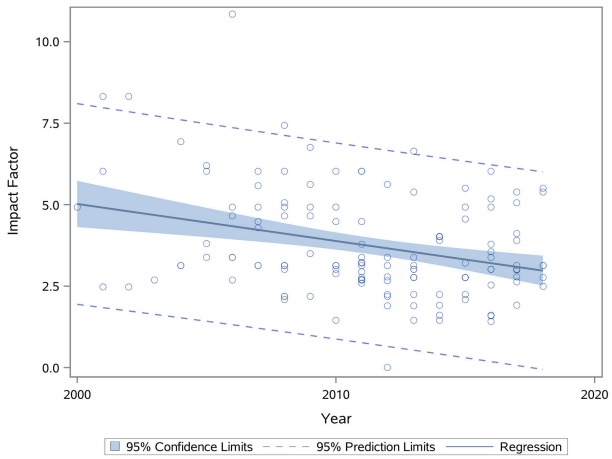


FIGURE 6 | Scatterplot showing relationship between journal impact factor and year of publication ($p = 0.005$). Spearman correlation coefficient was calculated to find correlation between these two variables. $p < 0.05$ is significant.

did not observe any differences in any of the four functional outcomes after BM-MSC therapy. These results show that there was likely conclusive evidence for the efficacy of BM-MSC many years ago. The data may also explain the negative correlation between year of publication of BM-MSC studies and impact factor, suggesting that redundant results showing functional improvement by BM-MSCs are being published in lower impact journals. The efficacy of BM-MSCs has also been shown across different countries and different laboratories. Animal protocols differ in different countries with their own review protocols and committees with different rules and regulations for animal surgeries. We found no differences in treatment effects across the globe except when using composite outcome scores where studies in Asia showed higher effect size as compared to studies in North America. To provide current up to date information that would be useful for the design of clinical trials, we performed a number of analyses on clinically relevant factors discussed below.

Timing of BM-MSCs Administration

We found that overall effect-size for all outcome categories was consistently greatest when BM-MSCs were administered before 7 days. Within 7 days of stroke, we could not find consistent effects to determine a clear optimal therapeutic window. Administering BM-MSCs between 2 to 7 days leads to greatest benefit on composite score outcomes. On the other hand, administering BM-MSCs between 0 and 6 h led to the most significant improvement in sensorimotor outcomes. Although there are far less studies, BM-MSC administered after 7 days (compared with earlier time points) showed no improvement in composite scores, while MSCs still led to treatment effects, howbeit to a lesser extent, on motor and sensorimotor outcomes. Our results suggest that earlier administration of BM-MSCs before 7 days in rodents may be optimal to enhance functional recovery (25, 26) but MSCs may still confer less benefit when administered between 7 and 30 days in rodents, a time-period which many would consider a subacute to chronic period after stroke.

Route of Administration

We found that intravenous administration resulted in significantly more improvement in motor function as compared to intracranial injection. Intravenous administration also showed a trend toward improving composite score and sensorimotor function, although this was not statistically significant. Intra-arterial administration showed similar trends of improving sensorimotor outcome as compared to intracranial administration. Our analyses do support that a systemic injection is preferable to intracranial injection within the early period after stroke, supporting the concept that MSCs act upon systemic responses after stroke. IV administration could direct MSCs to peripheral organs such as the lung and spleen (27–29), subsequently modulating release of trophic factors as well as immune responses from these organs (11, 12).

Dose

When we analyzed dose of cells administered, we saw some trends for each category of outcomes (**Figure S1**). There was a trend of inverse relation between effectiveness of BM-MSCs and dose. On the other hand, there was a trend of direct correlation for improvement of sensorimotor function by BM-MSCs and dose. However, none of these trends were significant (**Tables 2–4**). We conclude that we could not find any clear dose response.

Fresh vs. Cryopreserved BM-MSCs and Cell Passage

Various different passages, cryopreserved and fresh BM-MSCs exert functional benefits. Frozen BM-MSCs actually performed better than fresh BM-MSC in improving sensorimotor outcomes. These results do not support the belief that fresh cells are superior to frozen cells for studies involving focal ischemic stroke. Another surprising result was that BM-MSCs from passage 4 or higher produced significantly improved motor and sensorimotor outcome as compared to those from passage 2–4. However, we recommend to interpret these results with caution and recommend further detailed studies to analyze fresh vs. frozen and cell passage in future studies.

Age

Age is an important factor for determining stroke functional outcomes. The aged brain has less regenerative potential and increased inflammatory responses to stroke (30–32). Even spontaneous recovery after stroke is delayed in aged animals (33, 34). In our meta-analysis, BM-MSC administration was tested in old animals in only 5 out of 141 articles. While our sample size does not provide adequate power to conduct reliable meta-regression to evaluate the effect of age, our analyses found that the pooled effect size was significant for composite score as well as sensorimotor function in both young and old stroke animals. However, the 95% confidence interval was wider for old animals due to less number of studies and more variability.

Other Clinically Relevant Factors

Other factors that have not been studied well in prior meta-analyses were also examined. Labeling did not appear to alter the efficacy of BM-MSCs. These results support the use of labeled cells in clinical trials in order to track their migration and obtain

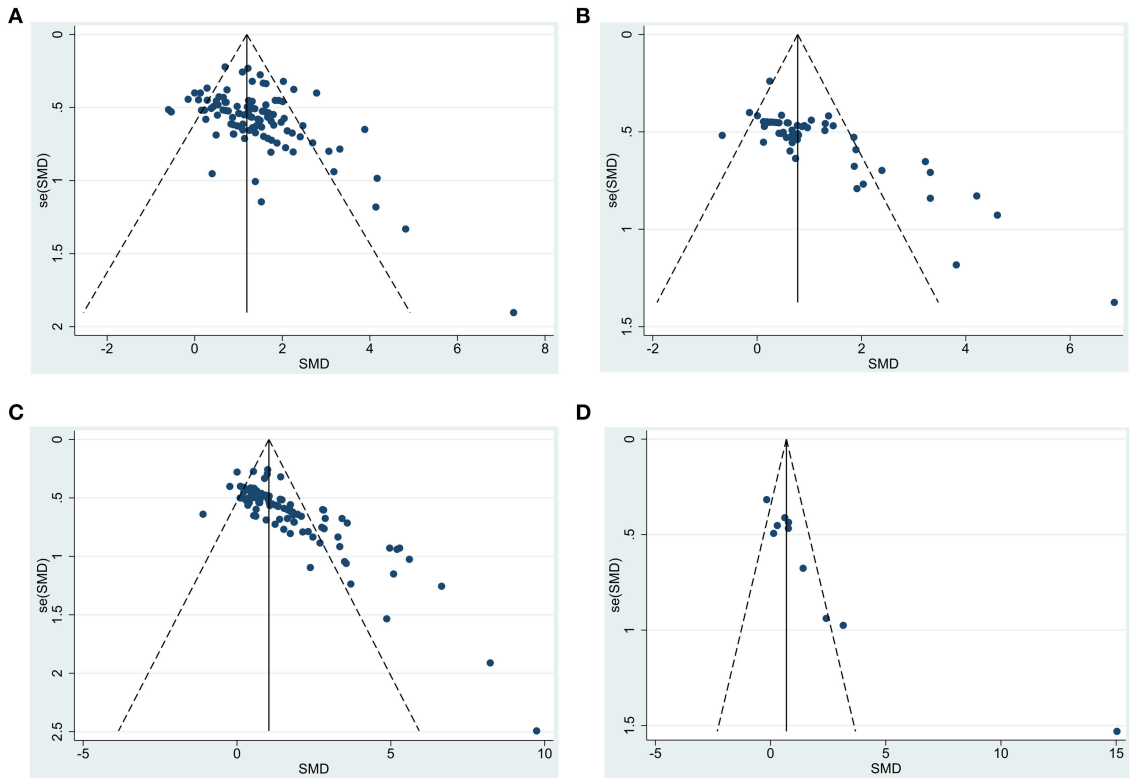


FIGURE 7 | Funnel plot to detect publication bias for functional outcomes after BM-MSC administration. **(A)** funnel plot to detect publication bias in all studies measuring composite scores; **(B)** funnel plot to detect publication bias in all studies measuring motor function; **(C)** funnel plot to detect publication bias in all studies measuring sensorimotor function; **(D)** funnel plot to detect publication bias in all studies measuring cognitive function.

needed data on biodistribution in patients. Not surprisingly, we found that animals without comorbidities improved significantly better after BM-MSCs treatment as compared to animals with co-morbidities. These results have implications for clinical trials and predicted effect sizes in patients with vascular risk factors and require further study. We hope that large clinical studies of BM-MSCs are funded that will permit sub hoc analyses to determine differential effects of vascular risk factors in patients treated with BM-MSCs. Lastly, we found intriguingly that female animals compared with males achieved better outcomes on sensorimotor outcomes after BM-MSC treatment but further studies will be needed to substantiate if there are true sex differences in the treatment effects of BM-MSCs.

CONCLUSION

Given the wealth of preclinical data supporting the efficacy of BM-MSCs over 18 years, we recommend that this cellular therapy should be tested extensively in well-designed clinical trials in different time windows using different delivery routes. BM-MSCs may exert different treatment effects at various time points after stroke from early acute stages to later subacute and even chronic stroke. Clinical trials should pave the path forward for the further development of BM-MSCs in stroke patients.

AUTHOR CONTRIBUTIONS

NS wrote the manuscript. CC conducted the statistical analysis and wrote the statistical section in manuscript. NS, KG, DM, SG, KP, DN, KV, and JR collected the data. NS and KP designed the study. NS, FV, and SS oversaw the project and edited the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.00405/full#supplementary-material

REFERENCES

1. Yang Q, Tong X, Schieb L, Vaughan A, Gillespie C, Wiltz JL, et al. Vital signs: recent trends in stroke death rates - United States. *MMWR.* (2017) 66:933–9. doi: 10.15585/mmwr.mm6635e1

2. Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, et al. Heart disease and stroke statistics–2017 update: a report from the american heart association. *Circulation.* (2017) 135:e146–e603. doi: 10.1161/CIR.0000000000000485

3. Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci.* (1999) 22:391–7.
4. Iadecola C, Anrather J. The immunology of stroke: from mechanisms to translation. *Nat Med.* (2011) 17:796–808. doi: 10.1038/nm.2399
5. Chen J, Li Y, Wang L, Zhang Z, Lu D, Lu M, et al. Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke.* (2001) 32:1005–11. doi: 10.1161/01.STR.32.4.1005
6. Chen J, Sanberg PR, Li Y, Wang L, Lu M, Willing AE, et al. Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. *Stroke.* (2001) 32:2682–8. doi: 10.1161/hs1101.098367
7. Li Y, Chen J, Wang L, Lu M, Chopp M. Treatment of stroke in rat with intracarotid administration of marrow stromal cells. *Neurology.* (2001) 56:1666–72. doi: 10.1212/WNL.56.12.1666
8. Lee JS, Hong JM, Moon GJ, Lee PH, Ahn YH, Bang OY, et al. A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke. *Stem Cells.* (2010) 28:1099–106. doi: 10.1002/stem.430
9. Honmou O, Houkin K, Matsunaga T, Niitsu Y, Ishiai S, Onodera R, et al. Intravenous administration of auto serum-expanded autologous mesenchymal stem cells in stroke. *Brain.* (2011) 134:1790–807. doi: 10.1093/brain/awr063
10. Shichinohe H, Kawabori M, Iijima H, Teramoto T, Abumiya T, Nakayama N, et al. Research on advanced intervention using novel bone marrOW stem cell (RAINBOW): a study protocol for a phase I, open-label, uncontrolled, dose-response trial of autologous bone marrow stromal cell transplantation in patients with acute ischemic stroke. *BMC Neurol.* (2017) 17:179. doi: 10.1186/s12883-017-0955-6
11. Nemeth K, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, et al. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med.* (2009) 15:42–9. doi: 10.1038/nm.1905
12. Satani N, Savitz SI. Is Immunomodulation a Principal Mechanism Underlying How Cell-Based Therapies Enhance Stroke Recovery? *Neurotherapeutics.* (2016) 13:775–82. doi: 10.1007/s13311-016-0468-9
13. Schmidt A, Wellmann J, Schilling M, Strecker JK, Sommer C, Schabitz WR, et al. Meta-analysis of the efficacy of different training strategies in animal models of ischemic stroke. *Stroke.* (2014) 45:239–47. doi: 10.1161/STROKEAHA.113.002048
14. Vu Q, Xie K, Eckert M, Zhao W, Cramer SC. Meta-analysis of preclinical studies of mesenchymal stromal cells for ischemic stroke. *Neurology.* (2014) 82:1277–86. doi: 10.1212/WNL.0000000000000278
15. Sarmah D, Agrawal V, Rane P, Bhute S, Watanabe M, Kalia K, et al. Mesenchymal stem cell therapy in ischemic stroke: a meta-analysis of preclinical studies. *Clin Pharmacol Ther.* (2018) 103:990–8. doi: 10.1002/cpt.927
16. Schaar KL, Brenneman MM, Savitz SI. Functional assessments in the rodent stroke model. *Exp Transl Stroke Med.* (2010) 2:13. doi: 10.1186/2040-7378-2-13
17. Higgins JP, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. *BMJ.* (2003) 327:557–60. doi: 10.1136/bmj.327.7414.557
18. Ioannidis JP, Patsopoulos NA, Evangelou E. Heterogeneity in meta-analyses of genome-wide association investigations. *PLoS ONE.* (2007) 2:e841. doi: 10.1371/journal.pone.0000841
19. Egger M, Davey Smith G, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. *BMJ.* (1997) 315:629–34.
20. Duval S, Tweedie R. Trim and fill: a simple funnel-plot-based method of testing and adjusting for publication bias in meta-analysis. *Biometrics.* (2000) 56:455–63. doi: 10.1111/j.0006-341X.2000.00455.x
21. Deng YB, Ye WB, Hu ZZ, Yan Y, Wang Y, Takon BF, et al. Intravenously administered BMSCs reduce neuronal apoptosis and promote neuronal proliferation through the release of VEGF after stroke in rats. *Neurol Res.* (2010) 32:148–56. doi: 10.1179/174313209X414434
22. Peng L, Jia Z, Yin X, Zhang X, Liu Y, Chen P, et al. Comparative analysis of mesenchymal stem cells from bone marrow, cartilage, and adipose tissue. *Stem Cells Dev.* (2008) 17:761–73. doi: 10.1089/scd.2007.0217
23. Hwang JH, Shim SS, Seok OS, Lee HY, Woo SK, Kim BH, et al. Comparison of cytokine expression in mesenchymal stem cells from human placenta, cord blood, and bone marrow. *J Korean Med Sci.* (2009) 24:547–54. doi: 10.3346/jkms.2009.24.4.547
24. Jin HJ, Bae YK, Kim M, Kwon SJ, Jeon HB, Choi SJ, et al. Comparative analysis of human mesenchymal stem cells from bone marrow, adipose tissue, and umbilical cord blood as sources of cell therapy. *Int J Mol Sci.* (2013) 14:17986–8001. doi: 10.3390/ijms140917986
25. Wang LQ, Lin ZZ, Zhang HX, Shao B, Xiao L, Jiang HG, et al. Timing and dose regimens of marrow mesenchymal stem cell transplantation affect the outcomes and neuroinflammatory response after ischemic stroke. *CNS Neurosci Ther.* (2014) 20:317–26. doi: 10.1111/cns.12216
26. Nam HS, Kwon I, Lee BH, Kim H, Kim J, An S, et al. Effects of Mesenchymal Stem Cell Treatment on the expression of matrix metalloproteinases and angiogenesis during ischemic stroke recovery. *PLoS ONE.* (2015) 10:e0144218. doi: 10.1371/journal.pone.0144218
27. Gao J, Dennis JE, Muzic RF, Lundberg M, Caplan AI. The dynamic *in vivo* distribution of bone marrow-derived mesenchymal stem cells after infusion. *Cells Tissues Organs.* (2001) 169:12–20. doi: 10.1159/000047856
28. Sensebe L, Fleury-Cappellesso S. Biodistribution of mesenchymal stem/stromal cells in a preclinical setting. *Stem Cells Int.* (2013) 2013:678063. doi: 10.1155/2013/678063
29. Leibacher J, Henschler R. Biodistribution, migration and homing of systemically applied mesenchymal stem/stromal cells. *Stem Cell Res Ther.* (2016) 7:7. doi: 10.1186/s13287-015-0271-2
30. Popa-Wagner A, Buga AM, Kokaia Z. Perturbed cellular response to brain injury during aging. *Ageing Res Rev.* (2011) 10:71–9. doi: 10.1016/j.arr.2009.10.008
31. Balseanu AT, Buga AM, Catalin B, Wagner DC, Boltze J, Zagrean AM, et al. Multimodal approaches for regenerative stroke therapies: combination of granulocyte colony-stimulating factor with bone marrow mesenchymal stem cells is not superior to G-CSF alone. *Front Aging Neurosci.* (2014) 6:130. doi: 10.3389/fnagi.2014.00130
32. Popa-Wagner A, Filfan M, Uzoni A, Pourgolafshan P, Buga AM. Poststroke cell therapy of the aged brain. *Neural Plast.* (2015) 2015:839638. doi: 10.1155/2015/839638
33. Buchhold B, Mogoanta L, Suofu Y, Hamm A, Walker L, Kessler C, et al. Environmental enrichment improves functional and neuropathological indices following stroke in young and aged rats. *Restor Neurol Neurosci.* (2007) 25:467–84.
34. Popa–Wagner A, Buga AM, Doeppner TR, Hermann DM. Stem cell therapies in preclinical models of stroke associated with aging. *Front Cell Neurosci.* (2014) 8:347. doi: 10.3389/fncel.2014.00347

Conflict of Interest Statement: As an employee of the institution (UTHealth), SS has served in the following roles: as a site investigator in clinical trials sponsored by industry companies—Athersys, Genentech, Pfizer, Dart Neuroscience, and SanBio, for which UTHealth receives payments on the basis of clinical trial contracts; as an investigator on clinical trials supported by National Institutes of Health (NIH) grants, Department of Defense, Let's Cure CP, the Texas Institute for Rehabilitation and Research Foundation, and the Cord Blood Registry Systems; as a principal investigator on NIH-funded grants in basic science research; as principal investigator for an imaging analysis center for clinical trials sponsored by SanBio. Whereas, UTHealth uses SS with expertise in stroke, UTHealth has served as a consultant to Neuralstem, SanBio, Mesoblast, ReNeuron, Lumosa, Celgene, Dart Neuroscience, BlueRock and Aldagen. All funding goes to the institution.

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.

Copyright © 2019 Satani, Cai, Giridhar, McGhiey, George, Parsha, Nghiem, Valenzuela, Riecke, Vahidy and Savitz. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Neurology

REVIEW
published: 29 April 2019
doi: 10.3389/fneur.2019.00422

Using Dental Pulp Stem Cells for Stroke Therapy

Maria R. Gancheva[1]*, *Karlea L. Kremer*[1], *Stan Gronthos*[2,3] and *Simon A. Koblar*[1,3,4]

[1] Stroke Research Programme Laboratory, Adelaide Medical School, Faculty of Health and Medical Sciences, University of Adelaide, Adelaide, SA, Australia, [2] Mesenchymal Stem Cell Laboratory, Adelaide Medical School, Faculty of Health and Medical Sciences, University of Adelaide, Adelaide, SA, Australia, [3] South Australian Health and Medical Research Institute, Adelaide, SA, Australia, [4] Central Adelaide Local Health Network, Adelaide, SA, Australia

Stroke is a leading cause of permanent disability world-wide, but aside from rehabilitation, there is currently no clinically-proven pharmaceutical or biological agent to improve neurological disability. Cell-based therapies using stem cells, such as dental pulp stem cells, are a promising alternative for treatment of neurological diseases, including stroke. The ischaemic environment in stroke affects multiple cell populations, thus stem cells, which act through cellular and molecular mechanisms, are promising candidates. The most common stem cell population studied in the neurological setting has been mesenchymal stem cells due to their accessibility. However, it is believed that neural stem cells, the resident stem cell of the adult brain, would be most appropriate for brain repair. Using reprogramming strategies, alternative sources of neural stem and progenitor cells have been explored. We postulate that a cell of closer origin to the neural lineage would be a promising candidate for reprogramming and modification towards a neural stem or progenitor cell. One such candidate population is dental pulp stem cells, which reside in the root canal of teeth. This review will focus on the neural potential of dental pulp stem cells and their investigations in the stroke setting to date, and include an overview on the use of different sources of neural stem cells in preclinical studies and clinical trials of stroke.

Keywords: cell-based therapy, ischaemic stroke, dental pulp stem cells, neural stem cells, multipotent, reprogramming, differentiation, neural crest

OPEN ACCESS

Edited by:
Andrew N. Clarkson,
University of Otago, New Zealand

Reviewed by:
Henry Ma,
Monash University, Australia
Bronwen Connor,
The University of Auckland,
New Zealand

***Correspondence:**
Maria R. Gancheva
maria.gancheva@adelaide.edu.au

Specialty section:
This article was submitted to Stroke, a section of the journal Frontiers in Neurology

Received: *20 December 2018*
Accepted: *08 April 2019*
Published: *29 April 2019*

Citation:
Gancheva MR, Kremer KL, Gronthos S and Koblar SA (2019) Using Dental Pulp Stem Cells for Stroke Therapy. Front. Neurol. 10:422. doi: 10.3389/fneur.2019.00422

INTRODUCTION

The central nervous system (CNS) functions through complex molecular and cellular interactions, and disruption by severe injury or disease leads to irreversible neuronal loss and associated functional deficits. This results in highly debilitating pathologies associated with significant health and economic burden for patients, their families, carers, and the health systems.

Stroke is a global health care problem and a leading cause of acquired adult neurological disability (1). With an aging population, the incidence and prevalence of stroke is predicted to rise. A stroke is characterised by reduced and insufficient blood supply to part of the brain. Inadequate oxygen and nutrients lead to tissue infarction, resulting in disability due to loss-of-function associated with the damaged area of the brain.

Abbreviations: BBB, blood-brain barrier; bFGF, basic fibroblast growth factor; BMSC, bone marrow stromal/stem cell(s); CNS, central nervous system; DPSC, dental pulp stem cell(s); EGF, epidermal growth factor; ESC, embryonic stem cell(s); MSC, mesenchymal stem cell(s); NSC, neural stem cell(s); OCT4, octamer-binding transcription factor 4; PSC, pluripotent stem cell(s); SDF1α, stromal cell-derived factor 1-alpha; SOX2, SRY (sex determining region Y)-box 2; VEGF, vascular endothelial growth factor.

There are two main types of stroke; haemorrhagic and ischaemic. Haemorrhagic strokes, accounting for 13 percent of strokes (1), result from bleeding when a blood vessel is ruptured. Ischaemic stroke is the most common presentation of stroke at 87 percent of all cases (1), and is due to an obstruction in the blood supply, which could be formed locally (thrombosis) or formed elsewhere in the body (embolism).

During an ischaemic stroke, a complex chain of events takes place at the molecular and cellular levels, which results in cell necrosis at the site of the vascular insult (the ischaemic core), while the region surrounding the core (the ischaemic penumbra) remains viable for some time due to collateral blood supply and can thus be salvaged. A strong inflammatory response is initiated within hours of stroke onset, characterised by reactive astrogliosis, microglial activation, disruption to the blood-brain barrier (BBB), and infiltration of neutrophils and monocytes/macrophages (2). Growth factors and inflammatory mediators, from local glial and inflammatory cells, alter the reaction of endogenous neural stem and progenitor cells. Over time, reorganisation of the neural network around the core takes place. If untreated, the penumbra will transform into ischaemic tissue, expanding the irreversibly damaged area of brain. There is an opportunity to save the penumbral tissue via acute recanalisation therapies.

The currently available therapeutic interventions, such as thrombectomy and thrombolysis, are limited to a narrow therapeutic window and eligibility criteria, and though they have a significant impact on stroke outcome, disability remains after any intervention. Thrombectomy refers to the mechanical removal of a blood clot, which has been effective when performed within 24 h post-stroke (3). The more common intervention is thrombolysis by intravenously administered recombinant tissue plasminogen activator, to breakdown the clot. This is currently the only approved pharmacological agent that shows significant benefits in acute ischaemic stroke, but is only applicable within a short time frame of 4.5 h from symptomatic onset (4). Unfortunately, many patients are ineligible for these reperfusion therapies. In addition, poor patient outcomes can still be observed. Once a stroke patient is stabilised, rehabilitation interventions are relied upon to promote neuroplasticity, as patients adapt to residual disability. Improvements are most significant in the first several months following a stroke (5). There is currently no therapy that can restore damaged neural tissue and its associated functions.

Cell-based therapies have the potential to promote functional recovery in patients affected by stroke and other neurological diseases. Stem cells are promising candidates, as they can act through multiple cellular and molecular mechanisms to provide support for endogenous cells, stimulate endogenous processes, and act as a source of cell replacement. Neural stem cells (NSC), which reside in specific areas of the CNS, are the most appropriate stem cells for brain repair. Research is focused on two therapeutic paradigms; enhancing and manipulating endogenous NSC, and implanting exogenous NSC. Reprogramming strategies are being applied to develop NSC from more easily accessible and abundant cell types (6). Dental pulp stem cells (DPSC) are adult stem cells obtained from the dental pulp tissue in the tooth chamber (7). These cells are easily sourced and have neurogenic potential. They are being investigated as an alternative source of neural cells and in preclinical models of neurological diseases, including stroke. This review will focus on the potential use of human DPSC for stroke therapy and will include an overview of different types of NSC being studied.

TYPES OF STEM CELLS

Stem cells are unique cells that have the ability to differentiate into a variety of cell types while maintaining the pool of unspecialised stem cells through their self-renewal capacity. They can be classified based on their differentiation potential. Totipotent stem cells can give rise to all the cells needed for embryonic development including the extra-embryonic tissues, while pluripotent stem cells are able to differentiate into cells of all three germinal lineages (ectoderm, mesoderm, endoderm) and hence any cell of the body (**Figure 1**). Multipotent stem cells are more restricted as they can differentiate into the cells of the tissue they reside in, whereas unipotent stem cells maintain the population of one cell type within their tissue. There are two broad types of stem cells; pluripotent embryonic stem cells and multipotent post-natal adult or somatic stem cells.

Embryonic stem cells (ESC) are derived from the early embryo, more specifically the inner cell mass of the blastocyst (**Figure 1**). They were first isolated from the mouse blastocyst in the 1980s (8, 9), then from human embryos in 1998 (10). They can be propagated indefinitely and are cultured as embryoid bodies or as a monolayer using feeder layers. Due to their pluripotent nature, ESC give rise to teratomas *in vivo*, which are tumours derived from multiple germ layers and consist of a mixture of differentiated cells (11). By applying specific molecules, ESC can be induced along the neural lineage, making them safer for transplantation (11, 12). However, ESC-derived cells are subject to ethical controversy as culturing ESC involves the destruction of embryos.

Adult stem cells are found in tissues throughout the developed body where they function to maintain homeostasis and repair by giving rise to tissue-specific specialised cells. Examples of multipotent adult stem cells are bone marrow-derived mesenchymal stem or stromal cells (BMSC) and haematopoietic stem cells. Adult stem cells are not subjected to the same ethical considerations as ESC, nor do they possess the same tumorigenic potential. They also allow for autologous cell therapy, to prevent immunocompatibility issues.

It was believed that lineage specification and terminal differentiation into specialised cells was an irreversible process. However, this classic notion that cellular identity is stable has been challenged in the past few decades by evidence of nuclear transfer, dedifferentiation, epigenetic modifications, and ectopic gene expression, suggesting that cells are plastic (13). We now know that somatic cells, such as fibroblasts, can be genetically reprogrammed to stem cells that resemble ESC, termed induced pluripotent stem cells (induced PSC) (14, 15) (**Figure 1**). Breakthrough research conducted by Yamanaka and colleagues more than a decade ago, demonstrated the underlying molecular mechanism permitting this plasticity (14, 15). Yamanaka's reprogramming method involves the use of specific transcription factors that activate/deactivate genes and modify signalling

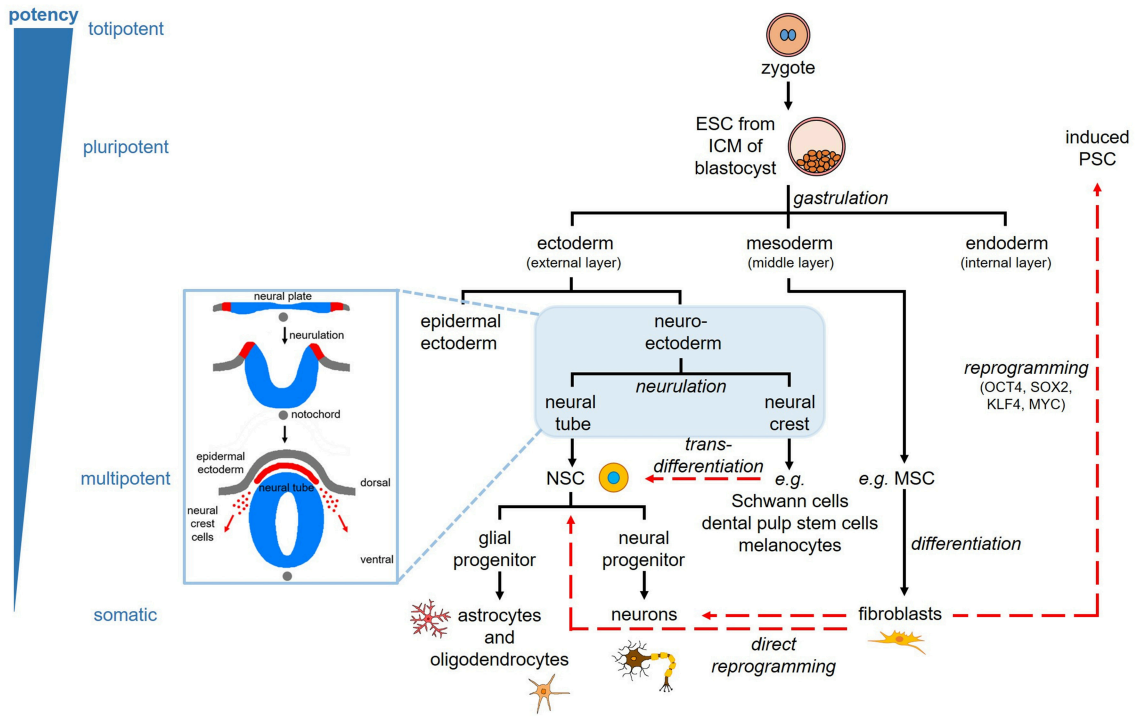


FIGURE 1 | Stem cell lineage and reprogramming. Pluripotent embryonic stem cells (ESC) are derived from the inner cell mass (ICM) of the blastocyst. The three germinal layers (ectoderm, mesoderm, endoderm) are specified through the process of gastrulation. The ectoderm gives rise to the skin and nervous system; the mesoderm gives rise to muscle, bone, blood, and fat; the endoderm gives rise to the respiratory, gastrointestinal, and urogenital tracts. The ectoderm consists of epidermal ectoderm forming the skin, and neuroectoderm, which forms the neural plate and undergoes neurulation to form the neural tube and neural crest cells. The neural tube gives rise to the central nervous system via neural stem cells (NSC), which give rise to neural and glial progenitors, and subsequently neurons, astrocytes, and oligodendrocytes. The neural crest cells migrate and contribute to tissues throughout the body, including the peripheral nervous system (e.g., Schwann cells), ectomesenchymal tissues of the head (e.g., dental pulp stem cells), and the pigmented cells of the skin known as melanocytes. Somatic cells, such as fibroblasts, can be reprogrammed to induced pluripotent stem cells (PSC) through the ectopic expression of four specific transcription factors. These cells can then be differentiated similar to ESC. Somatic cells can also be directly reprogrammed into neurons and NSC. Transdifferentiation is direct reprogramming of cells within the same germinal lineage, such as dental pulp stem cells into neural cells.

pathways. In this case, the transcription factors used consist of the pluripotency-associated factors; octamer-binding transcription factor 4 (OCT4), SRY (sex determining region Y)-box 2 (SOX2), KLF4, and MYC. These induced PSC resemble ESC with respect to their morphology, growth properties, gene/protein expression profiles, differentiation potential, teratoma formation *in vivo*, and potential to generate chimeras when injected into blastocysts to demonstrate germline transmission (14, 15). Induced PSC present a cell population with the same level of pluripotency as ESC but circumvent ethical issues, while also providing a source of cells for autologous cell therapies like adult stem cells. However, induced PSC too have a greater risk of tumorigenesis, thus giving adult stem cells, despite their more limited potential, an advantage in terms of safety and timing to the market. Nevertheless, this breakthrough technology has expanded the stem cell research field and helped enhance the progress of cell therapies by providing a cell source with unlimited proliferation and differentiation potentials, and more importantly, a strategy for manipulating cells across all cell lineages.

