BRAIN INJURY AND REPAIR FOLLOWING CEREBROVASCULAR DISEASES: FROM BENCH TO BEDSIDE

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BRAIN INJURY AND REPAIR FOLLOWING CEREBROVASCULAR DISEASES: FROM BENCH TO BEDSIDE

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Editorial: Brain injury and repair following cerebrovascular diseases: From bench to bedside

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Editorial on the Research Topic
Brain injury and repair following cerebrovascular diseases: From bench to bedside

Endogenous attempts of self-repair after cerebrovascular disorders are complex. Brain remodeling processes can therapeutically be stimulated. Underlying cellular and molecular mechanisms remain insufficiently characterized. This issue aims to clarify mechanisms of brain injury and endogenous repair in cerebrovascular disorders.

Clearance of waste products from the brain is important for self-repair under conditions of cerebrovascular injury. In their review, Jiachen et al. addressed the structural composition and main components of clearance systems in the brain, which include the glymphatic system and the meningeal lymphatic system that closely interact with neural cells, such as astrocytes and microglia, to carry out vital clearance functions. In another review on drainage systems, Sichao et al. concluded the glymphatic system plays a dual role in the process of cerebral edema after stroke with a harmful role in the early stage of edema formation and a beneficial role during edema resolution. The function of glymphatic system is supported by astrocytic AQP4. In another review, Rentang et al. discussed the dual roles of microglia in the brain after intracerebral hemorrhage (ICH). M1 microglia are widely considered as the deleterious phenotype which arouse acute inflammatory responses, oxidative stress, excitotoxicity, and cytotoxicity to cause neuron death, while M2 microglia is regarded as the beneficial phenotype which inhibits inflammation, clears hematoma and promotes tissue regeneration. Jiaxin et al. reviewed the mechanistic signaling pathways of erythrophagocytosis and highlights the potential of harnessing macrophage (Mϕ)

mediated phagocytosis for hematoma removal. This process is mediated by scavenger receptors and the upstream regulators for erythrophagocytosis such as PPARγ, Nrf-2, AMPK and others. Linqian et al. discussed the effects and mechanisms of oxidative stress after ICH and its relationship with inflammation and autophagy, as well as the current state of antioxidant therapies in ICH.

In an original article, Bing et al. investigated the role of mast cells (MCs) in subarachnoid hemorrhage (SAH) and showed that MC activation contributed to brain edema and neurological impairment after SAH. Furthermore, MC-derived tryptase exacerbates microglia-related neuroinflammation by interacting with microglial PAR-2. In another original article, Weijia et al. showed that L-F001 was an efficient antioxidant and ferroptosis inhibitor, which significantly restored RSL3-induced broken iron homeostasis, reduced lipid peroxidation, and JNK overactivation in HT22 cells.

Jie et al. reviewed the relationship between ICH and sepsis, which were mutually exacerbated via similar pathophysiological mechanisms, which involved systemic inflammation and vascular dysfunction. Zeyu et al. discussed the role of gut microbiota post stroke. They highlighted the possible utility of gut microbiota as a therapeutic target in ischemic stroke.

Shuo-Qi et al. summarized the role of sphingosine-1-phosphate (S1P) signaling in brain ischemia with specific focus on inflammation and immune responses, and discussed the current and future perspectives of targeting S1P for ischemic stroke treatment. Shuiping et al. reviewed the effects of low-frequency transcranial ultrasound stimulation (TUS) on ischemic brain injury, with specific focus on mechanical actions, microvascular flow, thrombus resolution, as well as infarct volume reduction post stroke. Hui et al. summarized the effects of physical exercise on ischemic injury with specific focus on blood-brain barrier integrity, angiogenesis, neuroprotection, and functional neurological recovery. Rui et al. evaluated evidence on the therapeutic potential of remote ischemic conditioning (RIC) under conditions of vascular cognitive impairment (VCI), with specific focus on blood pressure control, secondary stroke prevention, cerebral blood flow, microvascular integrity, white matter remodeling, oxidative stress, and brain inflammatory responses. RIC was judged to be a potential promising treatment of VCI.

In summary, the contributions of this Research Topic provide a timely and comprehensive overview on current research activities regarding mechanisms of brain injury and self-repair in the cerebrovascular field. Novel mechanisms and disease targets were identified. Preclinical studies pointed out the possible clinical potential of therapeutic interventions. Yet, the clinical utility of these strategies requires further scrutiny, before studies in human patients can be considered. As editors, we would like to thank the authors for their contributions. We hope that this Research Topic provides a useful stimulus to the readers for subsequent studies, which might pave the way for clinical studies in humans.

Author contributions

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

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Conflict of interest

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Therapeutic Potential of Remote Ischemic Conditioning in Vascular Cognitive Impairment

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Vascular cognitive impairment (VCI) is a heterogeneous disease caused by a variety of cerebrovascular diseases. Patients with VCI often present with slower cognitive processing speed and poor executive function, which affects their independence in daily life, thus increasing social burden. Remote ischemic conditioning (RIC) is a non-invasive and efficient intervention that triggers endogenous protective mechanisms to generate neuroprotection. Over the past decades, evidence from basic and clinical research has shown that RIC is promising for the treatment of VCI. To further our understanding of RIC and improve the management of VCI, we summarize the evidence on the therapeutic potential of RIC in relation to the risk factors and pathobiologies of VCI, including reducing the risk of recurrent stroke, decreasing high blood pressure, improving cerebral blood flow, restoring white matter integrity, protecting the neurovascular unit, attenuating oxidative stress, and inhibiting the inflammatory response.

Keywords: vascular cognitive impairment, remote ischemic conditioning, cerebral blood flow, white matter, neurovascular unit, inflammation, oxidative stress

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INTRODUCTION

Vascular cognitive impairment (VCI) is the second most common type of cognitive dysfunction following Alzheimer's disease (AD) among those aged 65 years or older (Rockwood et al., 2000). In developing countries such as China, vascular-related mild cognitive impairment (MCI) is the most common type, accounting for 42% of all MCI cases (Jia et al., 2014). Impairments in the processing speed and executive function are distinctive symptoms of VCI, which significantly interfere with the ability to maintain independent living and increase the burden on society (Sachdev et al., 2004; Wallin et al., 2018). With an increasingly aging population, VCI is causing an increasing social burden. To this end, we should explore more effective treatment methods to control VCI.

Currently, interventions for VCI are largely based on prevention. Pharmacotherapy for VCI shows mild improvement and is majorly limited to vascular dementia (VaD), the severe form of VCI (Gorelick et al., 2011), or VCI mixed with AD (Shaji et al., 2