Within a few years of discovery, the focus on cellular reprogramming turned to a direct strategy. The concept was to bypass the pluripotent state and reprogram cells directly to the desired cell type, thus providing an accelerated reprogramming pathway with a biologically safer product. This direct reprogramming pathway is sometimes referred to as transdifferentiation. In theory, transdifferentiation occurs within the same germinal lineage (**Figure 1**) (13), thereby requiring fewer epigenetic modifications and decreasing the risk of accumulating mutations and leading to tumorigenesis. Nevertheless, some studies have claimed that inter-lineage transdifferentiation is possible via various transient intermediates (16). Direct strategies therefore increase safety and efficiency, and result in terminally differentiated cells or multipotent stem/progenitor cells with limited proliferation and differentiation capacity (**Figure 1**). This strategy has been applied to different cell sources to induce cells along the neural lineage (6, 13).

NEURAL STEM CELLS

The CNS has an inherent, albeit limited capacity for repair. Neural stem cells, capable of self-renewal and differentiation

into neurons, astrocytes, and oligodendrocytes, reside in specific regions called neurogenic niches. The formation of the CNS is a series of complex developmental processes initiated early in embryonic development, following the specification of the three germ layers, the ectoderm, mesoderm and endoderm. The neuroectoderm is induced by the underlying mesoderm, through the inhibition of signals that specify epidermal ectoderm. Neurulation is then initiated when the neural plate folds in to form the neural tube, which is composed of neuroepithelial cells (**Figure 1**). These cells give rise to bipolar NSC called radial glia, which initially span the neural tube, multiply and act as scaffolds for migrating cells. They give rise to cells that respond to gradients of morphogens and mitogens, resulting in the patterning of the neural tube and ultimately giving rise to all the neurons and glia of the CNS (17).

The adult mammalian brain has two main neurogenic niches that contain NSC; the sub-ventricular zone of the lateral ventricles and the sub-granular layer of the dentate gyrus in the hippocampus (17). Neural stem cells give rise to intermediate glial and neural progenitors. Subsequently neuroblasts arise from neural progenitors, and migrate and differentiate into various neuronal sub-types that may integrate within the existing network. Under normal physiological conditions, neuroblasts in the sub-ventricular zone migrate to the olfactory bulb via the rostral migratory stream after which they migrate radially and differentiate into interneurons (granule cells and periglomerular cells), while those in the sub-granular layer migrate into the inner granule cell layer and differentiate into granule cells (17). Adult neurogenesis has been proposed to be involved in maintenance and repair of the neural network, memory formation, and olfaction (17).

Neural stem cells are mostly quiescent under normal physiological conditions but are induced to proliferate and migrate to the affected site in response to injury and disease, such as stroke (17). It has been shown that stroke induction stimulates NSC proliferation and neuroblasts are recruited to the ischaemic striatum (18, 19). This migratory response is mediated by molecules, such as the chemokine stromal cell-derived factor 1-alpha (SDF1α), secreted by glial, immune and endothelial cells (20–22). However, due to the hostile microenvironment, cell survival is low, thereby limiting the intrinsic repair potential (17).

Neural stem cells were described and isolated from the adult mouse brain in the late 1980s (23) and early 1990s (24), though there was evidence of mitogenic cells in the rodent brain from as early as the 1960s (25). In the presence of growth factors, including basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), these cells proliferated and formed cell clusters termed neurospheres, which could be passaged following single-cell dissociation to generate second, third and further generation neurospheres, or differentiate into neurons and glial cells (24). They were also capable of surviving, proliferating and differentiating when transplanted into the CNS (26, 27). In the late 1990s, analysis of post-mortem tissue confirmed the presence of NSC in the adult human brain (28, 29) and a few years later, NSC were isolated from the adult human olfactory bulb from patients undergoing invasive neurosurgery (30). They are rarely accessible from the living adult human brain and only through extremely invasive surgery, therefore NSC from rodents have been studied as well as human foetal brain-derived NSC (31, 32).

It is presumed that the optimum brain repair mechanism resides within the brain mediated by the local stem cells. Age is a risk factor for neurological diseases including stroke, but aging also attenuates brain repair potential, thus increasing susceptibility to disease. Neural stem cell proliferation rate, viability, and migration capacity can thus be restricted (17). For this reason, exogenous sources of NSC are being studied, including ESC-derived, foetal brain-derived, or reprogrammed from somatic cells either indirectly via the production of induced PSC or directly bypassing the pluripotent stage (6). Ethical and practical considerations limit the application of NSC from ESC and brain tissue, leaving reprogramming as an appealing alternative to provide a source of NSC for cellular therapy.

To be applicable for regenerative medicine, a cell source needs to be easily accessible, expandable under good manufacturing practices to provide sufficient cell numbers, and able to be differentiated/reprogrammed using an efficient and reproducible protocol with defined media. In this regard, stem cells have been investigated, and promising candidates include neural crest-derived DPSC (7, 33).

DENTAL STEM CELLS

The tooth is an ectomesenchymal tissue that contains, along with the supporting tissues, multiple stem cell niches. Several dental stem cell populations have been identified, which include; DPSC from the dental pulp tissue of permanent teeth (**Figure 2**) (7), stem cells from the dental pulp tissue of exfoliated deciduous teeth (34), stem cells from apical papilla isolated from the root canal of immature permanent teeth (35), and stem cells from tooth germs (36). Other stem cell populations from the supporting tissues in the oral cavity include; periodontal ligament stem cells (37), alveolar bone-derived MSC (38), gingival MSC (39), and dental follicle stem cells from the surrounding connective tissue of the tooth germ (40).

Dental stem cells follow the criteria that define multipotent mesenchymal stem/stromal cells (MSC), as described by the International Society for Cellular Therapy in 2006 (41). These criteria include; plastic adherence under standard culture conditions; ability to differentiate along the adipogenic, chondrogenic, and osteogenic lineages when cultured in appropriate inductive media; expression of the common MSC-associated markers CD73, CD90, CD105; and lack of expression of haematopoietic markers CD14, CD34, CD45, CD19, and HLA-DR. These dental stem cells are isolated similarly to MSC-like populations from other tissues, such as bone marrow and adipose tissue, and display a spindle-shaped morphology. This review will focus on DPSC, which were the first dental stem cells to be identified.

DENTAL PULP STEM CELLS

Human DPSC isolated from third molars, were first described by Gronthos et al. (7). They are a heterogeneous multipotent

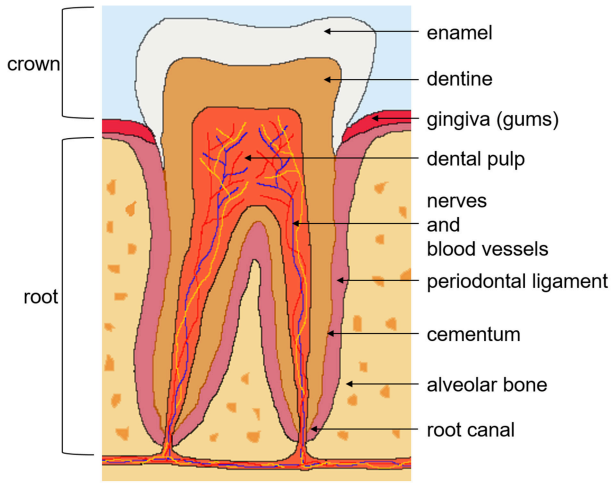


FIGURE 2 | Tooth anatomy and dental pulp stem cells. There are two anatomical parts of the tooth; the crown exposed to the oral cavity and the root embedded in the gum. The crown is composed of enamel on the outside, the underlying dentine, and pulp tissue, which contains nerves, blood vessels, and lymphatics. The root, covered with the bone-like tissue cementum, contains dentine and the root canal. The tooth is supported by the periodontal ligament, connecting it to the alveolar bone. Dental pulp stem cells reside in the perivascular niches of the dental pulp tissue of permanent teeth.

adult stem cell population that reside in the perivascular niche of the dental pulp (**Figure 2**) (42). The role of the dental pulp is to maintain tooth homeostasis and repair. The dental pulp cell population consists of dentine-generating odontoblasts on the outer side of the tissue, fibroblasts, immune cells, nerve and glial cells, and stem and progenitor cells in the perivasculature. It was demonstrated early on that DPSC can regenerate tooth structures, as they were capable of forming a dentin-pulp complex when transplanted into immunocompromised mice, which suggested the presence of stem and progenitor cells (7).

Dental pulp stem cells possess high proliferative and clonogenic capacity, and it is for this reason that they have attracted attention with regard to therapeutic applications (7). Limited cell proliferation and tendency to undergo senescence when cultured *ex vivo*, is a challenge that needs to be overcome if cells are to be used in the clinic. In comparison to some cell populations, such as BMSC, DPSC display a higher frequency of colony formation and greater proliferation potential, which is maintained throughout culture (7, 43).

Their multilineage differentiation potential has been demonstrated under *in vitro* inductive conditions and following implantation *in vivo*, along multiple lineages including the odontogenic, osteogenic, chondrogenic, adipogenic, neurogenic, and myogenic lineages (44, 45). This extensive array of lineages is an advantage over other stem cell populations, such as haematopoietic stem cells, which have a more limited differentiation capacity.

There is no known unique marker to identify and isolate DPSC. Extensive immunophenotyping of *ex vivo* expanded DPSC has demonstrated their expression of markers associated with; MSC-like populations; bone and dentine; and perivascular cells, including CD146 (7). Dental pulp stem cells may also express neural lineage markers, including low-affinity nerve growth factor receptor known as p75, the intermediate filament nestin, and glial fibrillary acidic protein, as well as more mature neuronal lineage markers, such as β-III tubulin and neuronal nuclear antigen (45). This immunophenotype reflects their origin and high level of heterogeneity.

Dental pulp stem cells originate from the embryonic neural crest. In vertebrate embryogenesis, during the formation of the neural tube, a transient population of multipotent cells arises at the junction between the neural tube and the epidermal ectoderm, termed the neural crest (**Figure 1**). These neural crest cells migrate from the dorsal margins of the neural tube, undergoing an epithelial to mesenchymal transition, and give rise to multiple neural and non-neural cell types throughout the body, including all of the neurons and glia of the peripheral nervous system, the ectomesenchymal derivatives of the craniofacial area and melanocytes of the skin (**Figure 1**). It is the migrating cranial neural crest cells that contribute to the dental pulp tissue, established using genetic lineage tracing (46–48). In addition, a more recent study demonstrated that a significant subpopulation of cells extracted from the dental pulp originates from peripheral nerve-associated glia during tooth development, homeostasis and regeneration (49). Thus, neural crest-derived stem and progenitor cells persist in adult tissues, and some, such as the glial Schwann cells lining peripheral nerves and melanocytes in the skin, are able to dedifferentiate into stem/progenitor-like cells (13).

It is therefore not surprising that DPSC express markers of the neural lineage, which substantiate their neural predisposition. However, they are heterogeneous and this quality can impact the differentiation efficiency (7). Analysis of individual colonies also demonstrated the different proliferation rates, suggesting that the more highly proliferating cells will dominate a multi-colony population, altering the composition (33).

Most studies have focused on using the whole DPSC population extracted from the dental pulp (50, 51). However, it may be beneficial to select subpopulations, using fluorescence- or magnetic-activated cell sorting, based on markers that may be associated with increased neurogenic potential. Using a more homogenous cell population could increase the differentiation efficiency. For example, stromal precursor cell surface marker STRO-1, known as an early marker of multiple MSC-like populations, has been used to purify DPSC (42). However, STRO-1 is downregulated very early during *ex vivo* expansion, creating a problem in obtaining sufficient cell numbers (42). The neural crest marker p75, co-expressed with STRO-1 on BMSC (52), may be suitable as $p75^{+}$ DPSC express higher levels of neural stem cell markers (53). It is however expressed by <10 percent of the overall DPSC population (53). Isolation methods may also make a difference. The common isolation method is to enzymatically release the cells from the dental pulp tissue using collagenase and dispase (7). Alternatively, cells can be allowed to migrate out using the explant method, which may select for specific subpopulations (54).

Dental pulp stem cells have also been shown to express the pluripotency-associated markers OCT4, SOX2 and MYC (55), which is uncommon to MSC. However, unlike ESC and induced PSC that express these markers, transplanted DPSC have not resulted in tumour formation (33). Additionally, both spontaneously and induced immortalised DPSC did not form tumours when transplanted into immunocompromised mice (56). Embryonic stem cells and induced PSC require complete differentiation prior to implantation due to tumorigenic risk.

These properties make DPSC promising candidates for stem cell therapy, in particular for neurological diseases due to their neurogenic predisposition. Most importantly, dental pulp presents an easily-accessible non-invasive source of adult stem cells with high proliferation potential. They are isolated from routinely extracted teeth that would otherwise be discarded as clinical waste, unlike the invasive aspiration of bone marrow required to obtain BMSC.

NEURONAL DIFFERENTIATION OF HUMAN DENTAL PULP STEM CELLS

Under defined neuronal inductive conditions, human DPSC are able to differentiate into functionally active neuronal cells. They acquire a neuronal morphology, displaying a rounded cell body with cytoplasmic extensions, and show an immunophenotype consisting of neuronal-associated markers (e.g., β-III tubulin, neuronal nuclear antigen) (50, 51). As outlined in **Table 1**, there have been multiple neuronal differentiation protocols applied to human DPSC, varying in media composition, use of supplements, growth factors, and small molecules, as well as seeding density, growth surfaces, number of stages, and duration. However, few studies have conducted functional analysis of their neuronally differentiated DPSC (**Table 1**), such as electrophysiological analysis for the presence of voltage-gated ion channels (Na^+, K^+, Ca^{2+}), which are required for the generation and propagation of action potentials in functionally mature neurons.

One of the earlier methods for neuronal induction of human DPSC, and the first to examine their functionality, resulted in neurons that displayed voltage-gated sodium channels, important for initiation of action potentials (50). A subpopulation of DPSC has been shown to display sodium currents in their normal state (57). These cells may have a role in sensory transduction within teeth. If this subpopulation differentiates into neuronal-like cells, enhanced electrophysiological properties would be expected. It is however uncertain, which subpopulations neuronally differentiate, and whether they initially display any voltage-dependent ion currents. A modified version of this neuronal differentiation protocol resulted in cells expressing both sodium and potassium currents (58).

Subsequently, a shorter, 2-week neuronal differentiation protocol for DPSC was developed, resulting in neurons displaying both voltage-dependent sodium and potassium currents and were sensitive to respective inhibitors (51). This method had a pre-induction step exposing the cells to the demethylating agent 5-azacytidine, thus modifying the epigenome to increase the ability of the cells to go down the specified lineage. These neuronally differentiated human DPSC localised to the neurogenic niches in the rodent brain and were detected in the lesioned cortex following injury (59). This demonstrated that the DPSC-derived neuronal cells survived and responded to the environmental signals, similar to results obtained in an avian embryo model using undifferentiated human DPSC (60).

Other groups have been interested in directing DPSC into specific neuronal types, including dopaminergic and spiral ganglion neurons (61–63). However, the investigations are few at the current time and more research is needed. Furthermore, xeno- and serum-free conditions from initial culture to neuronal differentiation have also been investigated (64). Removing animal-derived products and creating defined media is a critical step towards establishing a safe and consistent cell source for therapeutic purposes.

More recently, studies have begun to use neurosphere generation for neural induction of human DPSC (65). Neurospheres are a commonly used culture system for NSC, where cells are grown in conditions lacking adherent substrates and in the presence of growth factors, most commonly bFGF and EGF. They are heterogeneous, containing cells at different stages of differentiation and maturation, thus identifying the presence neural stem cells. Recent studies have incorporated this culture system in the neuronal differentiation of DPSC (65, 66). The cells were differentiated to functional neurons *in vitro*, through a two-step protocol, firstly by stimulating neurosphere formation, then going through a neuronal maturation stage. Patch-clamp analysis and calcium imaging were used to confirm the presence of functional properties. One research group observed that the cells not only expressed functional voltage-gated sodium and potassium channels but a subset of cells also generated single action potentials (65). Repeated action potentials were not observed nor were spontaneous action potentials, which would indicate fully functional neurons. The initial neurosphere culture step was further analysed by Pisciotta et al. (67), who demonstrated the prolonged expansion of the DPSC-derived spheres throughout which they maintained their neural crest properties.

These *in vitro* differentiation studies demonstrated that DPSC respond to local environmental cues, which is also true for the *in vivo* setting. When injected into an embryonic model at a time of active neurogenesis, DPSC followed the migratory pathway of endogenous cranial neural crest cells, reiterating their origin, and underwent neuronal differentiation (60).

HUMAN DENTAL PULP STEM CELLS IN STROKE MODELS

Despite the extensive *in vitro* research examining the neurogenic properties of DPSC, there have only been a hand-full of studies that have examined the efficacy of human DPSC-based therapies *in vivo* to treat stroke. **Table 2** summarises the use of human DPSC in animal stroke models, based on rodent models of focal

TABLE 1 | Neuronal induction protocols for human dental pulp stem cells with functional assessment.

Cells	Growth conditions					Functional assessment	References
	Basal media	Use of spheres	Coating	Growth factors	Duration		
$CD34^+/cKit^+/STRO\text{-}1^+$ DPSC	DMEM/F12	Yes	Poly-L-lysine	bFGF EGF BDNF NGF	>3 weeks	Patch-clamp analysis of Na^+ and K^+ currents.	Pisciotta et al. (67)
DPSC	DMEM/F12	Yes	Poly-L-ornithine, laminin	bFGF EGF BDNF GDNF NT-3	19–23 days	Fluorescent detection of Ca^{2+} influx following stimulation.	Gonmanee et al. (63)
DPSC	Neuro-basal	No		bFGF EGF BDNF	12 days	Fluorescent detection of Ca^{2+} influx following stimulation.	Singh et al. (62)
DPSC	DMEM/F12	No	Type I and IV collagen, laminin, fibronectin	bFGF EGF BDNF GDNF IGF-I	>3 weeks	Transplantation into neonatal rat brain and spinal cord injury rat model.	Jung et al. (64)
DPSC	Neuro-basal A	No	Geltrex	bFGF EGF	3 weeks	Patch-clamp analysis of Na^+ and K^+ currents.	Ullah et al. (58)
DPSC	DMEM/F12, Neuro-basal	Yes	Poly-L-ornithine, laminin	bFGF EGF NT-3	5 weeks	Patch-clamp analysis of Na^+ and K^+ currents, and action potential production.	Gervois et al. (65)
DPSC	Neuro-basal	No		bFGF FGF8 BDNF	9 days	Fluorescence detection of Ca^{2+} influx, and measurement of dopamine release with/without stimulation.	Kanafi et al. (61)
DPSC	Neuro-basal	Yes	Type IV collagen	bFGF EGF	2 weeks	Fluorescent detection of Ca^{2+} influx following stimulation.	Osathanon et al. (66)
DPSC	DMEM/F12, Neuro-basal A	No	Poly-L-lysine	bFGF NGF NT-3	9–13 days	Patch-clamp analysis of Na^+ and K^+ currents, transplantation into the cerebrospinal fluid of neonatal rats, transplantation into rats with lesions to the forelimb motor cortex.	Kiraly et al. (51, 59)
DPSC	Neuro-basal A	No	Poly-ornithine, laminin	bFGF EGF	3 weeks	Patch-clamp analysis of Na^+ currents.	Arthur et al. (50)

BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; DMEM, Dulbecco's Modified Eagle's Medium; DPSC, dental pulp stem cell(s); EGF, epidermal growth factor; F12, Ham's F12 nutrient mixture; FGF8, fibroblast growth factor 8; GDNF, glial cell-derived neurotrophic factor; IGF-I, insulin-like growth factor I; NGF, nerve growth factor; NT-3, neurotrophin-3.

cerebral ischaemia via the transient occlusion of the middle cerebral artery.

The first study to investigate DPSC in an animal model of stroke used a mechanical extraction method to obtain cells from human wisdom teeth (68). These cells expressed the nuclear receptor related 1 protein, which is essential for the dopaminergic system of the brain. Although the study involved a small sample size, it provided preliminary data for the therapeutic potential of dental pulp cells for stroke. Functional recovery was exhibited and cells were detected in the penumbra, however potential mechanisms were not investigated.

A subsequent study provided the first preclinical support for use of human DPSC in acute ischaemic stroke (69). Dental pulp stem cells were intracerebrally transplanted into the rat brain 24 h post-stroke, at two injection sites; in the cortex and in the striatum. At 4 weeks, significant neurobehavioural improvement was observed, however only 2.3 percent of engrafted cells were detected. These results suggest that the improvement was not mediated by a cell replacement mechanism rather through a DPSC-dependent paracrine effect, involving the many growth factors secreted by DPSC, such as nerve growth factor, brain-derived neurotrophic

TABLE 2 | Human dental pulp stem cells in stroke models.

Cells	Experimental model	Delivery method	Cell number	Timing	Outcome	References
DPSC	Rat Transient Middle Cerebral Artery occlusion (MCAo)	IV	1×10^6	Cells injected at 0 or 3 h post-stroke, analysis performed up to 14 days post-stroke.	Cells reversed motor deficits and reduced infarct volume by inhibiting microglial activation, pro-inflammatory cytokine production, and neuronal degeneration.	Nito et al. (71)
DPSC over-expressing hepatocyte growth factor		IV	1×10^6	Cells injected at 0 h post-stroke, analysis performed up to 14 days post-stroke.	Cells improved motor function and decreased infarct size via immunomodulation, enhanced angiogenesis, suppression of neuronal degeneration, and maintenance of blood-brain barrier integrity.	Sowa et al. (76)
$CD34^+$/c-Kit^+/ STRO-1^+ DPSC		IV	4×10^6	Cells injected 24 h post-stroke, analysis performed up to 28 days.	Cells stimulated functional recovery and decreased infarct volume via autocrine/paracrine mechanisms.	Song et al. (75)
DPSC		IC	6×10^5	Cells injected 24 hours post-stroke, analysis performed up to 28 days.	DPSC enhanced post-stroke forelimb sensorimotor recovery via DPSC-dependent Paracrine effects.	Leong et al. (69)
Dental pulp-derived nuclear receptor related 1 $protein^+$ stem cells		IC	$1–2 \times 10^5$	Analysis performed up to 28 days.	Transplanted cells survived and promoted functional recovery, due to hypo-immunogenic properties and immunomodulation ability.	Yang et al. (68)

DPSC, dental pulp stem cell(s); IC, intracerebral; IV, intravenous.

factor, glial-derived neurotrophic factor, and ciliary neurotrophic factor (70, 71). The low survival rate observed in this preclinical model may indicate a specific subpopulation of DPSC have greater neurogenic potential and ability to survive and differentiate, with the $p75^+$ subpopulation being a candidate. The DPSC displayed targeted migration towards the infarct, and differentiated into astrocytes within the vicinity of the infarct and neurons further from the infarct site. The targeted migration of the transplanted cells towards the ischaemic border zone is likely mediated by the SDF1 gradient produced by endogenous cells following stroke (20). This chemokine is upregulated for at least a month post-stroke (72). Furthermore, it was previously shown in an avian embryo model that DPSC induce endogenous axon guidance of host trigeminal ganglion axons via the production of SDF1 (60). In this preclinical study, a reduction in post-stroke corpus callosum atrophy was also observed, again indicating to the ability of DPSC to influence host axonal remodelling. These results demonstrate the role of SDF1 signalling in DPSC migration and neuroplasticity properties.

Of note, both of these studies used an intracerebral route of administration, which is a highly invasive procedure. Subsequent studies used an intravascular route, which may be clinically safer in the acute stroke setting (73). It has been shown that stem cells administered through this route can be detected in the brain but involves the transmigration of the BBB. This could be a result of the disrupted BBB post-stroke, however cellular trafficking occurs even after the period of increased BBB permeability (74), implicating other mechanisms at play.

In these DPSC studies, administration via the tail vein resulted in cells transmigrating the BBB and migrating towards the infarct (71, 75, 76), with a lack of detection in systemic organs (75). Winderlich et al. previously demonstrated that human DPSC stimulate BBB permeability via the production of the soluble factor vascular endothelial growth factor-alpha (VEGFα) (73), which is known to be produced following stroke and is secreted by stem cells, including DPSC (77). DPSC mediate their angiogenic effect via VEGFα (75, 76). In addition to its role in angiogenesis, VEGFα is involved in vascular permeability, and has been shown to decrease tight junction proteins thus increasing permeability (78). Interestingly, Sowa et al. showed that the integrity of the BBB was maintained via the DPSC attenuation of the decrease in tight junction proteins that results from stroke (76). This was observed with unmodified DPSC, but also with DPSC overexpressing the pleiotropic cytokine hepatocyte growth

factor, which showed an even greater effect. This factor may have a role in inhibiting BBB disruption (79). Hence, DPSC not only produce factors like VEGFα that allow them to transmigrate the BBB but they also have the counteracting potential to maintain BBB integrity. There are likely other factors involved in regulating BBB permeability and with more research, the complex mechanisms used by stem cells can be uncovered.

Dental pulp stem cells have been shown to modulate the immune response in several ways. They inhibit microglial activation, as evidenced by the decrease in cells expressing ionised calcium-binding adaptor molecule 1, a marker specifically expressed in the brain by activated microglial cells (71, 76). Analysis of DPSC-conditioned medium has shown their expression of various cytokines, including interleukin 10, which decreases pro-inflammatory cytokine production (71). Additionally, decreased levels of the pro-inflammatory cytokines tumour necrosis factor-alpha and interleukin 1-beta are observed in brain tissue and serum from DPSC-treated stroke animals (71, 76).

The BBB has a role in maintaining the immune privileged status of the CNS. It appears that DPSC are not entirely rejected, as these studies have not employed the use of immunosuppressive drugs (71, 75, 76). This could be mediated by the DPSC expression of the Fas ligand, which induces the apoptosis of T cells, and is also one of the mechanisms used to maintain immune privilege of certain tissues. Fas ligand is known to be expressed by stem cells including DPCS, even with prolonged culture and particularly using the neurosphere culture system (67). Thus, DPSC have the potential to evade the immune system, transmigrate the BBB, migrate towards the infarct and modulate the inflammatory response. The ability of DPSC to exert these therapeutic effects in the acute phase when inflammation occurs, demonstrates their high level of potency (71).

Dental pulp stem cells are also able to inhibit reactive astrogliosis (75), which leads to glial scar formation as a protective mechanism but impedes axonal regrowth. Attenuation of neuronal degeneration by DPSC is also evidenced by decreased numbers of degenerating neurons (71, 76). This neuroprotective effect is likely mediated by the array of neurotrophic factors produced by DPSC, such as nerve growth factor, brain-derived neurotrophic factor, glial-derived neurotrophic factor (70). Additionally, DPSC express the cytokine interleukin 3 (IL-3) (71), which promotes proliferation and differentiation of stem cells, suggesting that DPSC may have capacity to stimulate endogenous cells, though this mechanism has not been investigated in these studies.

These *in vivo* studies observed functional recovery and decreased infarct volumes (**Table 2**). Evidence from all preclinical studies suggest that the therapeutic effects of DPSC are most likely mediated through paracrine mechanisms and less so of a neural cell replacement mechanism. Dental pulp stem cells secrete growth factors, neurotrophins and cytokines, which promote angiogenesis, modulate the immune response, provide neuroprotection, and enhance neuroplasticity (**Figure 3**).

Even though cell survival and differentiation to astrocytes and neurons is observed, neural tissue replacement is unlikely to play a big part. Cell survival may be increased through the addition of neurotrophic factors, such as brain-derived neurotrophic factor (80). Alternatively, the culture method may be important, with a better outcome observed with DPSC-derived neurospheres, possibly due to greater amounts of neural progenitor cells (81). As previously mentioned, certain DPSC subpopulations may have greater neurogenic potential and survival. The perivascular cell marker CD146 is used to purify MSC-like populations from different tissues. However, it may actually select cells that are more committed to non-neural lineages, as $CD146^+$ cells demonstrate greater regeneration of the dentin-pulp complex than $CD146^-$ cells (82). The $CD146^-$ subpopulation may instead contain cells that are of neural crest origin, and may explain why it displayed positive outcomes in an ischaemic model (83). Another investigated subpopulation is $CD34^+$ DPSC, which despite their slower proliferation and loss of "stemness," have greater commitment to the neural lineage and express the neural markers p75 and nestin, compared to $CD34^-$ DPSC (75, 84). Although CD34 is believed to be a distinct marker for cells of haematopoietic origin and endothelial cells, this subpopulation improved functional recovery via the aforementioned mechanisms but also exhibited superior neuroprotective properties in comparison to BMSC (75).

There is strong experimental evidence that stem cell therapy can improve neurological function through a variety of cellular and molecular mechanisms, with growing evidence to support DPSC as an attractive source. These cells are appealing for use in autologous and allogeneic therapies of stroke. Stroke is more widely regarded as an aging disease, and improved healthcare suggests that a greater proportion of the older population retain their teeth. Nevertheless, to reach all stroke patients irrespective of having their own teeth, an allogeneic strategy may be required, and the immunosuppressive properties of DPSC make them attractive for this type of clinical application (85).

While promising, the efficacy of DPSC to deliver therapeutic outcomes based on their capacity to differentiate into functional neural cell populations remains the subject of intense research. The limitation in using undifferentiated and unselected DPSC is that they may differentiate along multiple lineages or only a small fraction would differentiate to neural cells, thereby decreasing the clinical efficacy. A neural cell population would be more suited to the environment and have greater potential to replace lost and damaged neural tissue. For example, neuronally pre-differentiated human DPSC responded well to the normal and injured *in vivo* environment (59). However, since neuronal cells lack the ability to self-renew, the number of cells that can actually mediate positive outcomes will be fewer than initially transplanted due to cell loss. Furthermore, in a disease like stroke where the ischaemic milieu affects multiple neural cell populations, administering neuronal cells would be targeting the replacement of only one affected population. Neural stem or progenitor cell populations would be a better alternative, allowing

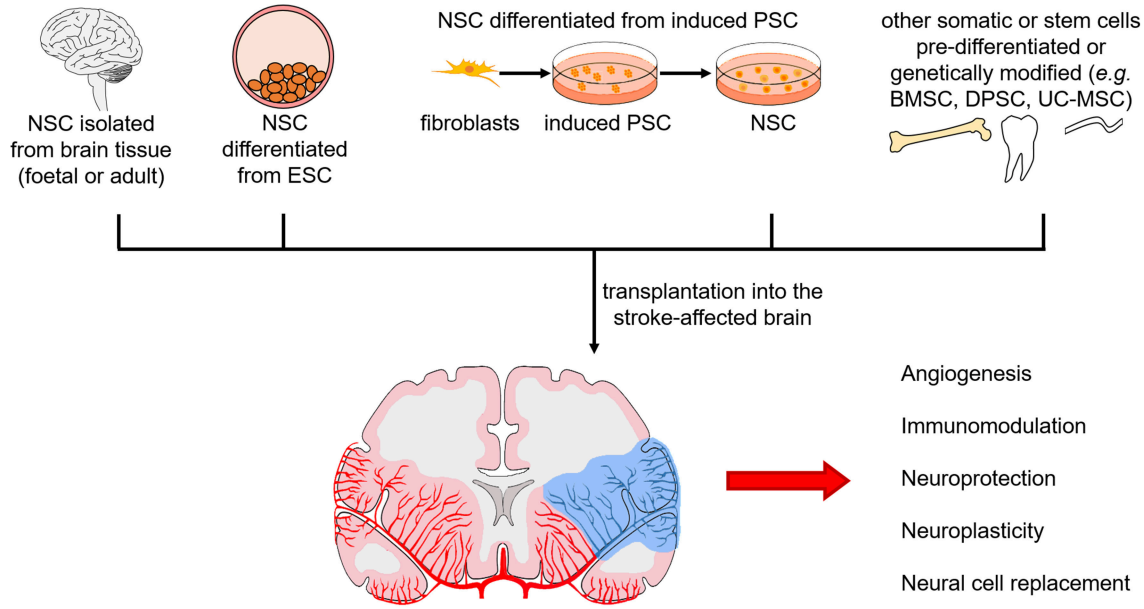


FIGURE 3 | Sources of neural stem cells for transplantation in the stroke-affected brain and mechanisms of action. Neural stem cells (NSC) from foetal and adult brain tissue, differentiated from pluripotent stem cells [embryonic stem cells (ESC) or induced pluripotent stem cells (PSC)], and differentiated or genetically modified from other somatic cells and stem cells [e.g., bone marrow stem cells (BMSC), dental pulp stem cells (DPSC), umbilical cord-derived mesenchymal stem cells (UC-MSC)], are being investigated. Mechanisms of action in the stroke-affected brain include stimulating angiogenesis, modulating the immune response, providing neuroprotection, inducing neuroplasticity, and replacing neural cells.

expansion to provide sufficient cell numbers and differentiation into various cells based on the endogenous environment.

REPROGRAMMING DENTAL PULP STEM CELLS

Finding an alternative source of NSC has been a major research focus for some years now, with much of the research investigating cellular reprogramming strategies. In this regard, DPSC might be a promising cell source. Due to their neural crest origin, they may require less manipulation or display greater efficiency than other cell types. As previously mentioned, cells can be directed along the neural lineage either indirectly, using induced PSC production, or directly to the desired cell type.

Dental pulp stem cells have successfully been reprogrammed to induced PSC (86, 87), even in the absence of the oncogenic MYC, and subsequently directed along the neural lineage (88). Dental pulp can thus provide an alternative cell source for establishing induced PSC banks for regenerative medicine. There has been little investigation in the area of converting DPSC into NSC, other than by the use of neurosphere culture methods, usually as a transient step in the pathway to neuronal differentiation (65). While there has been some evidence of the neurosphere culture system maintaining prolonged culture of DPSC-derived spheres (67), these spheres are not clonal nor consisting of only stem cells. Since DPSC are of neural crest origin, it may be more efficient to use a direct reprogramming strategy to convert them to NSC, in comparison to cells of a different germinal lineage. Transdifferentiation should be possible between these cell types, which share a common tissue of origin. Similar methods that have been applied to other somatic cell types to induce NSC can be applied to DPSC (89–92).

DIRECT REPROGRAMMING OF CELLS ALONG THE NEURAL LINEAGE

Direct reprogramming along the neural lineage was first investigated by Wernig et al. who demonstrated the production of induced neurons from mouse fibroblasts using the neural-specific transcription factors, ASCL1, BRN2 and MYT1L (93), and from human fibroblasts with the addition of the NEUROD1 transcription factor (94). Since fibroblasts are from a heterogeneous mesenchymal cell population potentially containing cells of neural crest origin, they further applied this transcription factor combination to a homogeneous non-neural-derived cell population (95). This demonstrated that reprogramming is not restricted to cells of the same germinal lineage. Thereafter, they established that the pro-neural factor ASCL1 alone was capable of reprogramming fibroblasts to neuronal cells (96); thus this gene may be viewed as a pioneer transcription factor (97). Reprogramming is associated with epigenetic modifications of the DNA. Pioneer transcription factors are first to occupy chromatin sites, leading to remodelling of the chromatin configuration at lineage-specific genes and subsequent recruitment of other transcription factors, demonstrating the hierarchical mechanisms in lineage

reprogramming. The achievement of generating induced neurons is of clinical importance with regard to addressing the safety issues associated with induced PSC. However, the post-mitotic nature of neurons may not be ideal for all applications.

Subsequent studies applied a direct reprogramming strategy to obtain expandable neural stem and progenitor cells, using pluripotency-associated and/or neural-specific transcription factors. The first study to derive induced neural stem or progenitor cells, applied transient expression of the Yamanaka factors (OCT4, SOX2, KLF4, MYC) in fibroblasts. However, these induced cells occurred at a low frequency of <1 percent, were expandable *ex vivo* for only three to five passages, giving rise to neurons and astrocytes but not oligodendrocytes (16). A similar study used the same factors but limited OCT4 expression to the first 5 days of reprogramming, resulting in tripotent NSC that could be stably expanded for more than 50 passages and exhibited a transcriptional profile similar to brain-derived NSC (92). These studies claimed to transdifferentiate the fibroblasts to the neural lineage using the same factors for induced pluripotency but under neural conditions, thus bypassing the pluripotent state. However, genetic lineage tracing studies demonstrated that these methods actually involved cells passing through a transient pluripotent state, before differentiating along the lineage specified by their environment (98, 99). This suggests that the pluripotent state is a requisite when reprogramming cells between different germinal lineages, although transcription factor combinations may also influence this.

Different research groups instead used neural-specific transcription factor combinations, including SOX2, which has a role in embryonic development and neural induction (89, 100). One combination included BRN2, a transcription factor in the same family as OCT4, which was necessary for tripotency, while SOX2 was essential for development of mature neurons (89). In another study, the Yamanaka factors were used but OCT4 was replaced with BRN4, again in the same family (90). These fibroblast-derived NSC survived in the mouse brain for up to 6 months and differentiated along the three neural lineages (101). More recently, the same group compared the transcriptome of these induced NSC with forebrain-derived NSC and ESC-derived NSC (102). They showed that all three groups of NSC were distinct from one another, but that induced NSC and forebrain-derived NSC had a greater similarity with each other than with ESC-derived NSC. Moreover, this transcription factor combination resulted in NSC that possessed mostly a caudal identity, while forebrain-derived NSC had a rostral identity and ESC-derived NSC had a less defined regional identity indicating their more immature state. This suggests that different transcription factor combinations could yield NSC suitable for different areas of the brain.

From these studies, it appears that members of the POU domain family of transcription factors, like OCT4, have a critical role in lineage conversion (103). This factor is a fundamental transcriptional regulator of pluripotency. The OCT4 and SOX2 transcription factors heterodimerise; SOX2 binds weakly to DNA while OCT4 contains the POU DNA-binding domain. Together, they govern the activation of genes for pluripotency and concurrently the suppression of genes for differentiation. It has been proposed that OCT4 acts as a pioneer transcription factor to induce plasticity of cells, with subsequent factors and environmental conditions directing the lineage differentiation (103, 104). Indeed OCT4 alone has been used to reprogram fibroblasts (105) and blood cells (106, 107) to NSC, which under neural conditions do not display hallmarks of induced pluripotency (104).

Other groups have demonstrated the production of induced neural cells from fibroblasts using only SOX2 (91), and from blood cells using SOX2 and MYC (108, 109). Like OCT4, SOX2 is a key regulator of pluripotency but is further involved in maintenance of multiple stem cell populations, including NSC, and may be crucial for reprogramming (110). The role of the proto-oncogene MYC in the reprogramming process may involve chromatin remodelling. This factor aids the reprogramming process since removal of MYC lowers the efficiency of induced PSC generation (14, 15).

Cellular reprogramming is a rather inefficient process, requiring ongoing investigations to better understand the process and methods to optimise it. Low efficiency may reflect the stoichiometry of the transcription factors or the need for integration of the transgenes in specific loci (14, 15). Furthermore, the process is stochastic; reprogramming occurs randomly so cells in the population can be at different stages of differentiation and maturity, while incomplete reprogramming may also occur, whereby cells retain epigenetic memory of the donor cells (111). The heterogeneity of the initial cell populations and the molecular state of the cells likely contribute to this process.

Rather than inducing the ectopic expression of transcription factors, microRNAs (112) and small molecules (113, 114) have been applied, which in effect target the enzymes for epigenetic modifications and/or transcription factors and other elements of their signalling pathways to result in similar cellular outcomes as transcription factor-mediated reprogramming. For example, the microRNA miR-124 has a role in regulating the epigenome as well as multiple neuronal differentiation genes (6, 112). Chemical cocktails for reprogramming usually include epigenetic modulators that improve efficiency, such as the histone deacetylase inhibitor valproic acid, and DNA and histone methyltransferase inhibitors (113, 114). Other modulators are used to activate or deactivate specific signalling pathways, such as the Wnt, MAPK/ERK, TGFβ, and hedgehog signalling pathways, to activate appropriate neural genes, promote self-renewal, enhance mesenchymal to epithelial transition, improve viability, decrease cell senescence, and increase efficiency (6, 113, 114). This suggests that multiple signalling pathways have important roles in the reprogramming process and a variety of small molecules can be used to achieve this.

With regard to DPSC, little research exists on transcription factor-mediated reprogramming. Induced overexpression of OCT4A, the OCT4 isoform thought to be the key regulator of pluripotency, enhanced expression of other pluripotency regulators and multilineage differentiation in human DPSC (115). However, the effect on differentiation along the neural lineage was not investigated. Overexpression of SOX2 in DPSC

was also investigated, focusing on proliferation, migration, and adhesion, but the effect on multilineage differentiation is unknown (116).

NEURAL STEM CELL THERAPY FOR ISCHAEMIC STROKE: PRECLINICAL STUDIES

Neural stem cells are a promising cell source for establishment of functional neuronal networks following disruption by neurological injury or disease. They would be an appropriate stem cell source for stroke treatment, where the ischaemic environment affects multiple cell types, with additional heterogeneity seen among patients. Preclinical studies using NSC in rodent models of ischaemic stroke have utilised human and rodent NSC derived from ESC, brain tissue, induced PSC and more recently via somatic cell transdifferentiation (**Figure 3**) (117–120). They have been shown to survive, migrate towards the lesion, differentiate into neurons and astrocytes, decrease infarct volume, integrate, induce neuroplasticity, and stimulate functional improvements via paracrine effects (117, 118, 121–125). Transplanted NSC also induce endogenous NSC to proliferate (119, 120, 126, 127).

Even though beneficial effects have been observed, similar to other stem cells, research is still focused on how to better improve survival of the engrafted cells. Neural stem cells are expected to be better suited for the neural environment, and as such, survival of transplanted human foetal NSC has been shown to be >30 percent at 1 month after transplantation (121, 123), in comparison to the survival of human DPSC at only 2.3 percent (69). Cell survival decreased as time of transplantation post-stroke increased, i.e., hours vs. weeks, however it was still more than 10 times greater than that of DPSC (128), demonstrating their ability to survive in the harsh acute stage. Interestingly, a study that examined the difference of foetal and adult mouse NSC showed that even though both showed therapeutic effects, foetal NSC had a higher survival rate (129), indicating that the adult NSC were mediating their effects through mechanisms other than cell replacement similar to observations with other adult stem cells, such as DPSC. This suggests that there is a difference in foetal and adult stem cells that governs their mechanisms of action.

Multiple studies have shown that survival of transplanted NSC is enhanced when modified to express neurotrophic factors, including nerve growth factor (130), VEGF (131), neurotrophin-3 (132, 133), glial-derived neurotrophic factor (134), brain-derived neurotrophic factor (135, 136), and bFGF (137), or when pre-treated prior to transplantation (138).

Ischaemia produces a harsh environment containing reactive oxygen species, which damage macromolecules in the cells. Some studies have demonstrated that hypoxic preconditioning of cells increases viability (139). The hypoxia-inducible factor 1-alpha (HIF1α) transcription factor is expressed under hypoxic conditions, and one of its downstream targets is VEGF, leading to increased angiogenesis. Alternatively, NSC were modified to express HIF1α to aid their survival (140). In another study, NSC that were modified to overexpress an anti-oxidant enzyme to eliminate reactive oxygen species, showed greater survival following transplantation (141). Thus, NSC may be better primed using mechanisms that activate pathways addressing survival under hypoxic conditions.

Interestingly, in addition to the role in angiogenesis, VEGF has been shown to be involved in transdifferentiation of astrocytes into mature neurons (142). This suggests that an additional beneficial effect of reactive astrogliosis, which has a role in neuroprotection following stroke, is neuronal cell replacement. Furthermore, studies often see differentiation of transplanted stem cells into neurons and astrocytes. Timing of transplantation post-stroke has also been shown to affect differentiation, with earlier time point leading to astrocyte differentiation while later time point leads to neuronal differentiation (143). Thus, astrocytes appear to have a crucial role in the acute stage. Overall, NSC exhibit therapeutic effects but there are multiple ways they can be modified, as well as transplantation parameters, which can alter their survivability and differentiation outcome.

NEURAL STEM CELL THERAPY FOR ISCHAEMIC STROKE: CLINICAL TRIALS

There have been few clinical trials for ischaemic stroke using human neural stem or progenitor cells, and no trials utilising DPSC to date.

The first clinical trial of cell therapy for chronic stroke in patients with fixed motor deficits was conducted using the NTERA2 human teratocarcinoma cell line as a source of neurons. When treated with retinoic acid, these cells that resemble neuroepithelial cells differentiate into post-mitotic neurons that are indistinguishable from ESC-derived neurons. Following the phase I trial consisting of 12 patients and demonstrating the feasibility, a phase II trial was carried out with 18 patients (50 percent ischaemic stroke) who participated in a subsequent stroke rehabilitation program. The safety and feasibility of using these neurons was demonstrated, and although functional improvements were observed, they did not appear to have an overall significant effect on motor function (144, 145).

Several clinical trials have examined multiple cell combination therapy, including neural stem/progenitor cells.

Rabinovich et al. used cells from human foetal nervous and hemopoietic tissues (146). The clinical trial consisted of 10 patients, 5 male and 5 female, aged between 35 to 56 years, with haemorrhagic or ischaemic stroke (70 percent ischaemic stroke) and stable disability 4 to 24 months post-stroke. All patients had received rehabilitation therapy but showed no improvement. Cells were administered in the subarachnoidal space through spinal puncture. There were no serious complications and positive outcomes were observed in all patients, with significant improvements at 6 months compared to the control group. Positive shifts in neurological status became apparent at 2–3 weeks and progressed in the subsequent 30–50 days.

Between 2003 and 2011, a pilot study using combinations of human foetal tissue olfactory ensheathing cells, neural progenitor cells, umbilical cord mesenchymal cells, and Schwann cells was

conducted. This trial consisted of 10 stroke patients (60 percent ischaemic stroke), 6 male and 4 female, aged between 42 to 87 years, 6 months to 20 years post-stroke. Cells were administered via several routes; intracranial parenchymal implantation, intrathecal implantation, or intravenous administration. Follow up was carried out 6 months to 2 years post-transplantation. The study demonstrated the clinical safety of this combination therapy, and every patient achieved improved neurological function (147).

In another clinical trial for ischaemic stroke patients, human foetal brain-derived neural stem/progenitor cells, and umbilical cord-derived MSC were intravenously administered (148). Neurological function and daily living abilities were improved, and no tumorigenesis was detected at 2 years. The study was small, but further provided evidence of safety and feasibility for cell combination therapy.

The first clinical trial to use NSC for chronic ischaemic stroke began in 2010, called the Pilot Investigation of Stem Cells in Stroke (PISCES) (149). This trial used a clonal cell line (ReNeuron's CTX0E03) of allogeneic human foetal brain-derived NSC genetically modified to express the immortalising gene, MYC. The expression is conditional and activated by tamoxifen, so proliferation and differentiation can be controlled. The phase I trial evaluated the safety of this cell line. Recruited patients were male, over the age of 60, 6 months to 5 years post-stroke, and with stable moderate to severe disability. They received a single dose of either 2, 5, 10, or 20 million cells in a single site (ipsilateral putamen), and data was collected over 2 years. No cell-related adverse events were observed up to the highest dose, and improved neurological function was exhibited. The trial involved a small number of patients, however it provided evidence of safety using this cell line and promising signs of efficacy. A phase II trial has now been started to assess safety and efficacy, and examine functional recovery of upper-limb movement. Patients enrolled include male and female, aged 40 and over, 3 months to 1 year post-stroke, with stable upper-limb disability. The dose of 20 million cells will be administered in a minimum cohort of 21 patients.

CONCLUSION AND FUTURE PERSPECTIVES

Stem cell-based therapy is a promising alternative for stroke treatment. While stem cells from different sources, including induced PSC, ESC, MSC, and NSC, have been investigated, using NSC and enhancing the natural mechanisms is most appropriate for brain repair. In preclinical models of stroke, stem cell transplantation has led to positive outcomes through a variety of cellular and molecular mechanisms, many being mediated by the array of beneficial factors produced by the cells. Recent advances in cellular reprogramming have provided alternative sources of NSC to be investigated, allowing for safer and more efficient induction of NSC. More closely related cells, such as DPSC, are potential candidates for manipulation along the neural lineage. They have provided encouraging outcomes in stroke models and their neural replacement potential may be enhanced if resembling NSC. With more research, the limitations of cellular reprogramming can be overcome, and efficient, safe and reproducible methods for NSC induction can be developed.

AUTHOR CONTRIBUTIONS

MG: research and writing of manuscript. KK, SG, and SK: critical revision of manuscript for important intellectual content.

FUNDING

MG is supported by an Australian Government Research Training Program Scholarship.

REFERENCES

1. Benjamin EJ, Virani SS, Callaway CW, Chamberlain AM, Chang AR, Cheng S, et al. Heart disease and stroke statistics - 2018 update: a report from the american heart association. *Circulation*. (2018) 137:e67–e492. doi: 10.1161/CIR.0000000000000558. [Epub ahead of print].
2. Barthels D, Das H. Current advances in ischemic stroke research and therapies. *Biochim Biophys Acta*. (2018). doi: 10.1016/j.bbadis.2018.09.012. [Epub ahead of print].
3. Nogueira RG, Jadhav AP, Haussen DC, Bonafe A, Budzik RF, Bhuva P, et al. Thrombectomy 6 to 24 hours after stroke with a mismatch between deficit and infarct. *N Engl J Med*. (2018) 378:11–21. doi: 10.1056/NEJMoa1706442
4. Lansberg MG, Bluhmki E, Thijs VN. Efficacy and safety of tissue plasminogen activator 3 to 4.5 hours after acute ischemic stroke: a metaanalysis. *Stroke*. (2009) 40:2438–41. doi: 10.1161/STROKEAHA.109.552547
5. Kelly-Hayes M, Wolf PA, Kase CS, Gresham GE, Kannel WB, D'Agostino RB. Time course of functional recovery after stroke: the Framingham study. *J Neuro Rehab*. (1989) 3:65–70. doi: 10.1177/136140968900300202
6. Mertens J, Marchetto MC, Bardy C, Gage FH. Evaluating cell reprogramming, differentiation and conversion technologies in neuroscience. *Nat Rev Neurosci*. (2016) 17:424–37. doi: 10.1038/nrn.2016.46
7. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. *Proc Natl Acad Sci USA*. (2000) 97:13625–30. doi: 10.1073/pnas.240309797
8. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA*. (1981) 78:7634–8. doi: 10.1073/pnas.78.12.7634
9. Evans MJ, Kaufman MH. Establishment in culture of pluripotent cells from mouse embryos. *Nature*. (1981) 292:154–6. doi: 10.1038/292154a0
10. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. *Science*. (1998) 282:1145–7. doi: 10.1126/science.282.5391.1145
11. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. *Nat Biotechnol*. (2000) 18:399–404. doi: 10.1038/74447
12. Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA. *In vitro* differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol*. (2001) 19:1129–33. doi: 10.1038/nbt1201-1129
13. Jopling C, Boue S, Izpisua Belmonte JC. Dedifferentiation, transdifferentiation and reprogramming: three routes to regeneration. *Nat Rev Mol Cell Biol*. (2011) 12:79–89. doi: 10.1038/nrm3043
14. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. (2006) 126:663–76. doi: 10.1016/j.cell.2006.07.024

15. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. (2007) 131:861–72. doi: 10.1016/j.cell.2007.11.019
16. Kim J, Efe JA, Zhu S, Talantova M, Yuan X, Wang S, et al. Direct reprogramming of mouse fibroblasts to neural progenitors. *Proc Natl Acad Sci USA*. (2011) 108:7838–43. doi: 10.1073/pnas.1103113108
17. Ming GL, Song H. Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron*. (2011) 70:687–702. doi: 10.1016/j.neuron.2011.05.001
18. Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med*. (2002) 8:963–70. doi: 10.1038/nm747
19. Jin K, Wang X, Xie L, Mao XO, Zhu W, Wang Y, et al. Evidence for stroke-induced neurogenesis in the human brain. *Proc Natl Acad Sci USA*. (2006) 103:13198–202. doi: 10.1073/pnas.0603512103
20. Imitola J, Raddassi K, Park KI, Mueller FJ, Nieto M, Teng YD, et al. Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. *Proc Natl Acad Sci USA*. (2004) 101:18117–22. doi: 10.1073/pnas.0408258102
21. Kojima T, Hirota Y, Ema M, Takahashi S, Miyoshi I, Okano H, et al. Subventricular zone-derived neural progenitor cells migrate along a blood vessel scaffold toward the post-stroke striatum. *Stem Cells*. (2010) 28:545–54. doi: 10.1002/stem.306
22. Thored P, Arvidsson A, Cacci E, Ahlenius H, Kallur T, Darsalia V, et al. Persistent production of neurons from adult brain stem cells during recovery after stroke. *Stem Cells*. (2006) 24:739–47. doi: 10.1634/stemcells.2005-0281
23. Temple S. Division and differentiation of isolated CNS blast cells in microculture. *Nature*. (1989) 340:471–3. doi: 10.1038/340471a0
24. Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science*. (1992) 255:1707–10. doi: 10.1126/science.1553558
25. Altman J, Das GD. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J Compar Neurol*. (1965) 124:319–36. doi: 10.1002/cne.901240303
26. Gage FH, Coates PW, Palmer TD, Kuhn HG, Fisher LJ, Suhonen JO, et al. Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc Natl Acad Sci USA*. (1995) 92:11879–83. doi: 10.1073/pnas.92.25.11879
27. Snyder EY, Deitcher DL, Walsh C, Arnold-Aldea S, Hartwieg EA, Cepko CL. Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. *Cell*. (1992) 68:33–51. doi: 10.1016/0092-8674(92)90204-P
28. Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn A, Nordborg C, Peterson DA, et al. Neurogenesis in the adult human hippocampus. *Nat Med*. (1998) 4:1313–7. doi: 10.1038/3305
29. Schwartz PH, Bryant PJ, Fuja TJ, Su H, O'Dowd DK, Klassen H. Isolation and characterization of neural progenitor cells from post-mortem human cortex. *J Neurosci Res*. (2003) 74:838–51. doi: 10.1002/jnr.10854
30. Pagano SF, Impagnatiello F, Girelli M, Cova L, Grioni E, Onofri M, et al. Isolation and characterization of neural stem cells from the adult human olfactory bulb. *Stem Cells*. (2000) 18:295–300. doi: 10.1634/stemcells.18-4-295
31. Carpenter MK, Cui X, Hu Z, Jackson J, Sherman S, Seiger A, et al. *In vitro* expansion of a multipotent population of human neural progenitor cells. *Exp Neurol*. (1999) 158:265–78. doi: 10.1006/exnr.1999.7098
32. Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, et al. Direct isolation of human central nervous system stem cells. *PNAS*. (2000) 97:14720–5. doi: 10.1073/pnas.97.26.14720
33. Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, et al. Stem cell properties of human dental pulp stem cells. *J Dent Res*. (2002) 81:531–5. doi: 10.1177/154405910208100806
34. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, et al. SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci USA*. (2003) 100:5807–12. doi: 10.1073/pnas.0937635100
35. Sonoyama W, Liu Y, Yamaza T, Tuan RS, Wang S, Shi S, et al. Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. *J Endod*. (2008) 34:166–71. doi: 10.1016/j.joen.2007.11.021
36. Yalvac ME, Ramazanoglu M, Rizvanov AA, Sahin F, Bayrak OF, Salli U, et al. Isolation and characterization of stem cells derived from human third molar tooth germs of young adults: implications in neo-vascularization, osteo-, adipo- and neurogenesis. *Pharmacogenomics J*. (2010) 10:105–13. doi: 10.1038/tpj.2009.40
37. Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet*. (2004) 364:149–55. doi: 10.1016/S0140-6736(04)16627-0
38. Park JC, Kim JC, Kim YT, Choi SH, Cho KS, Im GI, et al. Acquisition of human alveolar bone-derived stromal cells using minimally irrigated implant osteotomy: *in vitro* and *in vivo* evaluations. *J Clin Periodontol*. (2012) 39:495–505. doi: 10.1111/j.1600-051X.2012.01865.x
39. Tang L, Li N, Xie H, Jin Y. Characterization of mesenchymal stem cells from human normal and hyperplastic gingiva. *J Cell Physiol*. (2011) 226:832–42. doi: 10.1002/jcp.22405
40. Morsczeck C, Gotz W, Schierholz J, Zeilhofer F, Kuhn U, Mohl C, et al. Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. *Matrix Biol*. (2005) 24:155–65. doi: 10.1016/j.matbio.2004.12.004
41. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. (2006) 8:315–7. doi: 10.1080/14653240600855905
42. Shi S, Gronthos S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J Bone Miner Res*. (2003) 18:696–704. doi: 10.1359/jbmr.2003.18.4.696
43. Shi S, Robey PG, Gronthos S. Comparison of human dental pulp and done marrow stromal stem cells by cDNA microarray analysis. *Bone*. (2001) 29:532–9. doi: 10.1016/S8756-3282(01)00612-3
44. Zhang W, Walboomers XF, Van Kuppevelt TH, Daamen WF, Van Damme PA, Bian Z, et al. *In vivo* evaluation of human dental pulp stem cells differentiated towards multiple lineages. *J Tissue Eng Regen Med*. (2008) 2:117–25. doi: 10.1002/term.71
45. Govindasamy V, Abdullah AN, Ronald VS, Musa S, Ab Aziz ZA, Zain RB, et al. Inherent differential propensity of dental pulp stem cells derived from human deciduous and permanent teeth. *J Endod*. (2010) 36:1504–15. doi: 10.1016/j.joen.2010.05.006
46. Janebodin K, Horst OV, Ieronimakis N, Balasundaram G, Reesukumal K, Pratumvinit B, et al. Isolation and characterization of neural crest-derived stem cells from dental pulp of neonatal mice. *PLoS ONE*. (2011) 6:e27526. doi: 10.1371/journal.pone.0027526
47. Chai Y, Jiang X, Ito Y, Bringas P, Han J, Rowitch DH, et al. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development*. (2000) 127:1671–9.
48. Yamazaki H, Tsuneto M, Yoshino M, Yamamura K, Hayashi S. Potential of dental mesenchymal cells in developing teeth. *Stem Cells*. (2007) 25:78–87. doi: 10.1634/stemcells.2006-0360
49. Kaukua N, Shahidi MK, Konstantinidou C, Dyachuk V, Kaucka M, Furlan A, et al. Glial origin of mesenchymal stem cells in a tooth model system. *Nature*. (2014) 513:551–4. doi: 10.1038/nature13536
50. Arthur A, Rychkov G, Shi S, Koblar SA, Gronthos S. Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. *Stem Cells*. (2008) 26:1787–95. doi: 10.1634/stemcells.2007-0979
51. Kiraly M, Porcsalmy B, Pataki A, Kadar K, Jelitai M, Molnar B, et al. Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons. *Neurochem Int*. (2009) 55:323–32. doi: 10.1016/j.neuint.2009.03.017
52. Gronthos S, Simmons PJ. The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions *in vitro*. *Blood*. (1995) 85:929–40.
53. Pan W, Kremer KL, Kaidonis X, Ludlow VE, Rogers ML, Xie J, et al. Characterization of p75 neurotrophin receptor expression in human dental pulp stem cells. *Int J Dev Neurosci*. (2016) 53:90–8. doi: 10.1016/j.ijdevneu.2016.07.007
54. Hilkens P, Gervois P, Fanton Y, Vanormelingen J, Martens W, Struys T, et al. Effect of isolation methodology on stem cell properties and multilineage differentiation potential of hDPSCs. *Cell Tissue Res*. (2013) 353:65–78. doi: 10.1007/s00441-013-1630-x

55. Liu L, Wei X, Ling J, Wu L, Xiao Y. Expression pattern of Oct-4, Sox2, and c-Myc in the primary culture of human dental pulp derived cells. *J Endod*. (2011) 37:466–72. doi: 10.1016/j.joen.2010.12.012
56. Wilson R, Urraca N, Skobowiat C, Hope KA, Miravalle L, Chamberlin R, et al. Assessment of the tumorigenic potential of spontaneously immortalized and hTERT-immortalized cultured dental pulp stem cells. *Stem Cells Transl Med*. (2015) 4:905–12. doi: 10.5966/sctm.2014-0196
57. Davidson RM. Neural form of voltage-dependent sodium current in human cultured dental pulp cells. *Archs Oral Biology*. (1994) 39:613–20. doi: 10.1016/0003-9969(94)90137-6
58. Ullah I, Subbarao RB, Kim EJ, Bharti D, Jang SJ, Park JS, et al. *In vitro* comparative analysis of human dental stem cells from a single donor and its neuronal differentiation potential evaluated by electrophysiology. *Life Sci*. (2016) 154:39–51. doi: 10.1016/j.lfs.2016.04.026
59. Kiraly M, Kadar K, Horvathy DB, Nardai P, Racz GZ, Lacza Z, et al. Integration of neuronally predifferentiated human dental pulp stem cells into rat brain *in vivo*. *Neurochem Int*. (2011) 59:371–81. doi: 10.1016/j.neuint.2011.01.006
60. Arthur A, Shi S, Zannettino AC, Fujii N, Gronthos S, Koblar SA. Implanted adult human dental pulp stem cells induce endogenous axon guidance. *Stem Cells*. (2009) 27:2229–37. doi: 10.1002/stem.138
61. Kanafi M, Majumdar D, Bhonde R, Gupta P, Datta I. Midbrain cues dictate differentiation of human dental pulp stem cells towards functional dopaminergic neurons. *J Cell Physiol*. (2014) 229:1369–77. doi: 10.1002/jcp.24570
62. Singh M, Kakkar A, Sharma R, Kharbanda OP, Monga N, Kumar M, et al. Synergistic effect of BDNF and FGF2 in efficient generation of functional dopaminergic neurons from human mesenchymal stem cells. *Sci Rep*. (2017) 7:10378. doi: 10.1038/s41598-017-11028-z
63. Gonmanee T, Thonabulsombat C, Vongsavan K, Sritanaudomchai H. Differentiation of stem cells from human deciduous and permanent teeth into spiral ganglion neuron-like cells. *Arch Oral Biol*. (2018) 88:34–41. doi: 10.1016/j.archoralbio.2018.01.011
64. Jung J, Kim JW, Moon HJ, Hong JY, Hyun JK. Characterization of neurogenic potential of dental pulp stem cells cultured in xeno/serum-free condition: *in vitro* and *in vivo* assessment. *Stem Cells Int*. (2016) 2016:6921097. doi: 10.1155/2016/6921097
65. Gervois P, Struys T, Hilkens P, Bronckaers A, Ratajczak J, Politis C, et al. Neurogenic maturation of human dental pulp stem cells following neurosphere generation induces morphological and electrophysiological characteristics of functional neurons. *Stem Cells Dev*. (2015) 24:296–311. doi: 10.1089/scd.2014.0117
66. Osathanon T, Sawangmake C, Nowwarote N, Pavasant P. Neurogenic differentiation of human dental pulp stem cells using different induction protocols. *Oral Dis*. (2014) 20:352–8. doi: 10.1111/odi.12119
67. Pisciotta A, Bertoni L, Riccio M, Mapelli J, Bigiani A, La Noce M, et al. Use of a 3D floating sphere culture system to maintain the neural crest-related properties of human dental pulp stem cells. *Front Physiol*. (2018) 9:547. doi: 10.3389/fphys.2018.00547
68. Yang KL, Chen MF, Liao CH, Pang CY, Lin PY. A simple and efficient method for generating Nurr1-positive neuronal stem cells from human wisdom teeth (tNSC) and the potential of tNSC for stroke therapy. *Cytotherapy*. (2009) 11:606–17. doi: 10.1080/14653240902806994
69. Leong WK, Henshall TL, Arthur A, Kremer KL, Lewis MD, Helps SC, et al. Human adult dental pulp stem cells enhance poststroke functional recovery through non-neural replacement mechanisms. *Stem Cells Transl Med*. (2012) 1:177–87. doi: 10.5966/sctm.2011-0039
70. Nosrat IV, Smith CA, Mullally P, Olson L, Nosrat CA. Dental pulp cells provide neurotrophic support for dopaminergic neurons and differentiate into neurons *in vitro*; implications for tissue engineering and repair in the nervous system. *Eur J Neurosci*. (2004) 19:2388–98. doi: 10.1111/j.0953-816X.2004.03314.x
71. Nito C, Sowa K, Nakajima M, Sakamoto Y, Suda S, Nishiyama Y, et al. Transplantation of human dental pulp stem cells ameliorates brain damage following acute cerebral ischemia. *Biomed Pharmacother*. (2018) 108:1005–14. doi: 10.1016/j.biopha.2018.09.084
72. Hill WD, Hess DC, Martin-Studdard A, Carothers JJ, Zheng J, Hale D, et al. SDF-1 (CXCL12) is upregulated in the ischemic penumbra following stroke association with bone marrow cell homing to injury. *J Neuropathol Exp Neurol*. (2004) 63:84–96. doi: 10.1093/jnen/63.1.84
73. Winderlich JN, Kremer KL, Koblar SA. Adult human dental pulp stem cells promote blood-brain barrier permeability through vascular endothelial growth factor-a expression. *J Cereb Blood Flow Metab*. (2016) 36:1087–97. doi: 10.1177/0271678X15608392
74. Shen LH, Li Y, Chen J, Zacharek A, Gao Q, Kapke A, et al. Therapeutic benefit of bone marrow stromal cells administered 1 month after stroke. *J Cereb Blood Flow Metab*. (2007) 27:6–13. doi: 10.1038/sj.jcbfm.9600311
75. Song M, Lee JH, Bae J, Bu Y, Kim EC. Human dental pulp stem cells are more effective than human bone marrow-derived mesenchymal stem cells in cerebral ischemic injury. *Cell Transplant*. (2017) 26:1001–16. doi: 10.3727/096368916X694391
76. Sowa K, Nito C, Nakajima M, Suda S, Nishiyama Y, Sakamoto Y, et al. Impact of dental pulp stem cells overexpressing hepatocyte growth factor after cerebral ischemia/reperfusion in rats. *Mol Ther Methods Clin Dev*. (2018) 10:281–90. doi: 10.1016/j.omtm.2018.07.009
77. Bronckaers A, Hilkens P, Fanton Y, Struys T, Gervois P, Politis C, et al. Angiogenic properties of human dental pulp stem cells. *PLoS ONE*. (2013) 8:e71104. doi: 10.1371/journal.pone.0071104
78. Argaw AT, Gurfein BT, Zhang Y, Zameer A, John GR. VEGF-mediated disruption of endothelial CLN-5 promotes blood-brain barrier breakdown. *Proc Natl Acad Sci USA*. (2009) 106:1977–82. doi: 10.1073/pnas.0808698106
79. Shimamura M, Sato N, Oshima K, Aoki M, Kurinami H, Waguri S, et al. Novel therapeutic strategy to treat brain ischemia: overexpression of hepatocyte growth factor gene reduced ischemic injury without cerebral edema in rat model. *Circulation*. (2004) 109:424–31. doi: 10.1161/01.CIR.0000109496.82683.49
80. Zhang X, Zhou Y, Li H, Wang R, Yang D, Li B, et al. Intravenous administration of DPSCs and BDNF improves neurological performance in rats with focal cerebral ischemia. *Int J Mol Med*. (2018) 41:3185–94. doi: 10.3892/ijmm.2018.3517
81. Kumasaka A, Kanazawa K, Ohke H, Miura I, Miura Y. Post-ischemic intravenous administration of allogeneic dental pulp-derived neurosphere cells ameliorated outcomes of severe forebrain ischemia in rats. *Neurocrit Care*. (2017) 26:133–42. doi: 10.1007/s12028-016-0304-4
82. Matsui M, Kobayashi T, Tsutsui TW. CD146 positive human dental pulp stem cells promote regeneration of dentin/pulp-like structures. *Hum Cell*. (2018) 31:127–38. doi: 10.1007/s13577-017-0198-2
83. Sugiyama M, Iohara K, Wakita H, Hattori H, Ueda M, Matsushita K, et al. Dental pulp-derived CD31-/CD146- side population stem/progenitor cells enhance recovery of focal cerebral ischemia in rats.pdf>. *Tissue Eng A*. (2011) 17:1303–11. doi: 10.1089/ten.tea.2010.0306
84. Pisciotta A, Carnevale G, Meloni S, Riccio M, De Biasi S, Gibellini L, et al. Human dental pulp stem cells (hDPSCs): isolation, enrichment and comparative differentiation of two sub-populations. *BMC Dev Biol*. (2015) 15:14. doi: 10.1186/s12861-015-0065-x
85. Wada N, Menicanin D, Shi S, Bartold PM, Gronthos S. Immunomodulatory properties of human periodontal ligament stem cells. *J Cell Physiol*. (2009) 219:667–76. doi: 10.1002/jcp.21710
86. Tamaoki N, Takahashi K, Tanaka T, Ichisaka T, Aoki H, Takeda-Kawaguchi T, et al. Dental pulp cells for induced pluripotent stem cell banking. *J Dent Res*. (2010) 89:773–8. doi: 10.1177/0022034510366846
87. Yan X, Qin H, Qu C, Tuan RS, Shi S, Huang GT. iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. *Stem Cells Dev*. (2010) 19:469–80. doi: 10.1089/scd.2009.0314
88. Chang YC, Li WC, Twu NF, Li HY, Lo WL, Chang YL, et al. Induction of dental pulp-derived induced pluripotent stem cells in the absence of c-Myc for differentiation into neuron-like cells. *J Chin Med Assoc*. (2014) 77:618–25. doi: 10.1016/j.jcma.2014.08.009
89. Lujan E, Chanda S, Ahlenius H, Sudhof TC, Wernig M. Direct conversion of mouse fibroblasts to self-renewing, tripotent neural precursor cells. *Proc Natl Acad Sci USA*. (2012) 109:2527–32. doi: 10.1073/pnas.1121003109
90. Han DW, Tapia N, Hermann A, Hemmer K, Hoing S, Arauzo-Bravo MJ, et al. Direct reprogramming of fibroblasts into neural stem cells by defined factors. *Cell Stem Cell*. (2012) 10:465–72. doi: 10.1016/j.stem.2012.02.021
91. Ring KL, Tong LM, Balestra ME, Javier R, Andrews-Zwilling Y, Li G, et al. Direct reprogramming of mouse and human fibroblasts into multipotent

neural stem cells with a single factor. *Cell Stem Cell*. (2012) 11:100–9. doi: 10.1016/j.stem.2012.05.018
92. Thier M, Worsdorfer P, Lakes YB, Gorris R, Herms S, Opitz T, et al. Direct conversion of fibroblasts into stably expandable neural stem cells. *Cell Stem Cell*. (2012) 10:473–9. doi: 10.1016/j.stem.2012.03.003
93. Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Sudhof TC, Wernig M. Direct conversion of fibroblasts to functional neurons by defined factors. *Nature*. (2010) 463:1035–41. doi: 10.1038/nature08797
94. Pang ZP, Yang N, Vierbuchen T, Ostermeier A, Fuentes DR, Yang TQ, et al. Induction of human neuronal cells by defined transcription factors. *Nature*. (2011) 476:220–3. doi: 10.1038/nature10202
95. Marro S, Pang ZP, Yang N, Tsai MC, Qu K, Chang HY, et al. Direct lineage conversion of terminally differentiated hepatocytes to functional neurons. *Cell Stem Cell*. (2011) 9:374–82. doi: 10.1016/j.stem.2011.09.002
96. Chanda S, Ang CE, Davila J, Pak C, Mall M, Lee QY, et al. Generation of induced neuronal cells by the single reprogramming factor ASCL1. *Stem Cell Rep*. (2014) 3:282–96. doi: 10.1016/j.stemcr.2014.05.020
97. Wapinski OL, Vierbuchen T, Qu K, Lee QY, Chanda S, Fuentes DR, et al. Hierarchical mechanisms for direct reprogramming of fibroblasts to neurons. *Cell*. (2013) 155:621–35. doi: 10.1016/j.cell.2013.09.028
98. Bar-Nur O, Verheul C, Sommer AG, Brumbaugh J, Schwarz BA, Lipchina I, et al. Lineage conversion induced by pluripotency factors involves transient passage through an iPSC stage. *Nat Biotechnol*. (2015) 33:761–8. doi: 10.1038/nbt.3247
99. Maza I, Caspi I, Zviran A, Chomsky E, Rais Y, Viukov S, et al. Transient acquisition of pluripotency during somatic cell transdifferentiation with iPSC reprogramming factors. *Nat Biotechnol*. (2015) 33:769–74. doi: 10.1038/nbt.3270
100. Maucksch C, Firmin E, Butler-Munro C, Montgomery JM, Dottori M, Connor B. Non-viral generation of neural precursor-like cells from adult human fibroblasts. *J Stem Cells Regenerat Med*. (2012) 8:162–70.
101. Hemmer K, Zhang M, van Wullen T, Sakalem M, Tapia N, Baumuratov A, et al. Induced neural stem cells achieve long-term survival and functional integration in the adult mouse brain. *Stem Cell Reports*. (2014) 3:423–31. doi: 10.1016/j.stemcr.2014.06.017
102. Hallmann AL, Arauzo-Bravo MJ, Zerfass C, Senner V, Ehrlich M, Psathaki OE, et al. Comparative transcriptome analysis in induced neural stem cells reveals defined neural cell identities *in vitro* and after transplantation into the adult rodent brain. *Stem Cell Res*. (2016) 16:776–81. doi: 10.1016/j.scr.2016.04.015
103. Sterneckert J, Hoing S, Scholer HR. Concise review: Oct4 and more: the reprogramming expressway. *Stem Cells*. (2012) 30:15–21. doi: 10.1002/stem.765
104. Mitchell R, Szabo E, Shapovalova Z, Aslostovar L, Makondo K, Bhatia M. Molecular evidence for OCT4-induced plasticity in adult human fibroblasts required for direct cell fate conversion to lineage specific progenitors. *Stem Cells*. (2014) 32:2178–87. doi: 10.1002/stem.1721
105. Mitchell RR, Szabo E, Benoit YD, Case DT, Mechael R, Alamilla J, et al. Activation of neural cell fate programs toward direct conversion of adult human fibroblasts into tri-potent neural progenitors using OCT-4. *Stem Cells Dev*. (2014) 23:1937–46. doi: 10.1089/scd.2014.0023
106. Liao W, Huang N, Yu J, Jares A, Yang J, Zieve G, et al. Direct conversion of cord blood CD34+ cells into neural stem cells by OCT4. *Stem Cells Transl Med*. (2015) 4:755–63. doi: 10.5966/sctm.2014-0289
107. Lee JH, Mitchell RR, McNicol JD, Shapovalova Z, Laronde S, Tanasijevic B, et al. Single transcription factor conversion of human blood fate to NPCs with CNS and PNS developmental capacity. *Cell Rep*. (2015) 11:1367–76. doi: 10.1016/j.celrep.2015.04.056
108. Giorgetti A, Marchetto MCN, Li M, Yu D, Fazzina R, Mu Y, et al. Cord blood-derived neuronal cells by ectopic expression of Sox2 and cMyc. *PNAS*. (2012) 109:12556–61. doi: 10.1073/pnas.1209523109
109. Castano J, Menendez P, Bruzos-Cidon C, Straccia M, Sousa A, Zabaleta L, et al. Fast and efficient neural conversion of human hematopoietic cells. *Stem Cell Rep*. (2014) 3:1118–31. doi: 10.1016/j.stemcr.2014.10.008
110. Maucksch C, Jones KS, Connor B. Concise review: the involvement of SOX2 in direct reprogramming of induced neural stem/precursor cells. *Stem Cells Transl Med*. (2013) 2:579–83. doi: 10.5966/sctm.2012-0179
111. Hanna J, Saha K, Pando B, van Zon J, Lengner CJ, Creyghton MP, et al. Direct cell reprogramming is a stochastic process amenable to acceleration. *Nature*. (2009) 462:595–601. doi: 10.1038/nature08592
112. Yoo AS, Sun AX, Li L, Shcheglovitov A, Portmann T, Li Y, et al. MicroRNA-mediated conversion of human fibroblasts to neurons. *Nature*. (2011) 476:228–31. doi: 10.1038/nature10323
113. Han YC, Lim Y, Duffieldl MD, Li H, Liu J, Abdul Manaph NP, et al. Direct reprogramming of mouse fibroblasts to neural stem cells by small molecules. *Stem Cells Int*. (2016) 2016:4304916. doi: 10.1155/2016/4304916
114. Zheng J, Choi KA, Kang PJ, Hyeon S, Kwon S, Moon JH, et al. A combination of small molecules directly reprograms mouse fibroblasts into neural stem cells. *Biochem Biophys Res Commun*. (2016) 476:42–8. doi: 10.1016/j.bbrc.2016.05.080
115. Liu L, Wu L, Wei X, Ling J. Induced overexpression of Oct4A in human dental pulp cells enhances pluripotency and multilineage differentiation capability. *Stem Cells Dev*. (2015) 24:962–72. doi: 10.1089/scd.2014.0388
116. Liu P, Cai J, Dong D, Chen Y, Liu X, Wang Y, et al. Effects of SOX2 on proliferation, migration and adhesion of human dental pulp stem cells. *PLoS ONE*. (2015) 10:e0141346. doi: 10.1371/journal.pone.0141346
117. Yao H, Gao M, Ma J, Zhang M, Li S, Wu B, et al. Transdifferentiation-induced neural stem cells promote recovery of middle cerebral artery stroke rats. *PLoS ONE*. (2015) 10:e0137211. doi: 10.1371/journal.pone.0137211
118. Yuan T, Liao W, Feng N-H, Lou Y-L, Niu X, Zhang A-J, et al. Human induced pluripotent stem cell-derived neural stem cells survive, migrate, differentiate, and improve neurologic function in a rat model of middle cerebral artery occlusion. *Stem Cell Res Ther*. (2013) 4:1–10. doi: 10.1186/scrt224
119. Jin K, Xie L, Mao X, Greenberg MB, Moore A, Peng B, et al. Effect of human neural precursor cell transplantation on endogenous neurogenesis after focal cerebral ischemia in the rat. *Brain Res*. (2011) 1374:56–62. doi: 10.1016/j.brainres.2010.12.037
120. Mine Y, Tatarishvili J, Oki K, Monni E, Kokaia Z, Lindvall O. Grafted human neural stem cells enhance several steps of endogenous neurogenesis and improve behavioral recovery after middle cerebral artery occlusion in rats. *Neurobiol Dis*. (2013) 52:191–203. doi: 10.1016/j.nbd.2012.12.006
121. Kelly S, Bliss TM, Shah AK, Sun GH, Ma M, Foo WC, et al. Transplanted human fetal neural stem cells survive, migrate, and differentiate in ischemic rat cerebral cortex. *Proc Natl Acad Sci USA*. (2004) 101:11839–44. doi: 10.1073/pnas.0404474101
122. Chu K, Kim M, Park KI, Jeong SW, Park HK, Jung KH, et al. Human neural stem cells improve sensorimotor deficits in the adult rat brain with experimental focal ischemia. *Brain Res*. (2004) 1016:145–53. doi: 10.1016/j.brainres.2004.04.038
123. Darsalia V, Kallur T, Kokaia Z. Survival, migration and neuronal differentiation of human fetal striatal and cortical neural stem cells grafted in stroke-damaged rat striatum. *Eur J Neurosci*. (2007) 26:605–14. doi: 10.1111/j.1460-9568.2007.05702.x
124. Andres RH, Horie N, Slikker W, Keren-Gill H, Zhan K, Sun G, et al. Human neural stem cells enhance structural plasticity and axonal transport in the ischaemic brain. *Brain*. (2011) 134(Pt 6):1777–89. doi: 10.1093/brain/awr094
125. de la Rosa-Prieto C, Laterza C, Gonzalez-Ramos A, Wattananit S, Ge R, Lindvall O, et al. Stroke alters behavior of human skin-derived neural progenitors after transplantation adjacent to neurogenic area in rat brain. *Stem Cell Res Ther*. (2017) 8:59. doi: 10.1186/s13287-017-0513-6
126. Zhang P, Li J, Liu Y, Chen X, Lu H, Kang Q, et al. Human embryonic neural stem cell transplantation increases subventricular zone cell proliferation and promotes peri-infarct angiogenesis after focal cerebral ischemia. *Neuropathology*. (2011) 31:384–91. doi: 10.1111/j.1440-1789.2010.01182.x
127. Ryu S, Lee SH, Kim SU, Yoon BW. Human neural stem cells promote proliferation of endogenous neural stem cells and enhance angiogenesis in ischemic rat brain. *Neural Regen Res*. (2016) 11:298–304. doi: 10.4103/1673-5374.177739
128. Darsalia V, Allison SJ, Cusulin C, Monni E, Kuzdas D, Kallur T, et al. Cell number and timing of transplantation determine survival of human neural stem cell grafts in stroke-damaged rat brain. *J Cereb Blood Flow Metab*. (2011) 31:235–42. doi: 10.1038/jcbfm.2010.81
129. Takahashi K, Yasuhara T, Shingo T, Muraoka K, Kameda M, Takeuchi A, et al. Embryonic neural stem cells transplanted in middle cerebral artery occlusion model of rats demonstrated potent therapeutic effects,

compared to adult neural stem cells. *Brain Res*. (2008) 1234:172–82. doi: 10.1016/j.brainres.2008.07.086
130. Andsberg G, Kokaia Z, Bjorklund A, Lindvall O, Martinez-Serrano A. Amelioration of ischaemia-induced neuronal death in the rat striatum by NGF-secreting neural stem cells. *Eur J Neurosci*. (1998) 10:2026–36. doi: 10.1046/j.1460-9568.1998.00214.x
131. Zhu W, Mao Y, Zhao Y, Zhou LF, Wang Y, Zhu JH, et al. Transplantation of vascular endothelial growth factor-transfected neural stem cells into the rat brain provides neuroprotection after transient focal cerebral ischemia. *Neurosurgery*. (2005) 57:325–33. doi: 10.1227/01.NEU.0000166682.50272.BC
132. Park KI, Himes BT, Stieg PE, Tessler A, Fischer I, Snyder EY. Neural stem cells may be uniquely suited for combined gene therapy and cell replacement: evidence from engraftment of Neurotrophin-3-expressing stem cells in hypoxic-ischemic brain injury. *Exp Neurol*. (2006) 199:179–90. doi: 10.1016/j.expneurol.2006.03.016
133. Zhang ZH, Wang RZ, Wang RZ, Li GL, Wei JJ, Li ZJ, et al. Transplantation of neural stem cells modified by human neurotrophin-3 promotes functional recovery after transient focal cerebral ischemia in rats. *Neurosci Lett*. (2008) 444:227–30. doi: 10.1016/j.neulet.2008.08.049
134. Chen B, Gao XQ, Yang CX, Tan SK, Sun ZL, Yan NH, et al. Neuroprotective effect of grafting GDNF gene-modified neural stem cells on cerebral ischemia in rats. *Brain Res*. (2009) 1284:1–11. doi: 10.1016/j.brainres.2009.05.100
135. Zhu JM, Zhao YY, Chen SD, Zhang WH, Lou L, Jin X. Functional recovery after transplantation of neural stem cells modified by brain-derived neurotrophic factor in rats with cerebral ischaemia. *J Int Med Res*. (2011) 39:488–98. doi: 10.1177/147323001103900216
136. Chang DJ, Lee N, Choi C, Jeon I, Oh SH, Shin DA, et al. Therapeutic effect of BDNF-overexpressing human neural stem cells (HB1.F3.BDNF) in a rodent model of middle cerebral artery occlusion. *Cell Transplant*. (2013) 22:1441–52. doi: 10.3727/096368912X657323
137. Zhang J-J, Zhu J-J, Hu Y-B, Xiang G-H, Deng L-C, Wu F-Z, et al. Transplantation of bFGF-expressing neural stem cells promotes cell migration and functional recovery in rat brain after transient ischemic stroke. *Oncotarget*. (2017) 8:102067–77. doi: 10.18632/oncotarget.22155
138. Rosenblum S, Smith TN, Wang N, Chua JY, Westbroek E, Wang K, et al. BDNF pretreatment of human embryonic-derived neural stem cells improves cell survival and functional recovery after transplantation in hypoxic-ischemic stroke. *Cell Transplant*. (2015) 24:2449–61. doi: 10.3727/096368914X679354
139. Theus MH, Wei L, Cui L, Francis K, Hu X, Keogh C, et al. *In vitro* hypoxic preconditioning of embryonic stem cells as a strategy of promoting cell survival and functional benefits after transplantation into the ischemic rat brain. *Exp Neurol*. (2008) 210:656–70. doi: 10.1016/j.expneurol.2007.12.020
140. Wu W, Chen X, Hu C, Li J, Yu Z, Cai W. Transplantation of neural stem cells expressing hypoxia-inducible factor-1alpha (HIF-1alpha) improves behavioral recovery in a rat stroke model. *J Clin Neurosci*. (2010) 17:92–5. doi: 10.1016/j.jocn.2009.03.039
141. Sakata H, Niizuma K, Wakai T, Narasimhan P, Maier CM, Chan PH. Neural stem cells genetically modified to overexpress cu/zn-superoxide dismutase enhance amelioration of ischemic stroke in mice. *Stroke*. (2012) 43:2423–9. doi: 10.1161/STROKEAHA.112.656900
142. Shen SW, Duan CL, Chen XH, Wang YQ, Sun X, Zhang QW, et al. Neurogenic effect of VEGF is related to increase of astrocytes transdifferentiation into new mature neurons in rat brains after stroke. *Neuropharmacology*. (2016) 108:451–61. doi: 10.1016/j.neuropharm.2015.11.012
143. Rosenblum S, Wang N, Smith TN, Pendharkar AV, Chua JY, Birk H, et al. Timing of intra-arterial neural stem cell transplantation after hypoxia-ischemia influences cell engraftment, survival, and differentiation. *Stroke*. (2012) 43:1624–31. doi: 10.1161/STROKEAHA.111.637884
144. Kondziolka D, Wechsler L, Goldstein S, Meltzer C, Thulborn KR, Gebel J, et al. Transplantation of cultured human neuronal cells for patients with stroke. *Neurology*. (2000) 55:565–9. doi: 10.1212/WNL.55.4.565
145. Kondziolka D, Steinberg GK, Wechsler L, Meltzer CC, Elder E, Gebel J, et al. Neurotransplantation for patients with subcortical motor stroke: a phase 2 randomized trial. *J Neurosurg*. (2005) 103:38–45. doi: 10.3171/jns.2005.103.1.0038
146. Rabinovich SS, Seledtsov VI, Banul NV, Poveshchenko OV, Senyukov VV, Astrakov SV, et al. Cell therapy of brain stroke. *Cell Technol Biol Med*. (2005) 1:126–8. doi: 10.1007/s10517-005-0229-y
147. Chen L, Xi H, Huang H, Zhang F, Liu Y, Chen D, et al. Multiple cell transplantation based on an intraparenchymal approach for patients with chronic phase stroke. *Cell Transplant*. (2013) 22(Suppl. 1):S83–91. doi: 10.3727/096368913X672154
148. Qiao LY, Huang FJ, Zhao M, Xie JH, Shi J, Wang J, et al. A two-year follow-up study of cotransplantation with neural stem/progenitor cells and mesenchymal stromal cells in ischemic stroke patients. *Cell Transplant*. (2014) 23:65–72. doi: 10.3727/096368914X684961
149. Kalladka D, Sinden J, Pollock K, Haig C, McLean J, Smith W, et al. Human neural stem cells in patients with chronic ischaemic stroke (PISCES): a phase 1, first-in-man study. *Lancet*. (2016) 2016:1–10. doi: 10.1016/S0140-6736(16)30513-X

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.

Copyright © 2019 Gancheva, Kremer, Gronthos and Koblar. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Neurology

REVIEW
published: 25 June 2019
doi: 10.3389/fneur.2019.00656

Cell-Based Therapies for Stroke: Are We There Yet?

Mirja Krause[1,2]*, *Thanh G. Phan*[3], *Henry Ma*[3], *Christopher G. Sobey*[4] *and Rebecca Lim*[1,2,5]

[1] The Ritchie Centre, Hudson Institute of Medical Research, Melbourne, VIC, Australia, [2] Department of Obstetrics and Gynaecology, Monash University, Melbourne, VIC, Australia, [3] Department of Medicine, Monash University, Melbourne, VIC, Australia, [4] Department of Physiology, Anatomy and Microbiology, La Trobe University, Melbourne, VIC, Australia, [5] Australian Regenerative Medicine Institute, Monash University, Melbourne, VIC, Australia

Stroke is the second leading cause of death and physical disability, with a global lifetime incidence rate of 1 in 6. Currently, the only FDA approved treatment for ischemic stroke is the administration of tissue plasminogen activator (tPA). Stem cell clinical trials for stroke have been underway for close to two decades, with data suggesting that cell therapies are safe, feasible, and potentially efficacious. However, clinical trials for stroke account for <1% of all stem cell trials. Nevertheless, the resources devoted to clinical research to identify new treatments for stroke is still significant (53–64 million US$, Phase 1–4). Notably, a quarter of cell therapy clinical trials for stroke have been withdrawn (15.2%) or terminated (6.8%) to date. This review discusses the bottlenecks in delivering a successful cell therapy for stroke, and the cost-to-benefit ratio necessary to justify these expensive trials. Further, this review will critically assess the currently available data from completed stroke trials, the importance of standardization in outcome reporting, and the role of industry-led research in the development of cell therapies for stroke.

Keywords: stroke, clinical trial, stem cells, cell therapy, clinical trial costs

OPEN ACCESS

Edited by:
Olivier Detante,
Centre Hospitalier Universitaire de Grenoble, France

Reviewed by:
Cesar V. Borlongan,
University of South Florida, United States
Claire Rome,
Université Grenoble Alpes, France

***Correspondence:**
Mirja Krause
Mirja.krause@hudson.org.au

Specialty section:
This article was submitted to Stroke, a section of the journal Frontiers in Neurology

Received: *01 March 2019*
Accepted: *04 June 2019*
Published: *25 June 2019*

Citation:
Krause M, Phan TG, Ma H, Sobey CG and Lim R (2019) Cell-Based Therapies for Stroke: Are We There Yet? Front. Neurol. 10:656. doi: 10.3389/fneur.2019.00656

INTRODUCTION

Background

Stroke has a devastating effect on the society worldwide. In addition to its significant mortality rate of 50% as reported in 5-year survival studies (1), it affects as many as 1 in 6 people in their lifetimes, and is the leading cause of disability worldwide (2). A stroke results in a complex interplay of inflammation and repair with effects on neural, vascular, and connective tissue in and around the affected areas of the brain (3). Therefore, sequelae of stroke such as paralysis, chronic pain, and seizures can persist long term and prevent the patient from fully reintegrating into society. Stroke therefore remains the costliest healthcare burden as a whole (4). In 2012, the total cost of stroke in Australia was estimated to be about $5 billion with direct health care costs attributing to $881 million of the total (5).

Unfortunately, treatment options for stroke are still greatly limited. Intravenous recombinant tissue plasminogen activator (tPA) and endovascular thrombectomy (EVT) are currently the only effective treatments available for acute stroke. However, there is only a brief window of opportunity where they can be successfully applied. EVT is performed until up to 24 h of stroke onset (6), while tPA is applied within 4.5 h of stroke onset. Notably, the recent WAKE-UP (NCT01525290) (7) and EXTEND (NCT01580839) trials have shown that this therapeutic window can be safely extended to 9 h from stroke onset. Furthermore, advancements in acute stroke care and neurorehabilitation have shown to be effective in improving neurological function (8). However, there are no treatments

that offer restoration of function and as a result, many patients are left with residual deficits following a stroke. Cell-based therapies have shown promising results in animal models addressing the recovery phase following stroke (9). This is encouraging as currently, there are no approved treatment options addressing the reversal of neurological damages once a stroke has occurred (10).

The majority of data from animal studies and clinical trials demonstrate the therapeutic potential of stem cells in the restoration of central nervous system (CNS) function (11, 12), applicable to neurodegenerative diseases as well as traumatic brain injury. Transplanted stem cells were reportedly able to differentiate into neurons and glial cells, whilst supporting neural reconstruction and angiogenesis in the ischemic region of the brain (13). Previous work demonstrated the ability of mesenchymal stem cells (MSCs) to differentiate into neurons, astrocytes (14), endothelial cells (15, 16), and oligodendrocyte lineage cells (17) such as NG2-positive cells (18) *in vitro*, and undergo neuronal or glial differentiation *in vivo* (19). Bone marrow-derived mesenchymal stem cells (BMSCs) have shown potential to differentiate into endothelial cells *in vitro* (20). Additionally, both BMSCs and adipose stem cells (ASCs) have been shown to demonstrate neural lineage differentiation potential *in vitro* (21–23). Furthermore, stem cells are able to modulate multiple cell signaling pathways involved in endogenous neurogenesis, angiogenesis, immune modulation and neural plasticity, sometimes in addition to cell replacement (3). The delivery of stem cells from the brain, bone marrow, umbilical cord, and adipose tissue, have been reported to reduce infarct size and improve functional outcomes regardless of tissue source (9). While these were initially exciting reports, they raise the question as to the validity of the findings to date since these preclinical reports are almost uniformly positive. The absence of scientific skepticism and robust debate may in fact have negated progress in this field.

Cell-based therapies have been investigated as a clinical option since the 1990s. The first pilot stroke studies in 2005 investigated the safety of intracranial delivery of stem cells (including porcine neural stem cells) to patients with chronic basal ganglia infarcts or subcortical motor strokes (24, 25). However, since the publication of these reports, hundreds of preclinical studies have shown that a variety of cell types including those derived from non-neural tissues can enhance structural and functional recovery in stroke. Cell therapy trials, mainly targeted at small cohorts of patients with chronic stroke, completed in the 2000s, showed satisfactory safety profiles and suggestions of efficacy (10). Current treatments such as tPA and EVT only have a narrow therapeutic window, limited efficacy in severe stroke and may be accompanied by severe side effects. Specifically, the side effects of EVT include intracranial hemorrhage, vessel dissection, emboli to new vascular territories, and vasospasm (26). The benefit of tPA for patients with a severe stroke with a large artery occlusion can vary significantly (27). This is mainly due to the failure (<30%) of early recanalisation of the occlusion. Thus, despite the treatment options stroke is still a major cause of mortality and morbidity, and there is need for new and improved therapies.

Stem cells have been postulated to significantly extend the period of intervention and target subacute as well as the chronic phase of stroke. Numerous neurological disorders such as Parkinson's disease (12, 28), Alzheimer's disease (29), age-related macular degeneration (30), traumatic brain injury (31), and malignant gliomas (32) have been investigated for the applicability of stem cell therapy. These studies have partly influenced the investigation of stem cell therapies for stroke. A small fraction of stem cell research has been successfully translated to clinical trials. As detailed in **Table 1**, most currently active trials use neuronal stem cells (NSCs), MSCs or BMSCs (35–37), including conditionally immortalized neural stem-cell line (CTX-DP) CTX0E03 (38), neural stem/progenitor cells (NSCs/NPSCs) (e.g., NCT03296618), umbilical cord blood (CoBis2, NCT03004976), adipose (NCT02813512), or amnion epithelial cells (hAECs, ACTRN 1261800076279) (39).

BOTTLENECKS AND CHALLENGES OF CELL THERAPIES

The development of a cell therapy for stroke is challenging for a number of reasons and these are detailed in **Table 1**. Each cell type requires testing for safety and efficacy to mitigate risks such as tumor formation. Identifying the ideal cell type for stroke has been hampered by the lack of data around clinical efficacy, as well as by the complex logistics and ethical concerns. The latter being a great hurdle for the use of fetal and embryonic stem cells in particular. The mechanisms of action of each cell type (i.e., cell replacement, growth factor secretion, and/or sequestration of inflammation) must be considered when choosing the appropriate route and timing of administration as these can directly influence treatment efficacy.

A critical translational consideration for stroke is the identification of the optimal route of administration. Different routes have been used in animal models for the transplantation of stem cells, including intracerebral (40), intracranial (41, 42), intranasal (43) or via stereotaxic infusion (44), and it is worth noting that all of these studies reported improvement in functional outcomes. Several different routes have been used in clinical trials (see **Table 2**); where the most common routes are intravenous infusion and intracerebral transplant. While different routes of administration have been compared in several reviews (58, 59), the optimal route has yet to be defined. Nevertheless, there is no evidence that a specific route of administration has significant effect on clinical efficacy (60–63).

Multiple factors influence the efficacy of cell transplantation and treatment outcomes, and these considerations may be specific to the cell type. Therefore, thorough investigation must be undertaken in order to develop the most effective and safest combination of cell type, dosage, route of administration and timing of delivery (10, 64). Stroke type and hence infarct size and location need to be considered to enable targeted treatment. The choice of cell type and delivery method will enable the homing of the cells to the site of injury and the level of efficacy that can be achieved. Furthermore, there are additional considerations if the cell therapy is administered to treat a subacute or a chronic stroke

TABLE 1 | Challenges and bottlenecks of stem cell therapy and clinical trials using stem cells (33, 34).

Challenges/Bottlenecks	
TRIAL RELATED CHALLENGES	
High costs	- *Increases with each trial phase*
Lengthy timelines	- *Average of 3.5–4 years per trial phase (estimated from data listed in **Table 2**)*
Difficulties in recruitment and retention of patients	- *Especially for acute stroke with a treatment time frame of <24 h; increased drop-out rates for longterm follow-up (>1 year after treatment)*
Insufficiencies in clinical research workforce (lack of specific training)	- *Disconnect between clinical research and medical care*
Strict regulations and admin barriers	- *Variations between different regulatory bodies; Designing a trial to answer a scientific question while considering the well-being of the patient and also adhere to regulations*
Complexity/Difficulties in maintaining and monitoring safety	- *Unexperienced personnel due to lack of specific training;*
Data collection and interpretation	- *Missing standardization*
Missing standardization of outcome reporting	- *Leads to biases; Data from several trials cannot be analyzed and compared*
TREATMENT AND THERAPY RELATED CHALLENGES	
Limited source of stem cells	- *Decrease in number/function of BMSCs in aged persons* - *Allogeneic MSCs need to be passaged to cover demand*
Optimal time frame for treatment	- *Decrease SC tropism toward brain with time* - *Mechanistic targets for cell therapy differ depending on time point of treatment* - *Insufficient cell amounts if autologous samples are used (depending on cell type)*
Limitations of SCs	- *Low yield* - *Heterogenous populations with difference in potential and efficacy*
Limitation in production processes	- *Lack of enabling technologies for cell-therapy bioprocessing at scale*
Adverse effects	- *Tumor formation; Immune rejection; Cells trapped in brain vessels or lung*

since the blood brain barrier will be less permeable compared to the acute stages of stroke (65). In these instances, the cell delivery would have to be intrathecal. This approach is more invasive and would require the patient to stop anti-thrombotic medication, thereby risking the recurrence of recurrence during this period.

In addition to the clinical challenges to applying a cell therapy for stroke, there are the manufacturing challenges. There are limited enabling technologies for manufacturing cells at a commercial scale (33). This process must be developed for each cell type, and optimized for the production of a high yielding, quality product. Development of cell manufacturing processes is exhaustive, expensive, and time-consuming. Even when the processes is optimized, the use of autologous BMSCs can be limited by the fact that they require *in vitro* expansion for a week or longer to obtain a therapeutic dose of cells (53). This eliminates the possibility of autologous treatment within a few hours of stroke onset. This might be the reason that most of the active trials to date have focused on allogeneic cell therapies as detailed in **Table 2**. Many cell lines are additionally immortalized and/or otherwise modified (e.g., MASTERS trial, ACTIsSIMA trial; see section Importance of Standardization in Outcome Reporting). This eliminates the variability in yield and potency as would be the case with autologous cell lines and is amenable to a streamlined production process with predetermined product quality and yield.

For successful trials reaching a phase where patients will be recruited at multiple sites on an international scale another challenge arises. The regulatory frameworks and authorities differ between countries. For example in the US clinical trials are regulated by the Food and Drug Administration (FDA) whereas in Australia Therapeutic Goods Administration (TGA) and in the UK it is The Medicines and Healthcare Products Regulatory Agency (MHRA). Their authorities, tasks and processing times differ from country to country. In an attempt to support the planning and implementation of international clinical research the NIH offers an online database, ClinRegs (https://clinregs.niaid.nih.gov), which compares the country-specific research regulatory information between 20 different countries (e.g., US, Canada, UK, China, India, Australia, and South Africa). Commonly discussed challenges for sponsors of multiregional trials are planning and trial design, data recording and analysis (statistics), clinical (medical standards of care, access to care, and qualification of personel), regulatory operational, and ethical practices (66). Important points to consider such as differences in patient populations, efficacy, clinical investigator sites was recently summarized by Shenoy (67). The review gives examples with a focus on China and the United States.

In current agreement trials follow the principles of Good Clinical Practice (GCP). These have their origin in the World Medical Association's Declaration of Helsinki, 1964 and were used as a basis for the guidelines published by the International Council for Harmonization (www.ich.org) in 1996. These guidelines have been adopted by several regulatory agencies from different countries. They are recently being updated (67).

CELL THERAPY CLINICAL TRIALS FOR STROKE

Several reports which have set out to analyze the outcomes of different cell therapy clinical trials for stroke have pointed out the risk for biases (3, 68, 69). The most commonly found was attrition bias (incomplete outcome data), reporting bias (selective reporting of results), and selection bias (random sequence generation, allocation concealment bias). There is, therefore, a need for updated guidelines and the implementation of standardization of recording and reporting data from cell therapy trials for stroke and likely, other conditions. The formation of clinical trial networks attempts to address some of the challenges of running a clinical trial. It offers support

TABLE 2 | Completed and active clinical trials using cell therapy to treat stroke.

	Trial number	Trial name	Current status	Study start and end date	Duration [y]	Phase	Sponsor	No. of participants	Cell type	Cell dose	Route	Time from stroke onset	References
COMPLETED													
1	NCT00152113		Compl	2005–2008	3	1	Research Hospital	5	Hematopoietic Stem Cell	5×10^6 CD34+/kg, 1×10^6 CD3+/kg	IV	Prophylactic	(45)
2	NCT00473057		Compl	2005–2011	6	1	University/ College	12	Autologous BMSCs	500 million	IA	90 days	
3	NCT00535197		Compl	2007–2012	5	1,2	University/ College	5	Autologous BMSCs	Max. 1×10^8	IA	<7 days	(46)
4	NCT01501773		Compl	2008–2011	3	2	Industry	120	Autologous BMSCs	30–500 million	IV	7–30 days	(44, 47)
5	NCT00761982		Compl	2008–2011	3	1,2	University Hospital	20	Autologous BMSCs	1.59×10^8	IA	5–9 days	(48)
6	NCT00950521		Compl	2009–2010	1	2	University Hospital	30	Hematopoietic CD34+ stem cells	2–8 million	ICb	6–60 months	(49)
7	NCT02425670	InVeSt	Compl	2009–2010	1	2	Research Institute	120	Autologous BMSCs	30–500 million	IV	7–29 days	(47)
8	NCT00859014		Compl	2009–2013	4	1	University/ College	25	Autologous BMSCs	10×10^6/kg	IV	24–72 h	(50)
9	NCT00875654	ISIS-HERMES	Compl	2010–2017	7	2	Hospital	31	Autologous mesenchymal stem cells	100 or 300 million	IV	<6 weeks	
10	NCT01287936	SanBio	Compl	2011–2015	4	1,2	Industry	18	Autologous modified Stromal Cells (SB623)	2.5, 5.0, or 10 million	IC	6–60 months	(51)
11	NCT01436487	MASTERS-1	Compl	2011–2015	4	2	Industry	134	Allogenic BMSCs (MultiStem)	400 or 1200 million	IV	24–48 h	(52, 53)
12	NCT01297413		Compl	2011–2017	6	1,2	Research Institute	20	Allogenic BMSCs	$0.5–1.5 \times 10^6$/kg	IV	>6 months	
13	NCT02117635	PISCES-II	Compl	2014–2016	2	2a	Industry	23	Allogenic NSCs (CTX DP)	20 million cells	ICb	<4 weeks	(38)
14	NCT01678534	AMASCIS-01	Compl	2014–2018	4	2	University Hospital	19	Allogenic ADSCs	1 million units/kg	IV	<2 weeks	(54)
15	NCT03080571		Compl	2015–2016	1	1	Industry	38	Autologous intra-arterial BM-MNCs	–	IA	0–15 days	
16	NCT02397018	CoBIS1	Compl	2015–2017	2	1	Research Institute	124	Allogeneic umbilical cord blood stem cells	$0.5–5 \times 10^7$/kg	IV	3–10 days	(55)
17	NCT02813512		Compl	2017–2018	1	1	Industry	3	Autologous ADSCs	–	ICb	>6 months	

(Continued)

TABLE 2 | Continued

	Trial number	Trial name	Current status	Study start and end date	Duration [y]	Phase	Sponsor	No. of participants	Cell type	Cell dose	Route	Time from stroke onset	References
ACTIVE													
1	NCT01151124	PISCES	Not recr	2010–2023	13	1,2	Industry	12	Allogenic NSCs (CTX DP)	2, 5, 10, or 20 million	ICb	6 months to 5 years	(38)
2	NCT01716481	STARTING-2	Recr	2012–2017	5	3	Industry	60	Autologous MSCs	1×10^6 cells/kg	IV	<90 days	(56)
3	NCT03296618		Not recr	2012–2018	6	1	Industry	18	NSCs (NSI-566)	1.2×10^7-8 $\times 10^7$	IC	3–24 months	
4	NCT02178657	IBIS	Recr	2014–2018	4	2	Research Institute	76	Autologous bone marrow mononuclear cells	2 or 5 $\times 10^6$/kg	IA	1–7 days	(57)
5	NCT02448641	ACTIsSIMA	Not recr	2016–2019	3	2	Industry	156	Autologous modified Stromal Cells (SB623)	2.5 or 5 million	IC	6–90 months	
6	NCT02795052	NEST	Recr	2016–2020	4	n.d.	Industry	300	Autologous BMSCs	–	IV, IN	>6 months	
7	NCT03371329		Recr	2017–2018	1	1	Hospital	12	Allogenic BMSCs	0.5, 1, 2 $\times 10^6$/kg	IV, IT	<72 h	
8	NCT03004976	CoBIS2	Recr	2017–2019	2	2	Research Institute	100	Allogeneic umbilical cord blood infusion	0.5–5 $\times 10^7$/kg	IV	3–10 days	
9	NCT03629275	PISCESIII	Recr	2018–2019	1	2b	Industry	110	Allogenic NSCs (CTX0E03)	20 million	ICb	6–12 months	
10	NCT03545607	MASTERS-2	Recr	2018–2020	2	3	Industry	300	Allogenic BMSCs (MultiStem)	1.2 billion	IV	24–36 h	(53)
11	NCT03570450	RESSTORE-1	Recr	2018–2020	2	1	University Hospital	15	ADSCs	1.1, 2.1, 2.5, 3.1 $\times 10^6$/kg	IV	24–48 h	
12	NCT02961504	TREASURE	Recr	2017–2020	3	2,3	Industry	220	Allogenic BMSCs, HLCM051 (MultiStem)	1.2 billion	IV	18–36 h	
13	ACTRN12618000076279	I-ACT	Recr	2019–2021	3	1	Research Institute	15	Allogenic hAECs	2, 4, 8, 16, 32 million/kg	IV	<24 h	(39)

IV, intravenous; IT, intrathecal; IA, intra-arterial; IC, intracrainial; ICb, intracerebral; IN, intranasal.

with trial coordination in general, site and data management, statistical analysis, patient recruitment in particular (70). This may especially benefit investigator-initiated trials where the trial team has limited experience with cell-based therapies.

To date, several pre-clinical and clinical trials indicate that cell-based therapies are generally safe, however the mechanisms through which the cells exert their therapeutic efficacy requires further investigation (25, 35, 36, 38, 44, 52, 53, 71). Agreeable safety profiles with functional improvements in patients with stroke have been reported for example after transplantation of neuronal cells differentiated from a teratocarcinoma cell line (24), immortalized human neural stem cell (38), transformed allogeneic BMSCs (44), and autologous BMSCs (72). The guidelines on the development of cell therapies for stroke, Stem Cell as an Emerging Paradigm in Stroke (STEPS) (73–75) (see Chapter 4 for more details on STEPS) outlined the need for long-term safety testing when the cells used are highly proliferative and easily differentiate. In a follow-up study from a trial published in 2005 (76). Lee et al. (71) analyzed long-term safety in an open-label, placebo controlled trial with 85 patients who suffered from ischemic stroke within the last 90 days (both trials have no NCT number). 5×10^7 autologous BMSCs were administered intravenously twice; 4 and 6 weeks after bone marrow aspiration. Patients were followed up for 5 years, and it was found that the SC transplant was safe. Another trial by Fang et al. (77) followed up their patients for 4 years (NCT01468064). This trial was a two-center, randomized, placebo-controlled phase I/IIa trial treating 18 patients suffering from acute cerebral infarct within 7 days of stroke onset. 5×10^6 cells/kg body weight BMSCs or endothelial progenitor cells (EPCs) were administered intravenously in 2 doses 4 and 5 weeks after bone marrow aspiration. This study also found that the treatment was safe. Long-term studies are currently still the exception, and more data is needed to understand if safety can be guaranteed for every cell type used in a potential therapy.

Most results reported up to date, demonstrate safety, but do not show sufficient data for clinical efficacy. The trial mentioned above by Fang et al. (77) for example reported no significant improvement in functional outcome. Recently, adjunctive therapies have been discussed to be a way to tackle low efficacy. The combination of a stem cell therapy with a drug that is able to improve neurogenesis and angiogenesis and/or reduce inflammation and hence working along the same pathways as stem cells do, could amplify efficacy. Several drugs with such biological activity have been already identified (78). For example, for the treatment of stroke G-CSF (granulocyte-colony stimulating factor) has been proposed as an adjunct therapy to stem cell treatment of human umbilical cord blood cells (79). Further preclinical studies are required for the translation of combinatorial therapies into the clinic.

A successful stem cell therapy for stroke must be safe, effective, applicable to a broad spectrum of stroke patients, and economically viable. Current trials differ in cell type, route, dose, and time of administration, as well as patient recruitment criteria. However, independent of the heterogeneity between trials, the most noteworthy adverse events such as seizures, headaches, and administration procedure-related events have been similar.

Patient selection is a critical component in reducing heterogeneity within any given trial cohort, however, this is particularly the case for stroke which has a heterogeneous clinical presentation. As such, an investigator may wish to exclude patients with certain comorbidities, and this should be incorporated into the trial design in order to limit heterogeneity and more accurately report on efficacy. Given that biological markers of stroke recovery are currently unavailable, the methods for ascertaining clinical improvement must be carefully chosen in order to provide meaningful data. The following section describes three industry-sponsored and two investigator-initiated cell therapy clinical trials as examples of trial design, execution and evaluation.

MASTERS and Treasure Trials

The Athersys Inc. funded MASTERS clinical trial (NCT01436487) was a phase 2, randomized, double-blinded, placebo controlled, dose-escalation trial, using allogeneic, bone marrow-derived, multipotent adult progenitor cells (MultiStem) (52). The MASTERS trial concluded in 2015, after treating 126 patients (134 enrolled) diagnosed with moderate to moderate-severe ischemic stroke (53). Patients were divided into three treatment groups: 1. Treatment 24–36 h after stroke onset with 400 million cells or placebo, 2. Treatment 24–36 h after stroke onset with 1.2 billion cells or placebo, and 3. Treatment 24–48 h after stroke onset with 1.2 billion cells or placebo. Sixty-five patients received cells and 61 patients received the placebo. No dose-limiting toxicity events or treatment-emergent adverse events (TEAE) were recorded. The investigators concluded that intravenously administered MultiStem was safe and well-tolerated, even at the higher dose. While changes in pro-inflammatory cytokines were noted, there was significant clinical improvement (at 90 days: mRS $\leq$ 2, Barthel Index $\geq$95, NIHSS $\geq$75% improvement). A major learning from the MASTERS trial is perhaps that of the logistics around cell manufacturing and provision of a living biologic within a relatively short treatment window. The MASTERS trial was initially designed with treatment within 24–36 h. However, the investigators ultimately changed their protocol to include a cohort at 24–48 h due to the logistical challenges in delivering cell products within the original timeframe (53). Upon conclusion of the MASTERS trial, the investigators concluded that the timing of the treatment is absolutely crucial, such that clinical efficacy was lost within the 36–48 h time window, thereby supporting an earlier intervention (53).

Indeed, this concept of an earlier intervention is currently being investigated by Athersys in the MASTERS-2 (NCT03545607) trial which commenced recruitment in 2018. The MASTERS-2 trial is a phase 3 quadruple-blind, randomized control trial to study the safety and efficacy of MultiStem, in patients suffering from acute ischemic stroke. The treatment (1.2 billion cells) are administered intravenously within 18–36 h of stroke onset. In addition to the MASTERS-2 trial, a different sponsor, Helios, is currently running a placebo controlled, multicentre phase 2/3 trial, TREASURE (NCT02961504) (80) where patients are recruited exclusively from Japan. The TREASURE trial also administers the cells intravenously within

18–36 h of stroke onset where MultiStem is administered at the same dose of 1.2 billion. Both trials are currently recruiting as of the preparation of this review and are estimated to conclude in 2020.

ACTIsSIMA Trial

The ACTIsSIMA trial is a SanBio sponsored Phase 2 double-blinded, sham-surgery controlled trial using allogeneic BMSCs transfected with a plasmid coding for a Notch I domain (SB623, NCT02448641). The SB623 cells are administered via stereotactic, intracranial injection to eligible patients suffering from chronic ischemic stroke. Preclinical studies indicate that these gene-edited allogeneic BMSCs surpassed the outcome of unmanipulated BMSCs in rodent stroke models (44) and were tested in a phase I/II dose escalation trial (NCT01287936) (36, 81). The open-label phase 1/2a safety trial enrolled 18 patients having suffered from a subcortical stroke within the past 6–60 months. Doses of 2.5, 5, or 10 million SB263 cells were administered via a stereotactic placement within the margin of the site of the infarct. The only TEAE recorded were related to the procedure, rather than the cells. Of the 18 recruited patients, 16 have completed the 12 months follow-up. Significant improvements were recorded in this study—European Stroke Scale: mean increase 6.88; National Institutes of Health Stroke Scale (NIHSS): mean decrease 2.00; Fugl-Meyer total score: mean increase 19.20; Fugl-Meyer motor function total score: mean increase 11.40. No changes on the modified Rankin Scale (mRS) were recorded (36, 51). Based on the conclusion that SB623 cells were safe and associated with an improvement in clinical outcome, the Phase 2 ACTIsSIMA trial commenced in 2016 where two cohorts of patients received either a dose of SB623 cells at 2.5 or 5 million cells, or a sham placebo will be randomized in a 1:1:1 ratio. The trial is expected to conclude in 2019.

Pisces Trial

Another industry sponsored trial to show promise in the cell therapy space for stroke is the ReNeuron sponsored trial investigating the potential of genetically modified human fetal cortical neuroepithelial cells for stroke (82). This cell therapy product is genetically modified using a retro-viral insertion of the modified growth factor c-mycERTAM (CTX0E03 DP) which overcomes the manufacturing problem of slow growing MSC through the transient expression of c-myc using a tamoxifen-estrogen receptor system (38, 81). The Pilot Investigation of Stem Cells in Stroke Trial (PISCES) was a Phase 1/2 open-label, dose-escalation safety trial (NCT01151124). The trial was based on preclinical studies in rats. Specifically, the injection of CTX0E03 DP was tested in a rat model of stroke induced by middle cerebral artery obstruction, where 450,000, 45,000 or 4,500 CTX0E03 DP were injected 4 weeks after MCOA (middle cerebral artery occlusion). Functional outcomes were assessed 2 weeks after cell implantation. Notably, significant functional improvement was only noted at the highest dose (41) and only when cells were delivered via an intraparenchymal injection, but not when delivered via intracerebroventricular injection which failed to achieve graft survival or functional improvement (42). Positive outcomes were associated with endogenous neurogenesis (83) and angiogenesis (84).

In PISCES, 11 patients suffering from ischemic stroke received a stereotactic ipsilateral putamen injection of CTX0E03 DP 6–60 months after stroke onset. Doses of 2, 5, 10, or 20 million cells were administered. As was the case for the MASTERS trial, the investigators struggled with the logistics of cell therapies and were able to treat only two patients at the highest dose. Safety was assessed over a 2-year period and no TEAE were recorded. Overall, the investigators concluded that this cell therapy is feasible and safe. Following this study PISCES II was launched in 2014 (NCT02117635). PISCES II is a phase 2a, open-label, safety trial where 20 million CTX0E03 DP cells were administered intracranially via stereotaxic neurosurgery. As of the preparation of this review, 23 patients have been recruited to PISCES II and received CTX0E03 DP cells. Results to date are available on the ReNeuron website (www.reneuron.com) but not compiled in a peer-reviewed publication. The 12-month follow-up showed an improvement in mRS and Barthel Index in 50 and 41% of enrolled patients, respectively. It was concluded that the treatment is safe and feasible, and a placebo-controlled, randomized phase 2b trial was started in 2018—PISCES III (NCT03629275)—where a larger number of patients will be recruited (110) and is estimated to conclude by late 2019. No further detailed analysis of the trial data is currently available.

I-ACT Trial

The I-ACT trial is an investigator-initiated single site trial. I-ACT is a open-label, dose escalation safety phase 1 study (39). Patients will receive an intravenous infusion of 2, 4, 8, 16, and 32 million cells per kg body weight of allogeneic placenta-derived hAECs within 24 h of stroke onset. The study is based on preclinical data in several mouse and marmoset models of cerebral ischemia demonstrating neuroprotection and the facilitation of mechanisms of repair and recovery (85). The trial is estimated to conclude mid 2020 including a 1-year follow up.

CoBIS2 Trial

The CoBIS2 trial is a continuation of CoBIS1. CoBIS1 was an investigator-initiated, multicentre, open-label, phase 1 safety study where 10 patients were recruited (37, 55, 86, 87). Allogeneic umbilical cord blood (UCB) containing 0.5–50 million total nucleated cells per kg bodyweight was administered intravenously 3–10 days post-stroke onset. Patients showed functional improvement 3 months past stroke onset. Fifty percent of patients showed improvement of one grade of mRS (mean mRS of 2.8 $\pm$ 0.9). NIHSS improved by at least 4 points (mean 5.9 $\pm$ 1.4). All patients demonstrated improvement in activities of daily living (Barthel Index mean increase 52.0 $\pm$ 24.7). The results conclude that the treatment is safe, feasible and suggestive of functional improvement. Based on these outcomes CoBIS2 was initiated. CoBIS2 is a multicenter, placebo controlled, randomized, double blinded, phase 2 study. CoBIS2 plans to recruit 100 participants. Doses of UCB and time frame of treatment remained the same. The trial is expected to conclude in 2020.

As can be seen from the trials above, the choice of route of administration is either intracranial or intravenous. And while some trials deliver a cell dosage based on bodyweight (I-ACT and CoBIS2), others deliver a fixed dose of cells. The completed trials preceding these current trials have provided clear results on treatment safety, but clinical efficacy remains uncertain. The MASTERS trial (52, 53), the InVeSt trial (44) and another phase 1 trial administering autologous BMSCs (88), did not report significant functional improvement. The PISCES trial however, linked improved neurological function in patients with chronic stroke to the treatment with genetically modified, immortalized human NSCs (38). This is certainly encouraging despite the absence of significant efficacy. Furthermore, the treatment in some cases (e.g., MASTERS trial) was linked with lower rates of mortality and TEAE (52).

IMPORTANCE OF STANDARDIZATION IN OUTCOME REPORTING

In order to assess the overall potential and combined outcomes of stem cell therapy for stroke, it is important to assess the outcomes across the various completed trials. The need for quality standards and documentation of study outcomes for pre-clinical and clinical stem cell research in stroke was identified a decade ago. In 2009, experts from academia and industry, members of the National Institute of Health (NIH) and the Food and Drug Administration (FDA) published their first meeting report, Stem Cell as an Emerging Paradigm in Stroke (STEPS) as a consensus-based guideline on the development of cell therapies for stroke, with a focus on the translation of pre-clinical studies and the design and conduct of early- and late-stage clinical trials for acute and chronic stroke (73). These guidelines have since been updated in 2011 (74) and 2014 (75).

Many of the problems identified in this exercise were also reported in several meta-analyses, in particular the lack of consistent reporting of safety and efficacy data for combinatorial (89) or mono-therapies (3), as well as heterogeneity in study design [e.g., single-arm (90)], a cell type [e.g., MSCs, (91)] or a stroke type [ischemic (69)]. Recently, Nagpal et al. (3) compared several different early-phase cell therapy trials while disregarding the cell type, treatment administration or study design differences. Overall, they concluded that the administration of different types of stem cells was feasible and safe. None of the adverse events reported could be ascertained to be related to the respective cell therapies. Nevertheless, additional research is still needed in order to demonstrate efficacy and enable market approval. As full recovery is unlikely, the outcome of any given stroke trial is dependent on the estimation of functional neurological improvement and structural recovery. Nagpal et al. drew conclusions based on changes to the Barthell index, the modified Rankin scale, and NIH Stroke Scale values across different trials, where despite indications of improvement, the magnitude of impact was small. Currently, it is impossible to draw conclusions with regard to optimal treatment protocols due to the limited data available from a small number of clinical studies comprised of small cohorts in a notoriously heterogeneous disease.

COST-TO-BENEFIT OF CELL THERAPY TRIALS

More than 6,000 trials are registered with www.clinicaltrials.gov employing different types of stem cells, addressing different types of disorders and/or diseases (excluding unknown status trials). Currently, 41.2% of these stem cell clinical trials are active and 44.5% are completed. And of the registered stem cell trials, only 0.73% address stroke, with 38.6% being completed, and 27.3% currently active. Interestingly, 25% of stroke trials have been withdrawn and another 9.1% were terminated or suspended. See **Figure 1** for details. Most trials were withdrawn or terminated due to low recruitment rates or lack or termination of funding. Strikingly, all completed trials are phase 1 and 2 trials, with only 1 in 5 trials moving on to the next phase. Of the 13 currently active trials only two trials have reached phase 3. These are the MASTERS-2 trial (Athersys Inc.) investigating a stem cell treatment for adults who have suffered an acute ischemic stroke, and the STARTING-2 trial (Samsung Medical Center, Korea) determining the efficacy of intravenous transplantation of autologous MSCs to treat acute ischemic stroke. On average clinical trials took 3.3 years from start to completion. From the data collected (see **Table 2**), one can estimate that a cell therapy for stroke will take >10 years to progress from phase 1 to phase 3 without considering any preceding preclinical research or process development.

Given this extremely long "gestation" for translating cell therapies for stroke, it is important to also consider the cost-to-benefit ratio. The US Department of Health & Human Services published a comprehensive report on clinical trial costs as part of their analysis of current barriers for drug development (92). The top three cost drivers of clinical trial expenditures were clinical procedure costs (15–22% of total), administrative staff costs (11–29% of total), and site monitoring costs (9–14% of total). Generally, costs increase with every trial phase: phase 1 on average being about US$ 4 million, phase 2 about US$ 13 million and phase 3 and 4 about US$ 20 million each. This totals to an average of US$ 57 million to take a therapeutic through its clinical trial stages. The costs of a trial depend largely on the therapeutic area being targeted. The most expensive clinical trials (phase 1–3) focus on pain and anesthesia US$ 71.3 million, ophthalmology US$ 49.8 million, and anti-infectives US$ 41.2 million. Trials on treatments focused on the CNS are estimated to cost US$ 37 million, and US$ 34.4 million on cardiovascular diseases. In order to understand if the costs of a trial are economically justifiable, one has to consider the financial costs that stroke has on a society, which was analyzed on an international level in 2004 (93). This study was done over a decade ago, and an update would certainly be necessary as the prevalence of stroke has continued to rise. In 2012, the total cost of stroke to Australia was estimated at AU$ 5 billion with health cost being AU$ 881 million and productivity cost being AU$ 3 billion (5). The costs of stroke to the UK health and social services in the same year were estimated at AU$ 5.2

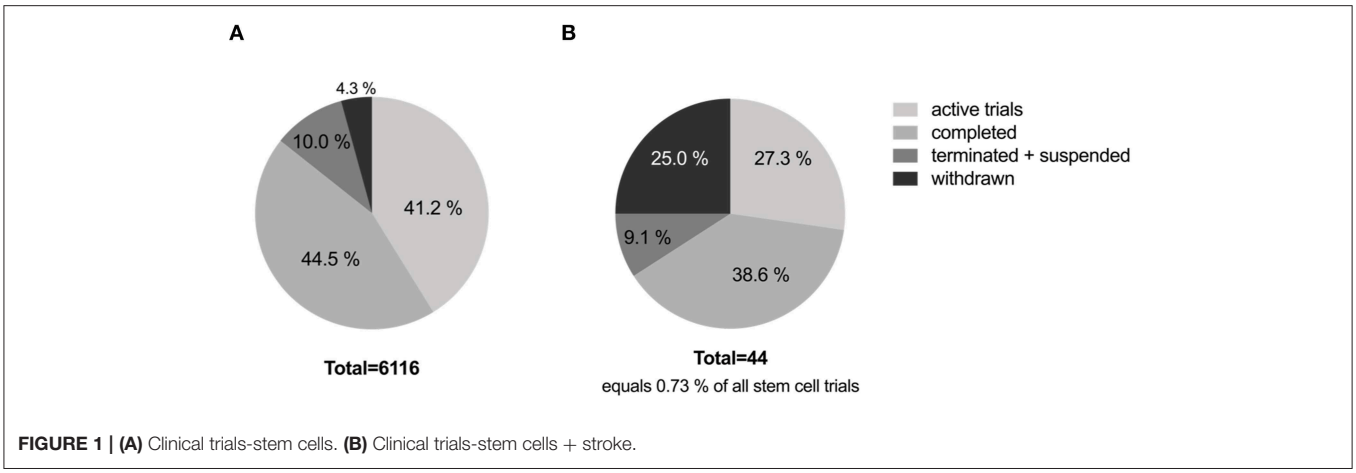


FIGURE 1 | (A) Clinical trials-stem cells. **(B)** Clinical trials-stem cells + stroke.

billion (£ 2.9 billion) (94). Efficacious stroke interventions are expected to significantly reduce the financial costs. However, this does not take into consideration the costs of stem cell-based therapies. This is despite attempts to assess the cost and benefits of medical research since the 1990's (95), with analysis of funds going into specific areas of medical research (e.g., stroke, cancer, and dementia) (94). Information on the actual costs of clinical trials in general or even specific clinical trials is scarce.

In 2017 the Australian Government published an economic evaluation of 25 clinical trials to assess the overall health and economic impact of investigator-initiated clinical trials (70). This report only included independent investigator-initiated trials that were part of clinical trial networks in Australia, in phase 2 and beyond. Included in this report were 7 trials conducted within the Australasian Stroke Trials Network between 2004 and 2014. The combined costs of 4 trials was estimated to be AU$ 32 million (excluding early phase trials, pilot and feasibility trials and observation studies). The gross economic benefit of these trials was estimated at AU$ 327 million. Thus, the benefit for these late-phase trials was estimated to be AU$ 10 per AU$ 1 invested. The report estimated a benefit of AU$ 5 for every AU$ 1 invested with a total gross benefit of AU$ 2 billion for all 25 trials analyzed, with a cumulative reduction in health service costs in the order of AU$ 580 million. Since none of the trials included in the report used any kind of cell therapy, an estimation of the benefit of cell-therapy clinical trials could not be made. However, it is clear that clinical trials are quantifiably beneficial and while the analysis covered a decade's worth of trials, it did not include any data from the early stage trial counterparts. This further emphasizes the length of time required for clinical development of any new treatment for stroke before clinical benefit is seen, let alone to see profit.

In 2018, the costs of producing for autologous cell therapies have been estimated to be US$ 94 per million cells (96). For a dose of 2 million cells per kg, assuming that a patient weighs 70 kg, the costs would be US$ 13,160 per dose. The costs of drug development influences the pricing of any clinical therapy, but several factors can also contribute to the cost, such as market size, competing products, the quality-adjusted life year (QALY), and what the consumer is willing to pay (97, 98). At the moment, there is no approved stem cell therapy for stroke. Stem cell therapies approved by the FDA include cord blood and a small number of cell lines (e.g., modified T-cells, chondrocytes, and fibroblasts). There are three different approved stem cell therapies on the market. The European commission approved a stem cell product, Alofisel (Takeda) in 2018, however FDA approval is still pending at the time of the writing of this review. Alofisel is an ASC treatment for perineal fistulas in patients with Crohn's disease. One course of treatment is US$ 61,000 (99). Another approved product is Holoclar (Holostem Terapie Avanzante), which was the first stem cell therapy to receive market authorization in the EU. Holoclar is comprised of corneal epithelial cells used to treat chemical burns of the eye, costing US$ 102,000 (99). TEMCELL (JCR Pharmaceuticals) is approved in Japan for the use of BMSCs to treat Graft vs. Host Disease, costing about US$ 170,000 per course (100). The price tags on these approved therapies can only hint at expected costs of cell therapies for stroke. Furthermore, every country has their own health care board deciding if a treatment would be covered by the national health care system, thus making it impossible to estimate the actual out-of-pocket costs a patient would incur. It is evident that a treatment which improves functional outcomes in stroke, as can be seen from other recent interventions, will have a significant benefit on health costs (5).

THE ROLE OF INDUSTRY-LED RESEARCH IN THE DEVELOPMENT OF CELL THERAPIES FOR STROKE

When assessing the success of clinical trials there is always the question if industry-led research is more successful than academic research. This has been critically analyzed and reviewed over the last decade as scientists and public are aware of the inherent risk of biases in industry funded studies (101–104). Recently, Lundh et al. (105) compared studies with and without industry funding from 75 papers on reported efficacy, conclusions and risk of bias. The papers selected were on primary research studies, empirical studies and randomized clinical trials (58 papers) focusing on drug and device development, but without a focus on a specific disease or type of therapy. Industry funded projects were found to demonstrate favorable efficacy

toward tested treatments, with less substantive conclusions. Over 60% of published findings from cell therapy clinical trials reported positive outcomes, with a trend toward a higher proportion of positive reports from industry funded trials (106). Expectedly, the more complete and detailed studies were published in higher impact factor journals where data are presumably subjected to a higher level of scrutiny.

Reporting according to CONSORT (107) or also SPIRIT (Standard Protocol Items: Recommendations for Interventional Trials) (108) identifies funding sources and possible conflicts of interest but are not without limits compared to more recent recommendations by Hakoum et al. (109) that specifically pay address the characteristics of funding of clinical trials. External influences exerted on research and clinical trials are difficult to trace and often remain unclear when the results are published at the end of a trial (104). Currently about 28.5% of all active, interventional clinical trials registered with www.clinicaltrials.gov are industry funded. In relation to current active, interventional trials focused on stem cell therapies for stroke, 66.7% are industry funded (**Table 2**). Late-stage trials (phase 2 and 3) are predominantly industry funded (58.3%) whereas early-stage trials (phase 1 and 1/2) are funded by other means (62.5%). This might be due to the fact that the later phases of clinical testing incur larger costs that are beyond the funding quanta of philanthropic or government bodies, or that promising late-stage trials may be of greater interest to industry.

CONCLUSION

It is clear that there are challenges in the use of cell therapies for stroke (**Table 1**) that remain to be addressed in the future. A major issue certainly is the need for standardized outcome reporting that is free of bias and enables comparison of different trials. Furthermore, optimized and more efficient bioprocesses need to be urgently developed to reduce the cost of production and in doing so, treatment costs. Most studies showed safety and feasibility for cell therapy for stroke independent of cell type and route of administration. However, there remains limited proof of efficacy. We and others will be watching closely for the outcomes of current stroke clinical trials utilizing cell therapies, as we await the evidence for clinical efficacy and impactful functional improvement that is desperately needed to spur this field ahead.

AUTHOR CONTRIBUTIONS

MK and RL drafted the manuscript. MK prepared all figures and tables. All authors provided input according to their expertise and discussed the manuscript.

FUNDING

This work was supported by the NHMRC CDF GNT1159277 (personal grant to RL) and the GNT1159277.

REFERENCES

1. Ingall T. Stroke-incidence, mortality, morbidity and risk. *J Insur Med.* (2004) 36:143–52.
2. Martínez-Garza D, Cantú-Rodríguez O, Jaime-Pérez J, Gutiérrez-Aguirre C, Góngora-Rivera J, Gómez-Almaguer D. Current state and perspectives of stem cell therapy for stroke. *Med Universit.* (2016) 18:169–80. doi: 10.1016/j.rmu.2016.07.005
3. Nagpal A, Choy FC, Howell S, Hillier S, Chan F, Hamilton-Bruce MA, et al. Safety and effectiveness of stem cell therapies in early-phase clinical trials in stroke: a systematic review and meta-analysis. *Stem Cell Res Ther.* (2017) 8:191. doi: 10.1186/s13287-017-0643-x
4. Teasell RW. Long-term sequelae of stroke: How should you handle stroke complications? *Can Fam Physician.* (1992) 38:381–8.
5. Foundation S. *The Economic Impact of Stroke in Australia*. (2013). Available online at: https://www2.deloitte.com/au/en/pages/economics/articles/economic-impact-stroke-australia.html (accessed April 30, 2019).
6. Nogueira RG, Jadhav AP, Haussen DC, Bonafe A, Budzik RF, Bhuva P, et al. Thrombectomy 6 to 24 hours after stroke with a mismatch between deficit and infarct. *N Engl J Med.* (2018) 378:11–21. doi: 10.1056/NEJMoa1706442
7. Thomalla G, Simonsen CZ, Boutitie F, Andersen G, Berthezene Y, Cheng B, et al. MRI-guided thrombolysis for stroke with unknown time of onset. *N Engl J Med.* (2018) 379:611–22. doi: 10.1056/NEJMoa1804355
8. Bushnell C, McCullough LD, Awad IA, Chireau MV, Fedder WN, Furie KL, et al. Guidelines for the prevention of stroke in women: a statement for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke.* (2014) 45:1545–88. doi: 10.1161/01.str.0000442009.06663.48
9. Savitz SI, Dinsmore JH, Wechsler LR, Rosenbaum DM, Caplan LR. Cell therapy for stroke. *NeuroRx.* (2004) 1:406–14. doi: 10.1602/neurorx.1.4.406
10. Kenmuir CL, Wechsler LR. Update on cell therapy for stroke. *Stroke Vasc Neurol.* (2017) 2:59–64. doi: 10.1136/svn-2017-000070
11. Lindvall O, Kokaia Z. Stem cells for the treatment of neurological disorders. *Nature.* (2006) 441:1094. doi: 10.1038/nature04960
12. Song CGY, Zhang ZH, Wu NX, Cao LC, Guo JY, Li QM, et al. Stem cells: a promising candidate to treat neurological disorders. *Neural Regener Res.* (2018) 13:1294–304. doi: 10.4103/1673-5374.235085
13. Ryu SS, Lee H, Kim SU, Yoon BW. Human neural stem cells promote proliferation of endogenous neural stem cells and enhance angiogenesis in ischemic rat brain. *Neural Regener Res.* (2016) 11, 298–304. doi: 10.4103/1673-5374.177739
14. Wislet-Gendebien S, Bruyère F, Hans G, Leprince P, Moonen G, Rogister B. Nestin-positive mesenchymal stem cells favour the astroglial lineage in neural progenitors and stem cells by releasing active BMP4. *BMC Neurosci.* (2004) 5:33. doi: 10.1186/1471-2202-5-33
15. Hess DC, Hill WD, Martin-Studdard A, Carroll J, Brailer J, Carothers J. Bone marrow as a source of endothelial cells and NeuN-expressing cells after stroke. *Stroke.* (2002) 33:1362–8. doi: 10.1161/01.STR.0000014925.09415.C3
16. Portalska KJ, Leferink A, Groen N, Fernandes H, Moroni L, van Blitterswijk C, et al. Endothelial differentiation of mesenchymal stromal cells. *PLoS ONE.* (2012) 7:e46842. doi: 10.1371/journal.pone.0046842
17. Steffenhagen CF, Dechant X, Oberbauer E, Furtner T, Weidner N, Küry P, et al. Mesenchymal stem cells prime proliferating adult neural progenitors toward an oligodendrocyte fate. *Stem Cells Develop.* (2011) 21:1838–51. doi: 10.1089/scd.2011.0137
18. Shen LH, Li Y, Chen J, Zhang J, Vanguri P, Borneman J, et al. Intracarotid transplantation of bone marrow stromal cells increases axon-myelin remodeling after stroke. *Neuroscience.* (2006) 137:393–9. doi: 10.1016/j.neuroscience.2005.08.092
19. Guzman R, Choi R, Gera A, De Los Angeles A, Andres RH, Steinberg GK. Intravascular cell replacement therapy for stroke. *Neurosurg Focus.* (2008) 24:E15. doi: 10.3171/FOC/2008/24/3-4/E14
20. Wang C, Li Y, Yang M, Zou Y, Liu H, Liang Z, et al. Efficient differentiation of bone marrow mesenchymal stem cells into endothelial cells *in vitro*. *Eur J Vasc Endovasc Surg.* (2018) 55:257–65. doi: 10.1016/j.ejvs.2017.10.012

21. Ashjian PH, Elbarbary AS, Edmonds B, DeUgarte D, Zhu M, Zuk PA, et al. *In vitro* differentiation of human processed lipoaspirate cells into early neural progenitors. *Plastic Reconstruct Surg.* (2003) 111:1922–31. doi: 10.1097/01.PRS.0000055043.62589.05
22. Cho KJ, Trzaska KA, Greco SJ, McArdle J, Wang FS, Ye JH, et al. Neurons derived from human mesenchymal stem cells show synaptic transmission and can be induced to produce the neurotransmitter substance P by interleukin-1α. *Stem Cells.* (2005) 23:383–91. doi: 10.1634/stemcells.2004-0251
23. Wislet-Gendebien S, Hans G, Leprince P, Rigo JM, Moonen G, Rogister B. Plasticity of cultured mesenchymal stem cells: switch from nestin-positive to excitable neuron-like phenotype. *Stem Cells.* (2005) 23:392–402. doi: 10.1634/stemcells.2004-0149
24. Kondziolka D, Steinberg GK, Wechsler L, Meltzer CC, Elder E, Gebel J, et al. Neurotransplantation for patients with subcortical motor stroke: a phase 2 randomized trial. *J Neurosurg.* (2005) 103:38–45. doi: 10.3171/jns.2005.103.1.0038
25. Savitz SI, Dinsmore J, Wu J, Henderson GV, Stieg P, Caplan LR. Neurotransplantation of fetal porcine cells in patients with basal ganglia infarcts: a preliminary safety and feasibility study. *Cerebrovasc Dis.* (2005) 20:101–7. doi: 10.1159/000086518
26. Behme D, Gondecki L, Fiethen S, Kowoll A, Mpotsaris A, Weber W. Complications of mechanical thrombectomy for acute ischemic stroke—a retrospective single-center study of 176 consecutive cases. *Neuroradiology.* (2014) 56:467–76. doi: 10.1007/s00234-014-1352-0
27. Bhatia R, Hill MD, Shobha N, Menon B, Bal S, Kochar P, et al. Low rates of acute recanalization with intravenous recombinant tissue plasminogen activator in ischemic stroke: real-world experience and a call for action. *Stroke.* (2010) 41:2254–8. doi: 10.1161/STROKEAHA.110.592535
28. Gandhi V, Burle S, Kosalge S. Stem cell therapy for Parkinson's disease: a review. *Pharma Tutor.* (2018) 6:1–8. doi: 10.29161/PT.v6.i6.2018.1
29. Hunsberger JG, Rao M, Kurtzberg J, Bulte JW, Atala A, LaFerla FM, et al. Accelerating stem cell trials for Alzheimer's disease. *Lancet Neurol.* (2016) 15:219–30. doi: 10.1016/S1474-4422(15)00332-4
30. Singh MS, MacLaren RE. Stem cell treatment for age-related macular degeneration: the ChallengesStem Cells for AMD treatment. *Investigat Ophthalmol Vis Sci.* (2018) 59:AMD78–82. doi: 10.1167/iovs.18-24426
31. Weston NM, Sun D. The potential of stem cells in treatment of traumatic brain injury. *Curr Neurol Neurosci Rep.* (2018) 18:1. doi: 10.1007/s11910-018-0812-z
32. Zhang Q, Xiang W, Yi DY, Xue BZ, Wen WW, Abdelmaksoud A, et al. Current status and potential challenges of mesenchymal stem cell-based therapy for malignant gliomas. *Stem Cell Res Ther.* (2018) 9:228. doi: 10.1186/s13287-018-0977-z
33. Ratcliffe E, Thomas RJ, Williams DJ. Current understanding and challenges in bioprocessing of stem cell-based therapies for regenerative medicine. *Br Med Bull.* (2011) 100:137–55. doi: 10.1093/bmb/ldr037
34. Bang OY, Kim EH, Cha JM, Moon GJ. Adult stem cell therapy for stroke: challenges and progress. *J Stroke.* (2016) 18:256. doi: 10.5853/jos.2016.01263
35. Kondziolka D, Wechsler L, Goldstein S, Meltzer C, Thulborn K, Gebel J, et al. Transplantation of cultured human neuronal cells for patients with stroke. *Neurology.* (2000) 55:565–9. doi: 10.1212/WNL.55.4.565
36. Steinberg GK, Kondziolka D, Wechsler LR, Lunsford LD, Coburn ML, Billigen JB, et al. Clinical outcomes of transplanted modified bone marrow–derived mesenchymal stem cells in stroke: a phase 1/2a study. *Stroke.* (2016) 47:1817–24. doi: 10.1161/STROKEAHA.116.012995
37. Laskowitz DT, Bennett ER, Durham RJ, Volpi JJ, Wiese JR, Frankel M, et al. Allogeneic umbilical cord blood infusion for adults with ischemic stroke: clinical outcomes from a phase I safety study. *Stem Cells Transl Med.* (2018) 7:521–9. doi: 10.1002/sctm.18-0008
38. Kalladka D, Sinden J, Pollock K, Haig C, McLean J, Smith W, et al. Human neural stem cells in patients with chronic ischaemic stroke (PISCES): a phase 1, first-in-man study. *Lancet.* (2016) 388:787–96. doi: 10.1016/S0140-6736(16)30513-X
39. Phan TG, Ma H, Lim R, Sobey CG, Wallace EM. Phase 1 trial of amnion cell therapy for ischemic stroke. *Front Neurol.* (2018) 9:198. doi: 10.3389/fneur.2018.00198
40. Chen J, Li Y, Wang L, Lu M, Zhang X, Chopp M. Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. *J Neurol Sci.* (2001) 189:49–57. doi: 10.1016/S0022-510X(01)00557-3
41. Stroemer P, Patel S, Hope A, Oliveira C, Pollock K, Sinden J. The neural stem cell line CTX0E03 promotes behavioral recovery and endogenous neurogenesis after experimental stroke in a dose-dependent fashion. *Neurorehabil Neural Repair.* (2009) 23:895–909. doi: 10.1177/1545968309335978
42. Smith EJ, Stroemer RP, Gorenkova N, Nakajima M, Crum WR, Tang E, et al. Implantation site and lesion topology determine efficacy of a human neural stem cell line in a rat model of chronic stroke. *Stem Cells.* (2012) 30:785–96. doi: 10.1002/stem.1024
43. Wei N, Yu SP, Gu X, Taylor TM, Song DX, Liu F, et al. Delayed intranasal delivery of hypoxic-preconditioned bone marrow mesenchymal stem cells enhanced cell homing and therapeutic benefits after ischemic stroke in mice. *Cell Transplant.* (2013) 22:977–91. doi: 10.3727/096368912X657251
44. Kameshwar Prasad D, Sharma A, Garg A, Mohanty S, Bhatnagar S, Sharat Johri DD, et al. Intravenous autologous bone marrow mononuclear stem cell therapy for ischemic stroke. *Stroke.* (2014) 45:3618–24. doi: 10.1161/STROKEAHA.114.007028
45. Dallas MH, Triplett B, Shook DR, Hartford C, Srinivasan A, Laver J, et al. Long-term outcome and evaluation of organ function in pediatric patients undergoing haploidentical and matched related hematopoietic cell transplantation for sickle cell disease. *Biol Blood Marrow Transplant.* (2013) 19:820–30. doi: 10.1016/j.bbmt.2013.02.010
46. Banerjee S, Bentley P, Hamady M, Marley S, Davis J, Shlebak A, et al. Intra-arterial immunoselected CD34+ stem cells for acute ischemic stroke. *Stem Cells Translat Med.* (2014) 3:1322–30. doi: 10.5966/sctm.2013-0178
47. Prasad K, Mohanty S, Bhatia R, Srivastava M, Garg A, Srivastava A, et al. Autologous intravenous bone marrow mononuclear cell therapy for patients with subacute ischaemic stroke: a pilot study. *Indian J Med Res.* (2012) 136:221.
48. Moniche F, Gonzalez AJ, Gonzalez-Marcos R, Carmona M, Piñero P, Espigado I, et al. Intra-arterial bone marrow mononuclear cells in ischemic stroke: a pilot clinical trial. *Stroke.* (2012) 43:2242–4. doi: 10.1161/STROKEAHA.112.659409
49. Mackie AR, Losordo DW. CD34-positive stem cells in the treatment of heart and vascular disease in human beings. *Texas Heart Inst J.* (2011) 38:474–85.
50. Savitz SI, Misra V, Kasam M, Juneja H, Cox CS Jr, Alderman S, et al. Intravenous autologous bone marrow mononuclear cells for ischemic stroke. *Ann Neurol.* (2011) 70:59–69. doi: 10.1002/ana.22458
51. Steinberg GK, Kondziolka D, Wechsler LR, Lunsford LD, Kim AS, Johnson JN, et al. Two-year safety and clinical outcomes in chronic ischemic stroke patients after implantation of modified bone marrow–derived mesenchymal stem cells (SB623): a phase 1/2a study. *J Neurosurg.* (2018) 1:1–11. doi: 10.3171/2018.5.JNS173147
52. Hess DC, Sila CA, Furlan AJ, Wechsler LR, Switzer JA, Mays RW. A double-blind placebo-controlled clinical evaluation of MultiStem for the treatment of ischemic stroke. *Int J Stroke.* (2014) 9:381–6. doi: 10.1111/ijs.12065
53. Hess DC, Wechsler LR, Clark WM, Savitz SI, Ford GA, Chiu D, et al. Safety and efficacy of multipotent adult progenitor cells in acute ischaemic stroke (MASTERS): a randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet Neurol.* (2017) 16:360–8. doi: 10.1016/S1474-4422(17)30046-7
54. Díez-Tejedor E, Gutiérrez-Fernández M, Martínez-Sánchez P, Rodríguez-Frutos B, Ruiz-Ares G, Lara ML, et al. Reparative therapy for acute ischemic stroke with allogeneic mesenchymal stem cells from adipose tissue: a safety assessment: a phase II randomized, double-blind, placebo-controlled, single-center, pilot clinical trial. *J Stroke Cerebrovasc Dis.* (2014) 23:2694–700. doi: 10.1016/j.jstrokecerebrovasdis.2014.06.011
55. Kurtzberg J, Troy JD, Bennett E, Durham R, Shpall EJ, Wiese J, et al. Allogeneic umbilical Cord Blood Infusion for Adults with Ischemic Stroke (CoBIS): clinical outcomes from a phase 1 study. *Biol Blood Marrow Transplant.* (2017) 23:S173–4. doi: 10.1016/j.bbmt.2016.12.288
56. Kim SJ, Moon GJ, Chang WH, Kim YH, Bang OY. Intravenous transplantation of mesenchymal stem cells preconditioned with early phase stroke serum: current evidence and study protocol for a randomized trial. *Trials.* (2013) 14:317. doi: 10.1186/1745-6215-14-317

57. Moniche F, Escudero I, Zapata-Arriaza E, Usero-Ruiz M, Prieto-León MJ, de la Torre J, et al. Intra-arterial bone marrow mononuclear cells (BM-MNCs) transplantation in acute ischemic stroke (IBIS trial): protocol of a phase II, randomized, dose-finding, controlled multicenter trial. *Int J Stroke.* (2015) 10:1149–52. doi: 10.1111/ijs.12520
58. Willing AE, Shahaduzzaman M. Delivery routes for cell therapy in stroke. In: Jolkkonen J, Walczak P, editors. *Cell-Based Therapies in Stroke.* Wien: Springer (2013). p. 15–28. doi: 10.1007/978-3-7091-1175-8_2
59. Rodríguez-Frutos B, Otero-Ortega L, Gutiérrez-Fernández M, Fuentes B, Ramos-Cejudo J, Díez-Tejedor E. Stem cell therapy and administration routes after stroke. *Translat Stroke Res.* (2016) 7:378–87. doi: 10.1007/s12975-016-0482-6
60. Iihoshi S, Honmou O, Houkin K, Hashi K, Kocsis JD. A therapeutic window for intravenous administration of autologous bone marrow after cerebral ischemia in adult rats. *Brain Res.* (2004) 1007:1–9. doi: 10.1016/j.brainres.2003.09.084
61. Janowski M, Walczak P, Date I. Intravenous route of cell delivery for treatment of neurological disorders: a meta-analysis of preclinical results. *Stem Cells Develop.* (2010) 19:5–16. doi: 10.1089/scd.2009.0271
62. Dulamea AO. The potential use of mesenchymal stem cells in stroke therapy—From bench to bedside. *J Neurol Sci.* (2015) 352:1–11. doi: 10.1016/j.jns.2015.03.014
63. Sun JM, Kurtzberg J. Cord blood for brain injury. *Cytotherapy.* (2015) 17:775–85. doi: 10.1016/j.jcyt.2015.03.004
64. Liska MG, Crowley MG, Borlongan CV. Regulated and unregulated clinical trials of stem cell therapies for stroke. *Transl Stroke Res.* (2017) 8:93–103. doi: 10.1007/s12975-017-0522-x
65. Garbuzova-Davis S, Haller E, Williams SN, Haim D, Tajiri N, Hernandez-Ontiveros G, et al. Compromised blood–brain barrier competence in remote brain areas in ischemic stroke rats at the chronic stage. *J Compar Neurol.* (2014) 522:3120–37. doi: 10.1002/cne.23582
66. Minisman G, Bhanushali M, Conwit R, Wolfe GI, Aban I, Kaminski HJ, et al. Implementing clinical trials on an international platform: challenges and perspectives. *J Neurol Sci.* (2012) 313:1–6. doi: 10.1016/j.jns.2011.10.004
67. Shenoy P. Multi-regional clinical trials and global drug development. *Perspect Clin Res.* (2016) 7:62–7. doi: 10.4103/2229-3485.179430
68. Lees JS, Sena ES, Egan KJ, Antonic A, Koblar SA, Howells DW, et al. Stem cell-based therapy for experimental stroke: a systematic review and meta-analysis. *Int J Stroke.* (2012) 7:582–8. doi: 10.1111/j.1747-4949.2012.00797.x
69. Bang OY. Clinical trials of adult stem cell therapy in patients with ischemic stroke. *J Clin Neurol.* (2016) 12:14–20. doi: 10.3988/jcn.2016.12.1.14
70. Australian Commission on Safety and Quality in Health Care (2017). *Economic Evaluation of Investigator-Initiated Clinical Trials Conducted by Networks.* Australian Clinical Science Alliance.
71. Lee JS, Hong JM, Moon GJ, Lee PH, Ahn YH, Bang OY. A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke. *Stem Cells.* (2010) 28:1099–106. doi: 10.1002/stem.430
72. Yavagal D, Huang D, Graffagnino C, Rappard G, Budzik R, Likosky W, et al. Intra-arterial delivery of autologous ALDHbr cells in ischemic stroke: final 1-year results of the recovery-stroke trial. *Int J Stroke.* (2015) 10:13.
73. Participants S. Stem cell Therapies as an Emerging Paradigm in Stroke (STEPS) bridging basic and clinical science for cellular and neurogenic factor therapy in treating stroke. *Stroke.* (2009) 40:510–5. doi: 10.1161/STROKEAHA.108.526863
74. Savitz SI, Chopp M, Deans R, Carmichael S, Phinney D, Wechsler L, et al. Stem cell therapy as an emerging paradigm for stroke (STEPS) II. *Stroke.* (2011) 42:825–9. doi: 10.1161/STROKEAHA.110.601914
75. Savitz SI, Cramer SC, Wechsler LS, Wechsler L, STEPS 3 Consortium Stem cells as an emerging paradigm in stroke 3: enhancing the development of clinical trials. *Stroke.* (2014) 45:634–9. doi: 10.1161/STROKEAHA.113.003379
76. Bang OY, Lee JS, Lee PH, Lee G. Autologous mesenchymal stem cell transplantation in stroke patients. *Ann Neurol.* (2005) 57:874–82. doi: 10.1002/ana.20501
77. Fang J, Guo Y, Tan S, Li Z, Xie H, Chen P, et al. Autologous endothelial progenitor cells transplantation for acute ischemic stroke: a 4-year follow-up study. *Stem Cells Translat Med.* (2019) 8:14–21. doi: 10.1002/sctm.18-0012
78. Napoli E, Borlongan CV. Recent advances in stem cell-based therapeutics for stroke. *Transl Stroke Res.* (2016) 7:452–7. doi: 10.1007/s12975-016-0490-6
79. dela Peña I, Borlongan CV. Translating G-CSF as an adjunct therapy to stem cell transplantation for stroke. *Translat Stroke Res.* (2015) 6:421–9. doi: 10.1007/s12975-015-0430-x
80. Osanai T, Houkin K, Uchiyama S, Minematsu K, Taguchi A, Terasaka S. *Treatment Evaluation of Acute Stroke for Using in Regenerative Cell Elements (TREASURE) Trial: Rationale and Design.* London: SAGE Publications Sage (2018). doi: 10.1177/1747493017743057
81. Borlongan CV. Preliminary reports of stereotaxic stem cell transplants in chronic stroke patients. *Mol Ther.* (2016) 24:1710–1. doi: 10.1038/mt.2016.186
82. Borlongan CV. Age of PISCES: stem-cell clinical trials in stroke. *Lancet.* (2016) 388:736–8. doi: 10.1016/S0140-6736(16)31259-4
83. Hassani Z, O'Reilly J, Pearse Y, Stroemer P, Tang E, Sinden J, et al. Human neural progenitor cell engraftment increases neurogenesis and microglial recruitment in the brain of rats with stroke. *PLoS ONE.* (2012) 7:e50444. doi: 10.1371/journal.pone.0050444
84. Hicks C, Stevanato L, Stroemer RP, Tang E, Richardson S, Sinden JD. *In vivo* and *in vitro* characterization of the angiogenic effect of CTX0E03 human neural stem cells. *Cell Transplant.* (2013) 22:1541–52. doi: 10.3727/096368912X657936
85. Evans MA, Lim R, Kim HA, Chu HX, Gardiner-Mann CV, Taylor KW, et al. Acute or delayed systemic administration of human amnion epithelial cells improves outcomes in experimental stroke. *Stroke.* (2018) 49:700–9. doi: 10.1161/STROKEAHA.117.019136
86. Kurtzberg J, Durham R, Laskowitz D, Balber AE, Bennett E. Allogeneic umbilical cord blood infusion for adults with ischemic stroke. *Biol Blood Marrow Transplant.* (2016) 22:S141–2. doi: 10.1016/j.bbmt.2015.11.481
87. Kurtzberg J, Troy JD, Bennett E, Belagaje S, Shpall EJ, Wiese J, et al. Allogeneic umbilical Cord Blood Infusion for Adults with Ischemic Stroke (CoBIS): clinical outcomes from a phase 1 safety study. *Am Soc Hematol.* (2016) 7:521–29.
88. Ghali AA, Yousef MK, Ragab OA, ElZamarany EA. Intra-arterial infusion of autologous bone marrow mononuclear stem cells in subacute ischemic stroke patients. *Front Neurol.* (2016) 7:228. doi: 10.3389/fneur.2016.00228
89. Unsworth DJ, Mathias JL, Dorstyn DS. Safety and efficacy of cell therapies administered in the acute and subacute stages after stroke: a meta-analysis. *Regenerat Med.* (2016) 11:725–41. doi: 10.2217/rme-2016-0063
90. Jeong H, Yim HW, Cho Y-S, Kim YI, Jeong S-N, Kim H-B, et al. Efficacy and safety of stem cell therapies for patients with stroke: a systematic review and single arm meta-analysis. *Int J Stem Cells.* (2014) 7:63–9. doi: 10.15283/ijsc.2014.7.2.63
91. Cao W, Li P. Effectiveness and safety of autologous bone marrow stromal cells transplantation after ischemic stroke: a meta-analysis. *Med Sci Monitor.* (2015) 21:2190–5. doi: 10.12659/MSM.895081
92. Sertkaya AH, Wong H, Jessup A, Beleche T. Key cost drivers of pharmaceutical clinical trials in the United States. *Clin Trials.* (2016) 13:117–26. doi: 10.1177/1740774515625964
93. Evers SM, Struijs JN, Ament AJ, van Genugten ML, Jager JC, van den Bos GA. International comparison of stroke cost studies. *Stroke.* (2004) 35:1209–15. doi: 10.1161/01.STR.0000125860.48180.48
94. Luengo-Fernandez R, Leal J, Gray A. UK research spend in 2008 and 2012: comparing stroke, cancer, coronary heart disease and dementia. *BMJ Open.* (2015) 5:e006648. doi: 10.1136/bmjopen-2014-006648
95. Drummond MF, Davies LM, Ferris FL III. Assessing the costs and benefits of medical research: the diabetic retinopathy study. *Soc Sci. Med.* (1992) 34:973–81. doi: 10.1016/0277-9536(92)90128-D
96. Lopes AG, Sinclair A, Frohlich B. Cost analysis of cell therapy manufacture: autologous cell therapies, part 1. *BioProcess Int.* (2018) 16:S3–S8.
97. Rafiq Q, Thomas R. The evolving role of automation in process development & manufacture of cell & gene-based therapies. *Cell Gene Ther Insights.* (2016) 2:473–9. doi: 10.18609/cgti.2016.058

98. Rafiq Q. Emerging automated approaches for cell and gene therapy manufacture. *Cell Gene Ther Insights.* (2018) 4:911–94. doi: 10.18609/cgti.2018.070
99. Abou-El-Enein M, Elsanhoury A, Reinke P. Overcoming challenges facing advanced therapies in the EU market. *Cell Stem Cell.* (2016) 19:293–7. doi: 10.1016/j.stem.2016.08.012
100. Galipeau J, Sensébé L. Mesenchymal stromal cells: clinical challenges and therapeutic opportunities. *Cell Stem Cell.* (2018) 22:824–33. doi: 10.1016/j.stem.2018.05.004
101. Bekelman JE, Li Y, Gross CP. Scope and impact of financial conflicts of interest in biomedical research: a systematic review. *JAMA.* (2003) 289:454–65. doi: 10.1001/jama.289.4.454
102. Patsopoulos NA, Ioannidis JP, Analatos AA. Origin and funding of the most frequently cited papers in medicine: database analysis. *BMJ.* (2006) 332:1061–4. doi: 10.1136/bmj.38768.420139.80
103. Ioannidis JP, Greenland S, Hlatky MA, Khoury MJ, Macleod MR, Moher D, et al. Increasing value and reducing waste in research design, conduct, and analysis. *Lancet.* (2014) 383:166–75. doi: 10.1016/S0140-6736(13)62227-8
104. Janiaud P, Cristea IA, Ioannidis JP. Industry-funded versus non-profit-funded critical care research: a meta-epidemiological overview. *Intensive Care Med.* (2018) 44:1613–27. doi: 10.1007/s00134-018-5325-3
105. Lundh A, Sismondo S, Lexchin J, Busuioc OA, Bero L. Industry sponsorship and research outcome. *Cochrane Database Syst Rev.* (2012) 12. doi: 10.1002/14651858.MR000033.pub2
106. Fung M, Yuan Y, Atkins H, Shi Q, Bubela T. Responsible translation of stem cell research: an assessment of clinical trial registration and publications. *Stem Cell Rep.* (2017) 8:1190–201. doi: 10.1016/j.stemcr.2017.03.013
107. Schulz KF, Altman DG, Moher D. CONSORT 2010 statement: updated guidelines for reporting parallel group randomized trials. *Ann Int Med.* (2010) 152:726–32. doi: 10.7326/0003-4819-152-11-201006010-00232
108. Chan A-W, Tetzlaff JM, Altman DG, Dickersin K, Moher D. SPIRIT 2013: new guidance for content of clinical trial protocols. *Lancet.* (2013) 381:91–2. doi: 10.1016/S0140-6736(12)62160-6
109. Hakoum MB, Jouni N, Abou-Jaoude EA, Hasbani DJ, Abou-Jaoude EA, Lopes LC, et al. Characteristics of funding of clinical trials: cross-sectional survey and proposed guidance. *BMJ Open.* (2017) 7:e015997. doi: 10.1136/bmjopen-2017-015997

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.

Copyright © 2019 Krause, Phan, Ma, Sobey and Lim. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Neurology

ORIGINAL RESEARCH
published: 13 August 2019
doi: 10.3389/fneur.2019.00786

Intra-arterial Administration of Human Umbilical Cord Blood Derived Cells Inversed Learning Asymmetry Resulting From Focal Brain Injury in Rat

Elzbieta Gornicka-Pawlak, Miroslaw Janowski, Aleksandra Habich, Anna Jablonska, Joanna Sypecka and Barbara Lukomska*

NeuroRepair Department, Mossakowski Medical Research Center, Polish Academy of Sciences, Warsaw, Poland

OPEN ACCESS

Edited by:
Dileep Yavagal, University of Miami, United States

Reviewed by:
Pallab Bhattacharya, National Institute of Pharmaceutical Education and Research, Ahmedabad, India
Cesar V. Borlongan, University of South Florida, United States

***Correspondence:**
Elzbieta Gornicka-Pawlak
elago@autograf.pl

Specialty section:
This article was submitted to Endovascular and Interventional Neurology, a section of the journal Frontiers in Neurology

Received: *25 March 2019*
Accepted: *08 July 2019*
Published: *13 August 2019*

Citation:
Gornicka-Pawlak E, Janowski M, Habich A, Jablonska A, Sypecka J and Lukomska B (2019) Intra-arterial Administration of Human Umbilical Cord Blood Derived Cells Inversed Learning Asymmetry Resulting From Focal Brain Injury in Rat. Front. Neurol. 10:786. doi: 10.3389/fneur.2019.00786

Background: Focal brain injury is a leading cause of serious disability significantly worsening patients' quality of life. Such damage disrupts the existing circuits, leads to motor, and cognitive impairments as well as results in a functional asymmetry. To date, there is still no therapy to effectively restore the lost functions. We examined the effectiveness of human umbilical cord blood (HUCB)-derived cells after their intra-arterial infusion following focal stroke-like brain damage.

Methods: The model of stroke was performed using ouabain stereotactic injection into the right dorsolateral striatum in rats. Two days following the brain injury 10^7 cells were infused into the right carotid artery. The experimental animals were placed into enriched environment housing conditions to enhance the recovery process. Behavioral testing was performed using a battery of tasks visualizing motor as well as cognitive deficits for 30 days following brain injury. We assessed animal asymmetry while they were moving forward at time of testing in different tasks.

Results: We found that intra-arterial infusion of HUCB-derived cells inversed lateralized performance resulting from the focal brain injury at the early stage of T-maze habit learning task training. The inversion was independent from the level of neural commitment of infused cells. The learning asymmetry inversion was observed only under specific circumstances created by the applied task design. We did not found such inversion in walking beam task, vibrissae elicited forelimb placing, the first exploration of open field, T-maze switching task as well as apomorphine induced rotations. Both the asymmetry induced by the focal brain injury and its inversion resulting from cell infusion decreased along the training. The inversion of learning asymmetry was also independent on the range of the brain damage.

Conclusions: Intra-arterial infusion of HUCB-derived cells inversed lateralized performance of learning task resulting from focal brain damage. The inversion was not

visible in any other of the used motor as well as cognitive tests. The observed behavioral effect of cell infusion was also not related to the range of the brain damage. Our findings contribute to describing the effects of systemic treatment with the HUCB-derived cells on functional recovery following focal brain injury.

Keywords: focal brain injury, human umbilical cord blood cells, intra-arterial infusion, functional recovery, asymmetry

INTRODUCTION

The focal brain injury resulting from vascular dysfunction is a leading cause of serious and long-term disabilities of patients (1). To date, there is no efficient therapy to repair damaged tissue as well as to restore the impaired functions (2). Natural endogenous mechanisms triggered to recover lost functions or to develop compensation are unfortunately limited, although they possess an incredible potential (3). Such state of art encourages scientists and clinicians to assess novel therapeutic approaches aimed to restore the lost functions.

To measure the potential effects of experimental therapy on functional recovery following focal brain injury, it is necessary to induce in experimental animals a unilateral damage to the brain tissue similar to those occurring in patients suffering from stroke (4). To accurately evaluate treatment benefit, the brain lesion should be reproducible, relatively small, and causing functional impairments, which would be easy to measure using behavioral tasks allowing to expose disabilities along observation period. To describe the quality of life after such onset followed by a therapeutic intervention, the designed set of behavioral tests should contain tasks revealing motor deficits as well as cognitive impairments providing information concerning the progress in animal recovery.

Cell therapy seems to be a promising approach to improve functions impaired due to brain injury (5–7). Such therapy could enhance the endogenous healing mechanisms (8), as well as to promote structural repair to obtain healthy brain tissue. The cells destined for therapeutic use should meet criteria of safety and ethical acceptance (9). The human umbilical cord blood seems to be such source of cells with potential for restorative therapies (10, 11). One of the major advantage of this cell reservoir is relatively easy accessibility by puncturing umbilical cord at a time of child birth (12). It was previously shown that mononuclear non-hematopoietic cells ($CD34^-$) isolated from human umbilical cord blood conversed into neural progenitors lineage after *in vitro* culture (13, 14). As previously described we examined three different populations of human umbilical cord blood-derived cells differing in their level of neural commitment (15). We hypothesized that neurally-committed cells can be more effective for treatment of brain lesion. We compared the therapeutic effectiveness of: (1) freshly isolated human umbilical cord blood mononuclear cells deprived of $CD34^+$ population (HUCB-MNC $CD34^-$), (D-0); (2) HUCB-MNC $CD34^-$ cultured for 3 days in media stimulating neural differentiation (D-3); (3) human umbilical cord blood derived neural stem cell line (NSC) established in our laboratory. The main idea concerning routes of cell delivery was to administer them by infusion into artery supplying blood to the damaged area of the brain. It would potentially maximize the amount of cells reaching the injured tissue. There is a rapidly growing interest in intra-arterial route of stem cell delivery to the CNS. The safety of intra-arterial injections has been solved through adjustment of cell dose and velocity of infusion (16, 17). Real-time MRI allows to make the procedure precise and predictable, what is of utmost importance for translation to clinical setting (18). Moreover, the expression of adhesion molecules is capable to further enhance homing of intra-arterially delivered stem cells (19, 20).

To induce a focal brain injury we used a stereotactic injection of ouabain (OUA) into the rat's right dorsolateral striatum (21). Ouabain is known to trigger the cell death via mechanism similar to those occurring in the absence of an energy supply, observed after an ischemia or a hemorrhage (22). Briefly, the ouabain blocks the Na^+/K^+ ATP-ase pump, which was shown ceasing to function after interruption of glucose and oxygen delivery. This leads to membrane depolarization and following molecular events. Such action triggers development of lesion which might be considered as a model of stroke free of vascular dysfunction component. While, in this model brain injury is not caused by the vascular blockade, it has several advantages. The stereotactic surgery is less invasive and much faster than obtaining access to carotid or cerebral vessels, therefore the procedure itself is not producing that much systemic inflammation, which can be a confounding factor in traditional stroke models. Moreover, ouabain model of stroke leaves carotid vessels intact, which facilitates surgery for intra-arterial cell infusion, while the use of carotid vessels for stroke induction complicates their subsequent use for cell infusion a couple days later due to many local, post-operative changes. The size of the stroke can also be easily controlled by the amount of injected ouabain, and the lesion size selected by us was devoid of any mortality. The dorsolateral striatum was chosen as a structure to induce stroke-like damage, since it was shown to be involved in a motor behavior (23), as well as in cognitive processes (24–26). In our model the ouabain injection into dorsolateral striatum was shown to produce significant motor deficits in walking beam task and changed patterns of exploratory behavior in the open field (27). This focal brain injury also caused the cognitive impairments i.e., habit learning and cognitive flexibility deficits (28). To maximize the effectiveness of cell treatment we used the large groups of experimental animals housed in enriched environment conditions. The applied design enables social interactions as well as the opportunity to explore and to exercise the impairments resulting from the brain injury (29). Providing the opportunities

of various activities to experimental animals would increase the amount of functional connections in a regenerating tissue and in this way to improve the effectiveness of cell therapy.

Focal brain injury is inseparably related to unilateral damage of the existing, functional circuits, and it results in animal disabilities and lateralization. These symptoms are diversified depending on severity and exact location of the brain damage as well as specific demands in different situations. Experimental therapy is desired to repair them. In the presented study, we evaluated the experimental rat's asymmetry while they were moving forward under various circumstances to quantify and compare effects of cell infusion following focal brain injury.

METHODS

Animals

Male Wistar rats weighing around 250 g were used for experiments. Animals were kept in enriched environment housing conditions which was introduced just after receiving rats from animal house, 4 days before brain injury, as previously described in detail (15, 27, 28). Enriched environment cages (70 × 41 × 56 cm) contained beams, platforms, ladders etc. Cage equipment was rearranged twice a week. Rats were housed in large groups (7–8 animals per enriched environment cage), 12/12 h light/dark cycle, *ad libidum* feeding with standard feed and tap, filtered water. For the purpose of immunosuppression, Cyclosporine A (Novartis) in dose of 1 mg/kg b.w. daily were administered i.p. to rats throughout the entire experiment. All of the animal handling and experimental procedures were approved by IV Local Ethics Committee on Animal Care and Use (Ministry of Science and Higher Education, Warsaw, Poland).

Cell Preparation

The isolation and preparation of cell populations derived from the human umbilical cord blood as well as their quantity was maintained according to the protocol described previously (15). Briefly, after delivering the placenta, cord blood was collected by puncturing umbilical cord veins. The mononuclear $CD34^-$ cells were obtained using Ficoll separation. HUCB MNC $CD34^-$ cells were washed (D-0) or cultured for 3 days in serum free medium (DMEM/F-12+B27+EGF+bFGF, 10^7 cells/1 ml) (D-3). Then the cells were resuspended in FBS/DMSO and stored in liquid nitrogen for future purposes. Prior to transplantation the cells were quickly thawed at 37°C, washed and resuspended in PBS (10^7cells/0.5 ml). Cells viability was confirmed by staining with TrypanBlue dye at time of manual cell counting it was no lower than 80%.

Human umbilical cord blood derived neural stem cell (NSC) line (13) is being continuously cultured in our laboratory in low serum medium: DMEM/F12 (Gibco) + ITS (1:100, Gibco) + FBS (2%, Gibco) + AAS (Gibco). Twenty-four hours prior to infusion, the cells were placed in serum free medium (Neurobasal, Gibco). For administration 10^7 cells were washed and resuspended in 0.5 ml of PBS.

Surgery

Focal Brain Injury

The stereotaxic ouabain injection into the right dorsolateral striatum (**Figure 1A**) was made like previously described (15, 27, 28). Briefly, 1.5 μl 5 nmol ouabain (OUA) has been injected with coordinates A: 0.5, L: 3.8, V: 4.7 at a speed 1 μl/min.

Cell Infusion

Cell infusions were made into right internal carotid artery (ICA) (**Figure 1A**). In supine position of experimental animal the skin incision has been made in cervical area followed by the dissection of carotid arteries and after ligation of external carotid artery (ECA), the ICA was punctured and either 10^7 cells suspended in 0.5 ml of PBS or 0.5 ml PBS was injected over a period of 3–5 min. The needle has been withdrawn and after careful hemostasis the ICA has been left patent.

Behavioral Testing

Walking Beam Task

The rats were trained, like previously described (27) to walk along a narrow wooden beam (14 mm wide, 800 mm long, elevated 50 cm above the cushioned floor) to reach other 3 experimental animals being in the standard cage located at the end of the beam. To evaluate quality of using both the left and right limbs simultaneously while walking along beam, a mirror was set up a side of the apparatus. Each experimental rat performing the task was video recorded from the left side together with its right side mirror view. The rats were tested before brain injury and then 2, 7, 15, and 30 days following lesion. The quality of walking was evaluated quantitatively in developed previously 8-points scale (27). Briefly: unable to move - score 0, able to shift with severe difficulties - score 1, traversing whole beam length, both contralateral paws placed aside of the beam with deep sleeps - score 2, deep sleeps of the left hind limb, placing feet aside of the beam much lower than top of the beam - score 3, slight sleeps of the left hind limb, placing aside close to the upper edge - score 4, irregular consistently asymmetrical left hind limb stepping that is shorter steps, or another shape of moving limb or placing left hind paw with fingers aside of the top of the beam in compare with right hind limb moving and placing straightly - score 5, irregular, symmetric stepping, both left and right limbs incidentally shorter steps, or fingers placed aside of the top of the beam - score 6, perfect walk completely symmetric - score 7. Rats were evaluated from video records by experimenter blind for treatment.

Vibrissae Elicited Forelimb Placing

Same side vibrissae elicited forelimb placing test was performed accordingly (30). Briefly, Each rat was handled to feel relaxed. Then the animal was held by hind limbs and one of forelimbs were constrained. Only a single forelimb was free to move. The experimenter moved each rat toward the edge of a table to touch it with its vibrissae same side to unconstrained fore limb. The rat moved extending free forelimb to defend its head. The animals were tested for both left and right forelimbs, using accordingly both left and right vibrissae, before, 2, 7, 15, and 30 days following

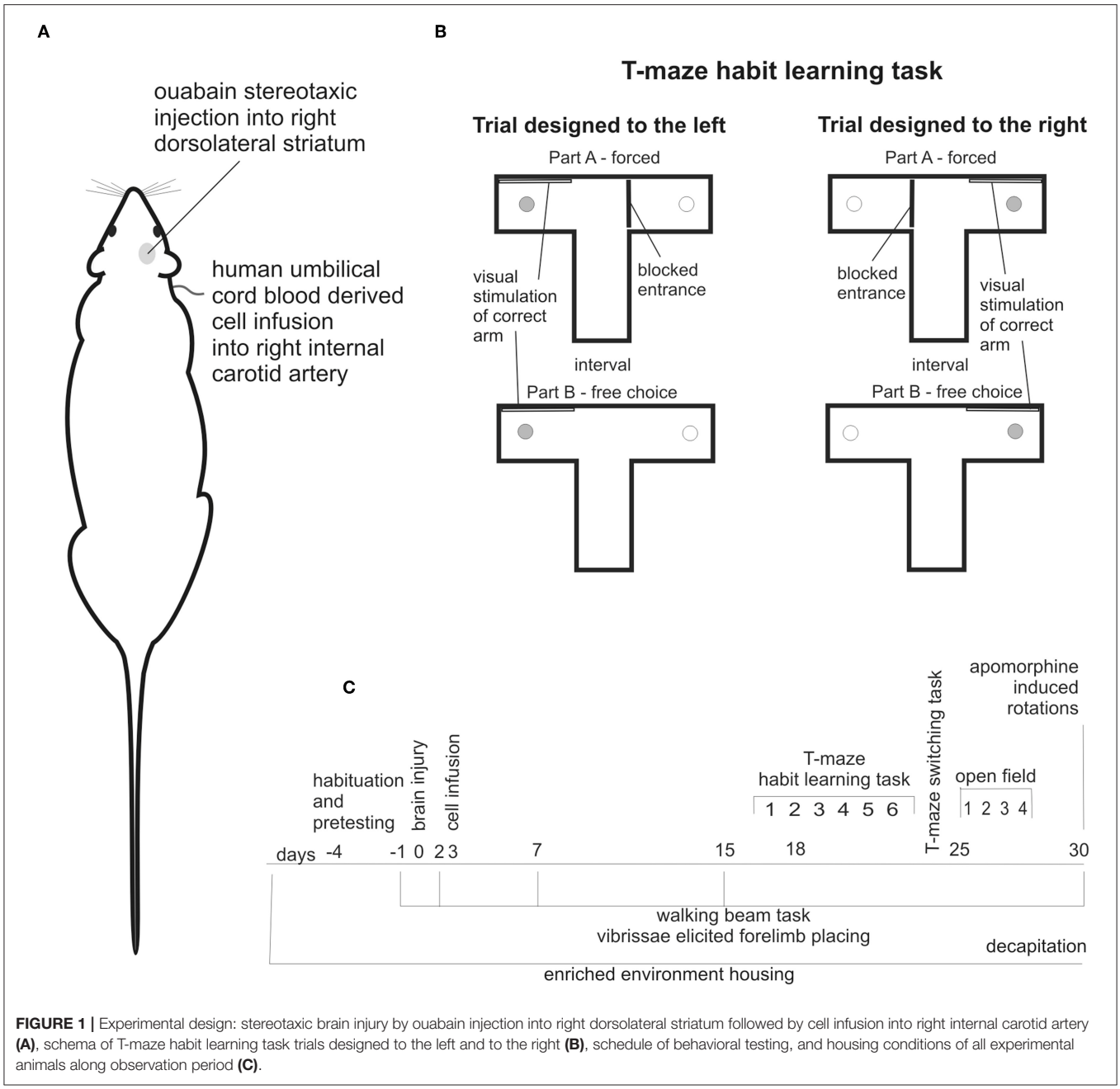


FIGURE 1 | Experimental design: stereotaxic brain injury by ouabain injection into right dorsolateral striatum followed by cell infusion into right internal carotid artery **(A)**, schema of T-maze habit learning task trials designed to the left and to the right **(B)**, schedule of behavioral testing, and housing conditions of all experimental animals along observation period **(C)**.

brain injury. The scores were noted as able or unable to move free forelimb to place at the incoming edge.

T-Maze Habit Learning Task

The T-maze habit learning task was performed like previously described (28). Briefly, the T-maze was made of black wood. Each arm contained a 1 cm deep food well at the end, so a rat was unable to see the cookie from the choice point. Before the surgery, the rats were habituated to the T-maze and food reward. The cognitive testing began 16 days following brain injury. At this time the rats did not show any symptoms like weakness, strong need to sleep, or lack of interest in surrounding environment, which would worsen cognitive performance independently on impairments resulting from the brain damage. We did not use any food restriction. On the 16th day following brain injury, the rats performed six trials of the T-maze habit learning task to familiarize them with the test protocol. Regular training begun on the 17th day following the brain lesion. At time of testing rats were kept in waiting boxes close to the apparatus. Each trial consisted of two parts: forced and free choice (**Figure 1B**). In the forced part, one arm of the maze was blocked at its entry with guillotine door and the only one bitted with food reward was available for the rat released from starting compartment of the maze. Next, after consuming the cookie rat was moved

outside the maze for interatrial interval, food well was refilled with piece of cookie, guillotine door was removed and the free choice part was conducted, in which the released rat could enter both arms. After consuming the cookie rat was moved back to the waiting box. The correct arm in the free choice part was the same as in the forced part. In addition, the correct arm in both parts was indicated by a visual clue - a white tablet on the wall. Rats were trained one by one. The daily sessions consisted of 10 trials: 5 designed to the left and 5 designed to the right arranged randomly with unpredictable order (**Figure 1B**). No more than two consecutive trials in the same direction were allowed. The T-maze habit learning task training was performed for 6 days. The number of correct responses made by each experimental animal during daily training that is turn first of all into correct arm in free choice part was noted as a positive result. In case of an incorrect choice, that is firs entry into unmarked, unvisited during forced part arm, the rat was allowed to find and consume the food reward in the correct arm and the result was noted as negative score. Single rat was handled to avoid too much scare as well as too much arousal prior to each trial.

To evaluate the unilateral lesion-induced lateralization, we analyzed separately the number of correct responses when trials were designed to the left and to the right (**Figure 1B**). To quantitatively describe T-maze habit learning task lateralization we expressed the number of correct responses made when trial was designed to the left as % of total correct responses.

T-Maze Switching Task

Just after completion of the habit-learning training, the rats were tested on the switching task using the same maze. The T-maze switching task consisted only of free choice trials in which visual stimulation was the only relevant clue. The rats needed to switch not to use information concerning which arm was previously visited. We used 20 trials performed along single day, an equal number of trials designed to the left and to the right and no more than two consecutive turns in the same direction. Similarly as in the T-maze habit learning task, if rat entered the incorrect arm, it was left in the T-maze until reward was found. The results and lateralization were scored as in the preceding task.

Open Field Test

The open field test was performed 25 days following the surgery. Each rat was placed in the center of the large, circular, black arena (1 m diameter surrounded by 30 cm height wall). On the first day individual rats sessions lasted 30 min. On the succeeding 3 days sessions 10 min were performed. The sessions were video recorded and the body center point of the freely moving rat was captured by the EthoVision video tracking system (Noldus). Only first 10 min of first session were used here. To analyze the raw xy coordinates of the rat body center SEE Workshop was applied (31). To evaluate tendency of each rat turning, a parameter Median Curvature of Progression Segments was multiplied by −1 and named locomotor curvature.

Apomorphine Induced Rotations

Thirty days following the surgery the rats were injected with apomorphine (0.75 mg/kg b.w., s.c.) and placed in individual circular arenas. The animals were video recorded for 60 min. The number and direction of rotations were scored from video recording by experimenter blind for treatment.

Histological Analysis of Rat Brain Tissue

Histological analysis was made like we previously descried (15). Briefly, 30 days following brain injury the rats were anesthetized with halothane and decapitated. The brains were quickly removed and immediately frozen in powdered dry ice and stored at −70°C. Every 5th of 20 μm thick coronal sections was stained with a standard hematoxylin and eosin (H&E) method. For quantitative measure of lesion volume images of every fifth of stained sections, visualized in the Discovery v. 1.2 (Zeiss) stereoscope microscope, were analyzed using Corel PHOTOPAINT software. Then values of cavity extent of preceding sections of each rat's brain were multiplied by corresponding brain block thickness (which was 500 μm) and summarized. For analysis of exact location and extend of the damaged tissue we chose the brain area ranging to end of striatum in rostral direction and at hippocampus level in caudal direction. Within this part of the brain the 5 coronal, subsequent planes were selected. Then the corresponding images of H&E stained sections of the brain of each rat were collected and arranged one by one. In next step, the printed representative juxtapositions of the injured brains of all animals included in one experimental group were arranged side by side. Then the minimal and maximal lesion extension were manually overdrawn on brain structures schemas. For the vector graphics Corel DRAW software were used.

Data Analysis and Statistics

Statistical analyses were performed for all experimental animals, which completed every designed procedures (**Figure 1C**), i.e., performed walking beam task before brain injury without visible asymmetry, successfully underwent surgery, showed motor impairments in walking beam task 2 days following brain injury, obtained cell intra-arterial infusion, were tested in all behavioral tasks without technical difficulties and their brains were stained for histological analysis. We did not reject any of experimental animals which met above criteria.

To evaluate the data, a non-parametric statistical analysis was applied. To find if one of compared groups was significantly different from other ones the Friedman ANOVA analyses were used. To estimate which of experimental groups is significantly different form other ones the Mann-Whitney *U*-test for non-matched pairs was calculated. Data on all graphs are medians ±25 and 75%.

RESULTS

T-Maze Habit Learning Task

We assessed T-maze habit learning task training of experimental animals. Acquisition of the task was juxtaposed with its lateralization to visualize animal asymmetry at different stages of learning process. As we previously described (15, 28) OUA injection into the right dorsolateral striatum impaired acquisition of T-maze habit learning task (**Figure 2A**). Injured rats treated

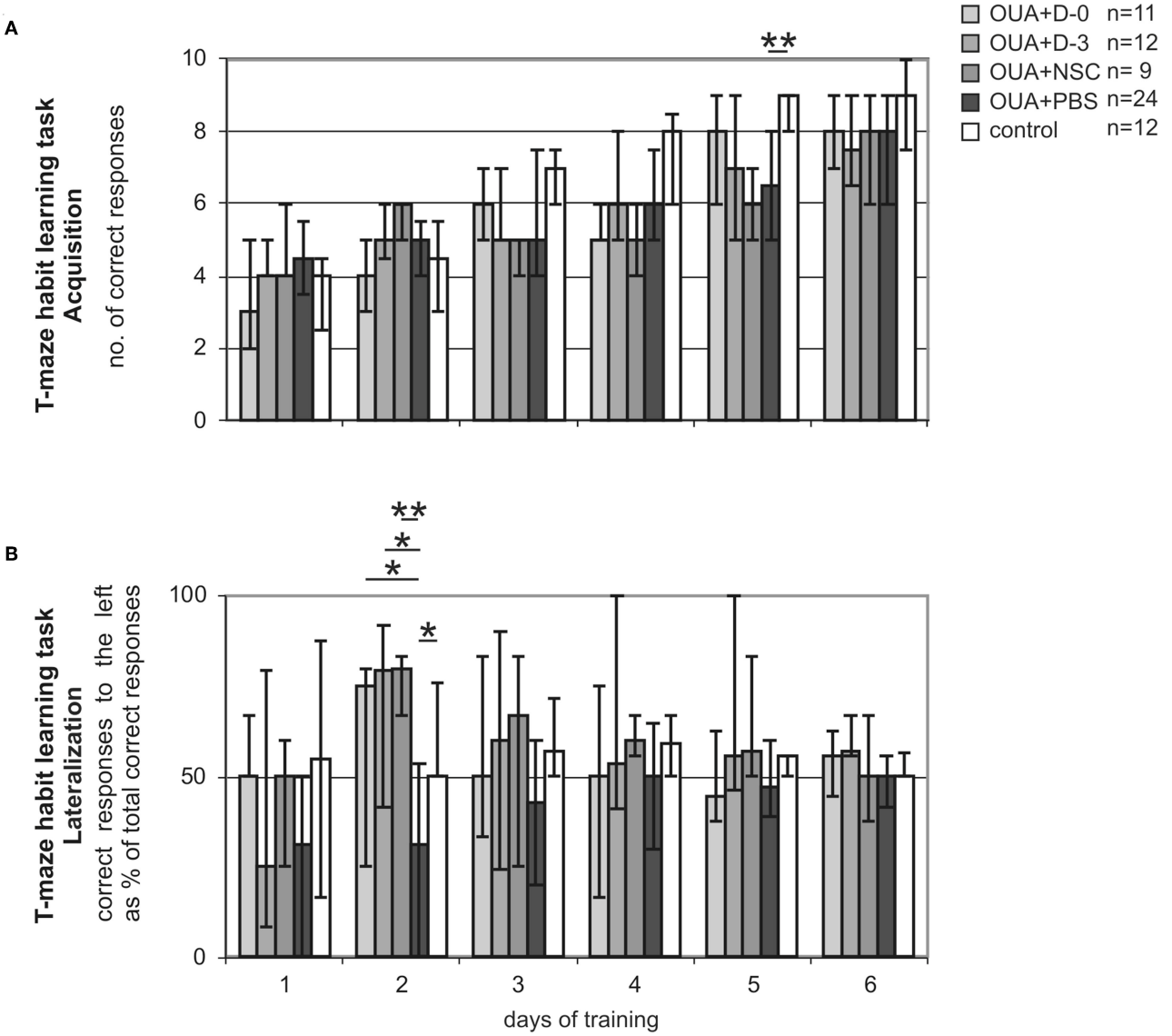


FIGURE 2 | T-maze habit learning task training. Acquisition **(A)** expressed as the number of correct responses made by rats treated either with human umbilical cord blood derived cells being at different stages of neural commitment or PBS and control ones. Lateralization **(B)** expressed as the amount of correct responses made by experimental animals when trial was designed to the left conveyed as a % of all correct responses along consecutive days of training. Line marked on the graph at value of 50% points out the direction of learning asymmetry (median ± 25 and 75%; $^{*}p < 0.05$, $^{**}p < 0.01$ in Whitney-Mann test for non-matched pairs).

with PBS made significantly less correct responses at 5th day of training than control ones ($p < 0.01$). OUA animals treated with either D-0 or D-3 cells showed tendency to improve impaired by the brain injury task acquisition, but this difference did not reach statistical significance.

T-maze habit learning task Lateralization, that is amount of correct responses made by experimental rats when trial was designed to the left expressed as percent of total correct responses was significantly shifted by focal brain injury (**Figure 2B**). OUA injection into the right dorsolateral striatum resulted in performing significantly less correct responses when trial was designed to the left in compare with those designed to the right at the early stage of acquisition that is second day of training. Treatment with all cells populations inversed the direction of task lateralization. D-0. D-3 ($p < 0.05$) and NSC ($p < 0.01$) cell treated groups of rats showed significantly more correct responses when trial was designed to the left then to the right in comparison with OUA+PBS group at second day of training. Control animals performed the task at comparable amount of correct responses made when trial was designed to the left and to the right through all training.

T-maze habit learning task Lateralization was the most inclined at 2nd day of training in both OUA+PBS and mirror view inversed in cell treated groups. Lateralized task performance

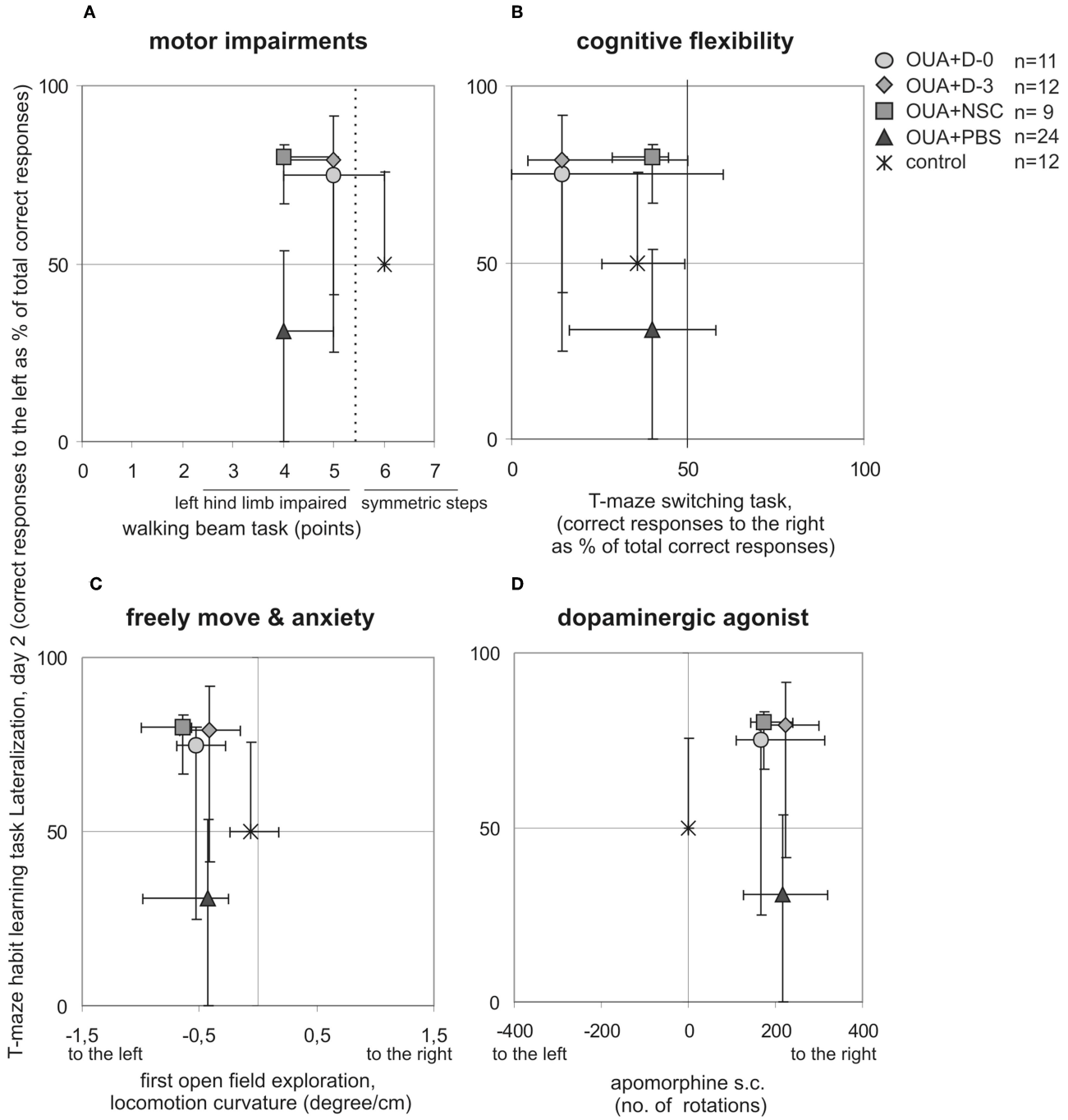


FIGURE 3 | Comparison of experimental animals asymmetries visible in different behavioral tasks. T-maze habit learning task lateralization at 2nd day of training was juxtaposed with: motor impairments of left hindlimb in walking beam task 15 days following brain injury **(A)**, cognitive flexibility in T-maze switching task **(B)**, turning tendency when rat was freely moving under anxiety in first open field task session **(C)**, number and direction of rotations induced by dopaminergic agonist apomorphine s.c. injection **(D)**, (median ± 25 and 75%, lines marked on the graphs point out direction of presented asymmetries).

at the early stage of training improved with time along consecutive sessions in all OUA groups. At the last day of training that is 6th, all experimental groups of rats performed the task at comparable amount of correct responses made when trial was designed to the left and to the right.

Juxtapositions of Rat Asymmetries Visible in Various Behavioral Tasks

T-maze habit learning task Lateralization at 2nd day of training was juxtaposed with asymmetries visible in other behavioral tasks performed in single animals to describe functional impairments

resulting from focal brain injury and potential recovery induced by cell infusion (**Figure 3**). Learning asymmetry was compared with: motor deficits, that is level of left hind limb impairments visible in walking beam test performed 15 days following brain injury; cognitive flexibility lateralization measured in T-maze switching task; turning tendency in the open field first 10 min exploration when rat was freely moving in novel empty arena and number as well as direction of rotations induced by non-selective dopaminergic agonist apomorphine s.c. injection. We also compared T-maze habit learning task Lateralization at 2nd day of training with vibrissae elicited left forelimb placing permanent deficit across all observation period. None of juxtaposed asymmetries of experimental animals under various circumstances were inversed by cell infusion like it was shown for T-maze habit learning task Lateralization at 2nd day of training.

Lateralization of T-maze habit learning task performance along training was also juxtaposed with turning tendency of freely moving single rats during consecutive open field 10 min sessions (**Figure 4**). In contrast to learning asymmetry at 2nd day of training, when all of examined treatments with cell populations induced inversion, at 2nd day of open field testing only OUA+D-0 rats showed opposite direction of median locomotor curvature, that is to the right (which however was statistically insignificant). Both compared asymmetries induced by focal brain injury and modified by cell treatment decreased with preceding sessions. Turning tendency at the end of open field testing remained a little to the left in contrast with T-maze habit learning task performance, reaching in the last training session comparable amount of correct responses to the left and to the right in all experimental groups.

Learning Asymmetry and Brain Injury Range

T-maze habit learning task Lateralization at 2nd day of training was also compared with range of brain damage (**Figure 5**). In contrast with statistically significant difference between OUA+PBS rats and all of three cell treated groups, in the level of learning asymmetry there were no significant differences between experimental groups in both lesion volume as well as cavity location and extension.

DISCUSSION

We found that the direction of considerable learning asymmetry resulting from focal brain injury was significantly inversed following intra-arterial infusion by each of the three examined populations of the human umbilical cord blood derived cells. Such common functional effect of applied therapy was observed although cell fractions differed in level of their neural commitment. Both the lateralization of T-maze habit learning task performance resulting from focal brain injury and the inversion of its direction following the cell infusion were observed only under specific circumstances, that is at the early stage of the task training. Such an inversion resulting from the cell infusion was not observed in any of asymmetric motor impairments that is left hind limb disabilities while walking along balance beam and vibrissae elicited left forelimb placing deficit. The inversion of asymmetry induced by unilateral brain injury followed by experimental treatment was also not observed in another cognitive task measuring animals behavioral flexibility and possibility to adapt to novel rules in the same environment. The asymmetry inversion resulting from the applied therapy in injured animals also did not concern the direction of forward movement of freely moving rats under anxiety as well as the direction of rotations induced by dopaminergic agonist apomorphine administration. Insignificant tendency to inverse the median direction of forward movement of freely moving rats at the early stage of open field testing sequence was observed only in OUA+D-0 rats. This is in agreement with our previous results that D-0 freshly isolated cells were the most effective in restoration of impaired function and decreasing lesion volume (15). Both the learning asymmetry and the freely move curvature improved along T-maze habit learning task training and open field consecutive testing.

In the present study we applied criteria of the experimental animal selection analysis. That is all included rats underwent testing in all of the considered behavioral tasks and subsequent histological analysis. Such approach seems to decrease experimental bias. It should be taken into consideration that each of the uninjured rat possess its natural asymmetry while solving T-maze task (32). Therefore, the individual animal asymmetry was overlapped by the individual vulnerability for cytotoxic brain injury which corresponded with the variable lesion volume observed in our study. Rats also differ in their natural capacity to restore or to compensate the impaired functions following brain damage, which corresponded with a lack of the correlation between the lesion volume and the behavioral tasks scores in our experiment. Such diversity was also influenced by the individual animal reactivity to xenogenic cell infusion. An evaluation of the cohort of experimental animals meeting selection criteria would provide possibility to compare more accurately the asymmetry of rats behaving under various circumstances.

To promote recovery of healthy brain tissue we used enriched environment housing conditions (29). Such housing, due to natural need of animals to explore surroundings, enables motor and cognitive exercising in three dimensional complex space equipped with various beams, ladders, platforms etc. differing in width, shape, and their position in space. Abundant social interactions additionally ensure establishment of proper functional recovery. Rearrangement of equipment within the cages during the experiment promoted rats abilities to find them serf in partially novel environment. Altogether, the above processes would facilitate a functional restoration and improve the cell infusion effectiveness by reducing focal brain injury induced asymmetry occurring under various circumstances. This is due to experimental rats were freely moving in enriched environment home cage and all activities, movements, paying attention or noting that something appeared were possible to the left as well as to the right side.

T-Maze Habit Learning Task Training

T-maze habit learning task employed "win-stay" paradigm rules in T-maze, which is rat needed to choose the arm of maze

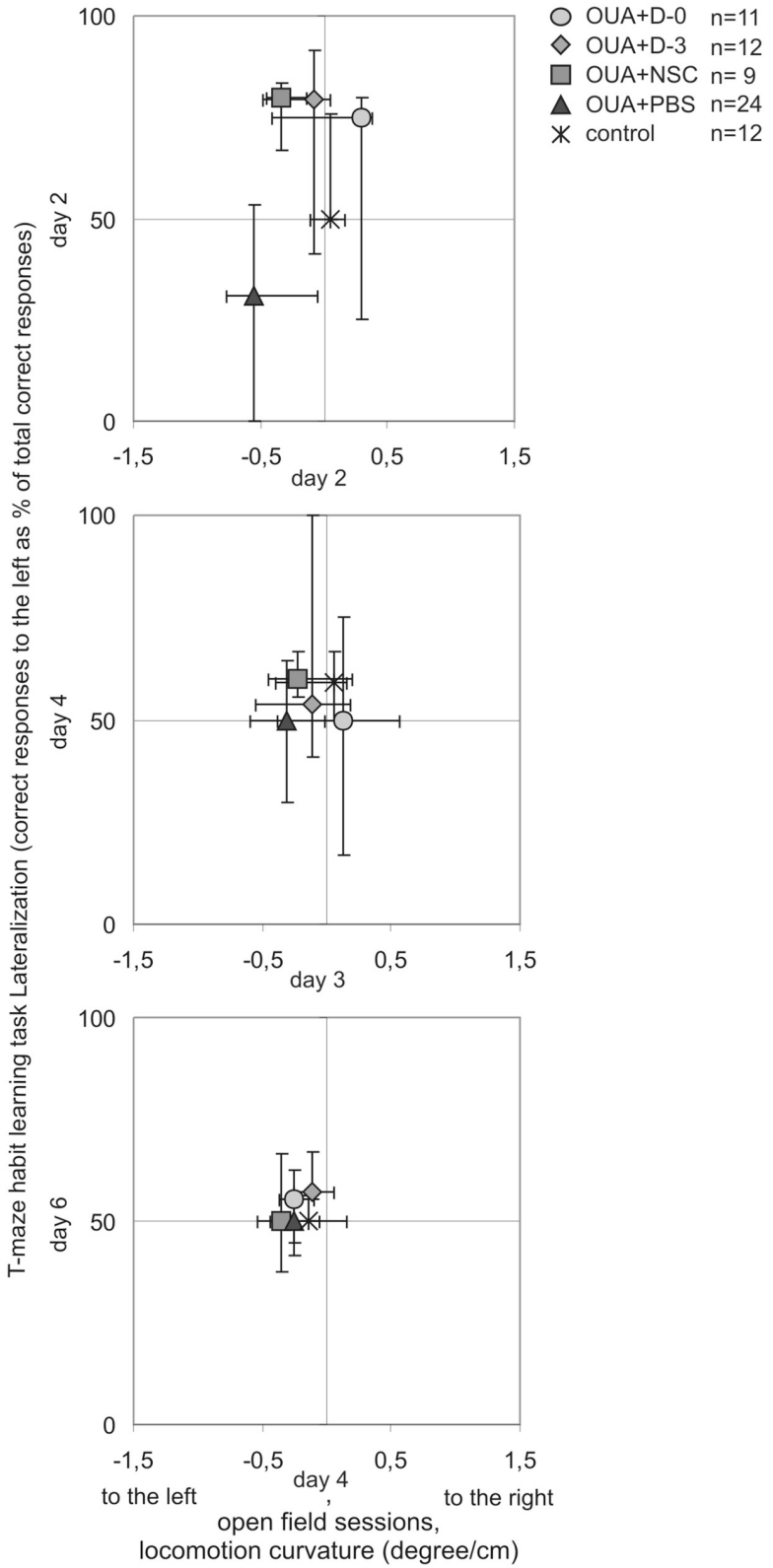


FIGURE 4 | Comparison of asymmetries by experimental animals along T-maze habit learning task training and consecutive open field sessions. Lateralization of cognitive task performance was juxtaposed with turning tendency of freely moving rats in corresponding subsequent days of testing (median ± 25 and 75%).

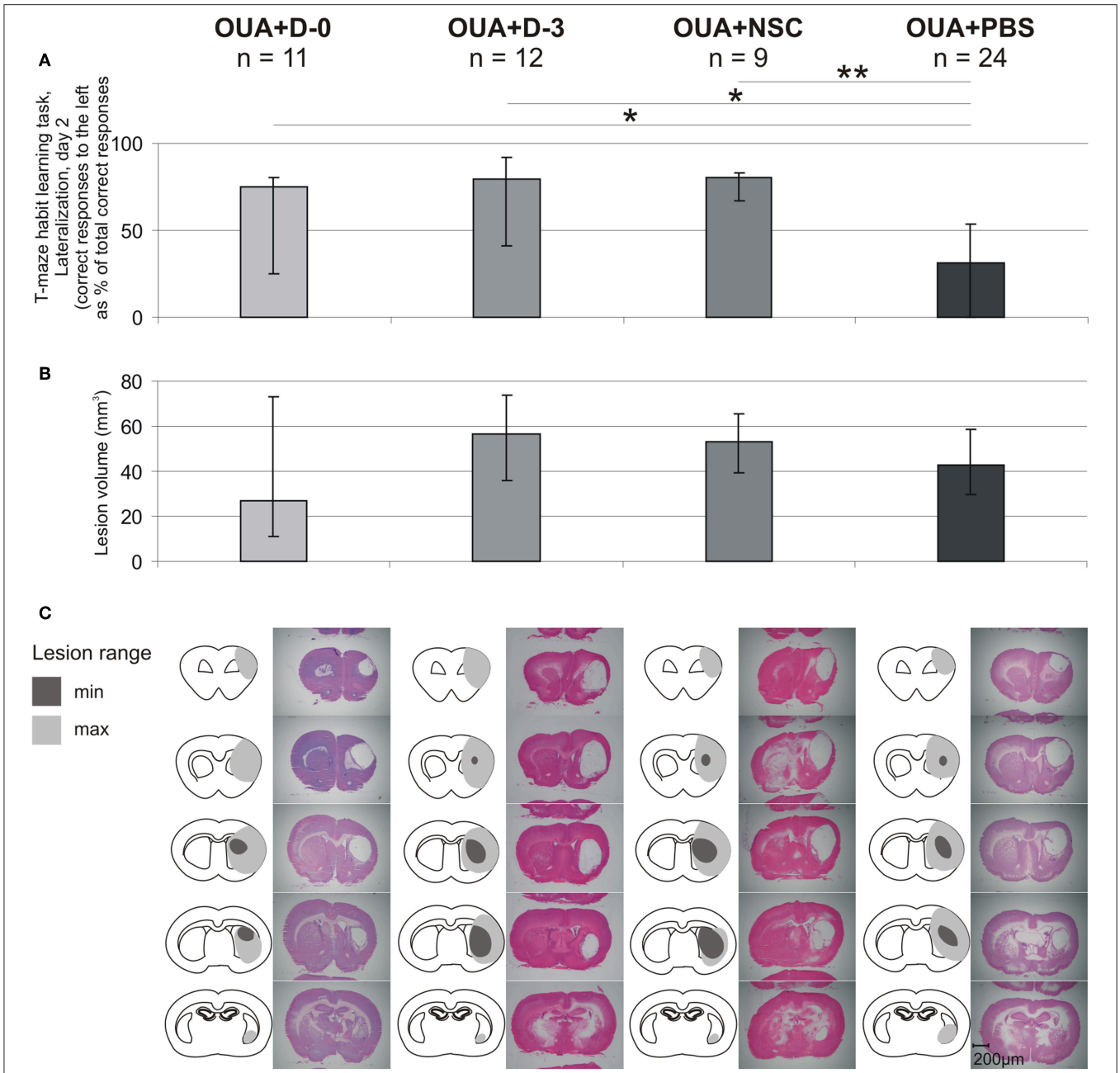


FIGURE 5 | Comparison of learning asymmetries and brain injury range. T-maze habit learning task Lateralization at 2nd day of training **(A)**, lesion volume **(B)**, lesion extent and representative images of H&E stained coronal brain slices **(C)** for each of experimental groups (median ± 25 and 75%; $^{*}p < 0.05$, $^{**}p < 0.01$ in Whitney-Mann test for non-matched pairs).

visited a while ago (28). On one hand the task requires that rat has to overcome natural preference to choose novel, previously unvisited environment. On the other hand if rat is anxious it prefer familiar, previously visited environment. Along 6 days of T-maze habit learning task training, rats at first were scared but explore the maze, learned that food reward was possible to find in one of food wells located at the end of each maze arms. Then the animals started to find out that the task was required from them. It occurred approximately on the second day of training. Then rats learned the task by the method of trials and mistakes. It seems to be raised up by natural drive to find the reward as result of first choice. Injured rats presented difficulties to acquire task design on about 5th day of training.

The most inclined task performance lateralization observed at second day of training and its inversion by the cell infusion would reflect how much the set of emotions at this stage

of training influenced decision making at the T-maze choice point. Accordingly, such set of processes would facilitate in injured animals turning into ipsilateral side, while as the effect of cell therapy turning into contralateral side were easier for experimental rats. Another explanation is that more correct responses when trial was designed to the left would reflect the positive therapy effect on social preference. This is due to experimental oversight, that means waiting boxes in which all experimental animals were placed at time of daily task training were located at left side of the T-maze. Such distribution of experimental equipment in space would influence rat's choice inside of enclosed training apparatus. It actually means that cell infusion with each of three examined populations would increase the choosing by experimental rat under specific circumstances T maze arm located closer to other animals, promoting this way preferences for being in company.

Experimental Animals Asymmetries Following Focal Brain Injury and Human Umbilical Cord Blood Derived Cells Systemic Treatment

The effects of systemic treatment of human umbilical cord blood derived cells on functional recovery following focal brain injury were studied before. Vendrame and coworkers assessed effectiveness of human umbilical cord blood derived cells doses (33). They showed, that direction as well as quantity of spontaneous rotations may significantly differ between 2 and 4 weeks following surgery depending on assessed cell dose.

The results of systemic treatment of human umbilical cord blood derived cells on cognitive impairments were studied in passive avoidance task (34), and water maze task (35). The authors showed cell treatment effects on learning deficits resulting from focal brain injury. Because of both tasks were designed not to be affected by eventual rat asymmetry resulting from focal brain injury, the issue of turning direction at time of tasks performance were not shown.

Effectiveness of systemic treatment with administration of the human umbilical cord blood derived cells on recovery of motor functions were also studied (35–37). In all experiments, the authors reported contralateral limbs impairments and tendency to improvement of impaired side of body following applied therapy. This is in agreement with our results concerning motor recovery following cell infusion.

Often used behavioral task was Neurological Severity Score (38–40). Such task allows to evaluate in details rat's reflexes, ability to move, body/muscle quality, possibility to flex. Because of such detailed assessing NSS is an abundant source of information concerning experimental animal asymmetry in various circumstances. The results are usually expressed as one graph describing overall rat recovery making possible to easy follow therapy effects on rats well-being. Such way of presenting shows general improvement, but unfortunately makes impossible to conclude and to compare the amount and the direction of experimental animal single asymmetries.

Our findings seem to contribute to understanding the effects of HUCB-derived cells treatment following focal brain injury, emphasizing the performance asymmetry of complex impaired by the brain lesion cognitive task at time of acquiring it. Knowledge concerning possible therapeutic effects of HUCB-derived cell treatment would to be beneficial in connection with public interest in umbilical cord tissue banking and stem cell transplantation. However, before translating our results into clinics, it should be taken into consideration that we assessed effects of human umbilical cord blood derived cells using animal model of man disease.

There are several limitations in our study. Our previous paper provided more extensive assessment of outcomes including cell survival in the same experimental set up. Here, we have focused on a very time-consuming and meticulous behavioral task, which revealed that therapeutic benefits can be also related to a very fine cognitive tasks, which may actually have a high impact on a human life. However, still we did not study the influence of intra-arterial xenograft delivery on the host immune response as well as we did not assess whole-body biodistribution of transplanted cells.

CONCLUSIONS

Intra-arterial infusion of cells derived from human umbilical cord blood inversed learning asymmetry caused by focal brain injury. The resulting effect of the applied therapy was independent on the infused cell progress in their neural commitment. Inversion of the asymmetry direction was observed only under specific circumstances, that is at the early stage of training in T-maze habit learning task. Cell infusion did not change significantly the direction of unilateral brain injury induced asymmetry visible in motor tests, cognitive flexibility task as well as while rats were freely moving in novel open arena or administered with dopaminergic agonist apomorphine. Insignificant tendency to shift the median direction of forward movement was only observed in rats treated with undifferentiated cells at the early stage of open field tests sequence. The asymmetries decreased along consecutive testing and familiarization of experimental animals with tasks in both T-maze habit learning task and open field test. Inversion of learning asymmetry due to the applied therapy was also independent on range of the brain damage.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

All of the animal handling and experimental procedures were approved by IV Local Ethics Committee on Animal Care and Use (Ministry of Science and Higher Education, Warsaw, Poland).

AUTHOR CONTRIBUTIONS

EG-P carried out animals behavioral testing and histological analysis. MJ performed surgeries. AH carried out cell preparation

for infusion. AJ participated in surgery and cell preparation. JS promoted manuscript drafting and English proofing. BL supervised the project.

FUNDING

The study was supported by MSR&HE grant no. 0394/B/P01/2010/38 and the MMRC statutory grant no. 6.

ACKNOWLEDGMENTS

We would like to thank Prof. Krystyna Domanska-Janik for open-minded supervising the Project, introducing therapeutic designs as well as principles of medical approaches. We also would like to thank Dr. Robert Kuba Filipkowski for supplying the idea to evaluate amount of correct responses made by experimental rats when T-maze habit learning task trial was designed to the left.

REFERENCES

1. Hara Y. Brain plasticity and rehabilitation in stroke patients. *J Nippon Med Sch*. (2015) 82:4–13. doi: 10.1272/jnms.82.4
2. Fitch MT, Silver J. CNS injury, glial scars, and inflammation: inhibitory extracellular matrices and regeneration failure. *Exp Neurol*. (2008) 209:294–301. doi: 10.1016/j.expneurol.2007.05.014
3. Schallert T, Leasure JL, Kolb B. Experience-associated structural events, subependymal cellular proliferative activity, and functional recovery after injury to the central nervous system. *J Cereb Blood Flow Metab.* (2000) 20:1513–28. doi: 10.1097/00004647-200011000-00001
4. Canazza A, Minati L, Boffano C, Parati E, Binks S. Experimental models of brain ischemia: a review of techniques, magnetic resonance imaging, and investigational cell-based therapies. *Front Neurol*. (2014) 5:19. doi: 10.3389/fneur.2014.00019
5. Hicks A, Jolkkonen J. Challenges and possibilities of intravascular cell therapy in stroke. *Acta Neurobiol Exp.* (2009) 69:1–11.
6. Janowski M, Date I. Systemic neurotransplantation–a problem-oriented systematic review. *Rev Neurosci*. (2009) 20:39–60. doi: 10.1515/REVNEURO.2009.20.1.39
7. Nagpal A, Choy FC, Howell S, Hillier S, Chan F, Hamilton-Bruce MA, et al. Safety and effectiveness of stem cell therapies in early-phase clinical trials in stroke: a systematic review and meta-analysis. *Stem Cell Res Ther*. (2017) 8:191. doi: 10.1186/s13287-017-0643-x
8. Satani N, Savitz SI. Is immunomodulation a principal mechanism underlying how cell-based therapies enhance stroke recovery? *Neurotherapeutics*. (2016) 13:775–82. doi: 10.1007/s13311-016-0468-9
9. Tandon PN, Seth P. Cell therapy for neurological disorders: the elusive goal. *Neurol India*. (2016) 64:612–23. doi: 10.4103/0028-3886.185418
10. Domanska-Janik K, Habich A, Sarnowska A, Janowski M. Neural commitment of cord blood stem cells (HUCB-NSC/NP): therapeutic perspectives. *Acta Neurobiol Exp.* (2006) 66:279–91.
11. Park DH, Borlongan CV, Willing AE, Eve DJ, Cruz LE, Sanberg CD, et al. Human umbilical cord blood cell grafts for brain ischemia. *Cell Transpl*. (2009) 18:985–98. doi: 10.3727/096368909X471279
12. Peberdy L, Young J, Kearney L. Health care professionals' knowledge, attitudes and practices relating to umbilical cord blood banking and donation: an integrative review. *BMC Pregn Childbirth*. (2016) 16:81. doi: 10.1186/s12884-016-0863-6
13. Buzanska L, Jurga M, Stachowiak EK, Stachowiak MK, Domanska-Janik K. Neural stem-like cell line derived from a non-hematopoietic population of human umbilical cord blood. *Stem Cells Dev*. (2006) 15:391–406. doi: 10.1089/scd.2006.15.391
14. Habich A, Jurga M, Markiewicz I, Lukomska B, Bany-Laszewicz U, Domanska-Janik K. Early appearance of stem/progenitor cells with neural-like characteristics in human cord blood mononuclear fraction cultured *in vitro*. *Exp Hematol.* (2006) 34:914–25. doi: 10.1016/j.exphem.2006.03.010
15. Gornicka-Pawlak el B, Janowski M, Habich A, Jablonska A, Drela K, Kozlowska H, et al. Systemic treatment of focal brain injury in the rat by human umbilical cord blood cells being at different level of neural commitment. *Acta Neurobiol Exp.* (2011) 71:46–64.
16. Janowski M, Lyczek A, Engels C, Xu J, Lukomska B, Bulte JW, et al. Cell size and velocity of injection are major determinants of the safety of intracarotid stem cell transplantation. *J Cereb Blood Flow Metab.* (2013) 33:921–7. doi: 10.1038/jcbfm.2013.32
17. Cui LL, Kerkela E, Bakreen A, Nitzsche F, Andrzejewska A, Nowakowski A, et al. The cerebral embolism evoked by intra-arterial delivery of allogeneic bone marrow mesenchymal stem cells in rats is related to cell dose and infusion velocity. *Stem Cell Res Ther*. (2015) 6:11. doi: 10.1186/scrt544
18. Walczak P, Wojtkiewicz J, Nowakowski A, Habich A, Holak P, Xu J, et al. Real-time MRI for precise and predictable intra-arterial stem cell delivery to the central nervous system. *J Cereb Blood Flow Metab*. (2017) 37:2346–58. doi: 10.1177/0271678X16665853
19. Jablonska A, Shea DJ, Cao S, Bulte JW, Janowski M, Konstantopoulos K, et al. Overexpression of VLA-4 in glial-restricted precursors enhances their endothelial docking and induces diapedesis in a mouse stroke model. *J Cereb Blood Flow Metab*. (2018) 38:835–46. doi: 10.1177/0271678X17703888
20. Andrzejewska A, Nowakowski A, Grygorowicz T, Dabrowska S, Orzel J, Walczak P, et al. Single-cell, high-throughput analysis of cell docking to vessel wall. *J Cereb Blood Flow Metab*. (2018). doi: 10.1177/0271678X18805238. [Epub ahead of print].
21. Janowski M, Gornicka-Pawlak E, Kozlowska H, Domanska-Janik K, Gielecki J, Lukomska B. Structural and functional characteristic of a model for deep-seated lacunar infarct in rats. *J Neurol Sci*. (2008) 273:40–8. doi: 10.1016/j.jns.2008.06.019
22. Veldhuis WB, van der Stelt M, Delmas F, Gillet B, Veldink GA, Vliegenthart JF, et al. *In vivo* excitotoxicity induced by ouabain, a Na+/K+-ATPase inhibitor. *J Cereb Blood Flow Metab*. (2003) 23:62–74. doi: 10.1097/01.WCB.0000039287.37737.50
23. Suvorov NF, Shuvaev VT. The role of the basal ganglia in organizing behavior. *Neurosci Behav Physiol.* (2004) 34:229–34. doi: 10.1023/B:NEAB.0000012800.96010.5e
24. Kantak KM, Green-Jordan K, Valencia E, Kremin T, Eichenbaum HB. Cognitive task performance after lidocaine-induced inactivation of different sites within the basolateral amygdala and dorsal striatum. *Behav Neurosci.* (2001) 115:589–601. doi: 10.1037//0735-7044.115.3.589
25. Yin HH, Knowlton BJ. Contributions of striatal subregions to place and response learning. *Learn Mem.* (2004) 11:459–63. doi: 10.1101/lm.81004
26. D'Amore DE, Tracy BA, Parikh V. Exogenous BDNF facilitates strategy set-shifting by modulating glutamate dynamics in the dorsal striatum. *Neuropharmacology.* (2013) 75:312–23. doi: 10.1016/j.neuropharm.2013.07.033
27. Gornicka-Pawlak E, Jablonska A, Chylinski A, Domanska-Janik K. Housing conditions influence motor functions and exploratory behavior following focal damage of the rat brain. *Acta Neurobiol Exp.* (2009) 69:62–72.
28. Gornicka-Pawlak E, Janowski M, Jablonska A, Sypecka J, Domanska-Janik K. Complex assessment of distinct cognitive impairments following ouabain injection into the rat dorsoloateral striatum. *Behav Brain Res*. (2015) 289:133–40. doi: 10.1016/j.bbr.2015.03.061
29. Benefiel AC, Dong WK, Greenough WT. Mandatory "enriched" housing of laboratory animals: the need for evidence-based evaluation. *ILAR J*. (2005) 46:95–105. doi: 10.1093/ilar.46.2.95
30. Woodlee MT, Asseo-Garcia AM, Zhao X, Liu SJ, Jones TA, Schallert T. Testing forelimb placing "across the midline" reveals distinct, lesion-dependent patterns of recovery in rats. *Exp Neurol*. (2005) 191:310–7. doi: 10.1016/j.expneurol.2004.09.005

31. Drai D, Golani I. SEE: a tool for the visualization and analysis of rodent exploratory behavior. *Neurosci Biobehav Rev*. (2001) 25:409–26. doi: 10.1016/S0149-7634(01)00022-7
32. Schwarting RK, Borta A. Analysis of behavioral asymmetries in the elevated plus-maze and in the T-maze. *J Neurosci Methods*. (2005) 141:251–60. doi: 10.1016/j.jneumeth.2004.06.013
33. Vendrame M, Cassady J, Newcomb J, Butler T, Pennypacker KR, Zigova T, et al. Infusion of human umbilical cord blood cells in a rat model of stroke dose-dependently rescues behavioral deficits and reduces infarct volume. *Stroke*. (2004) 35:2390–5. doi: 10.1161/01.STR.0000141681.06735.9b
34. Borlongan CV, Hadman M, Sanberg CD, Sanberg PR. Central nervous system entry of peripherally injected umbilical cord blood cells is not required for neuroprotection in stroke. *Stroke*. (2004) 35:2385–9. doi: 10.1161/01.STR.0000141680.49960.d7
35. Makinen S, Kekarainen T, Nystedt J, Liimatainen T, Huhtala T, Narvanen A, et al. Human umbilical cord blood cells do not improve sensorimotor or cognitive outcome following transient middle cerebral artery occlusion in rats. *Brain Res*. (2006) 1123:207–15. doi: 10.1016/j.brainres.2006.09.056
36. Huang L, Liu Y, Lu J, Cerqueira B, Misra V, Duong TQ. Intraarterial transplantation of human umbilical cord blood mononuclear cells in hyperacute stroke improves vascular function. *Stem Cell Res Ther*. (2017) 8:74. doi: 10.1186/s13287-017-0529-y
37. Karlupia N, Manley NC, Prasad K, Schafer R, Steinberg GK. Intraarterial transplantation of human umbilical cord blood mononuclear cells is more efficacious and safer compared with umbilical cord mesenchymal stromal cells in a rodent stroke model. *Stem Cell Res Ther*. (2014) 5:45. doi: 10.1186/scrt434
38. Chen J, Sanberg PR, Li Y, Wang L, Lu M, Willing AE, et al. Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. *Stroke*. (2001) 32:2682–8. doi: 10.1161/hs1101.098367
39. Hocum Stone LL, Xiao F, Rotschafer J, Nan Z, Juliano M, Sanberg CD, et al. Amelioration of ischemic brain injury in rats with human umbilical cord blood stem cells: mechanisms of action. *Cell Transplant*. (2016) 25:1473–88. doi: 10.3727/096368916X691277
40. Xiao J, Nan Z, Motooka Y, Low WC. Transplantation of a novel cell line population of umbilical cord blood stem cells ameliorates neurological deficits associated with ischemic brain injury. *Stem Cells Dev*. (2005) 14:722–33. doi: 10.1089/scd.2005.14.722

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.

Copyright © 2019 Gornicka-Pawlak, Janowski, Habich, Jablonska, Sypecka and Lukomska. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership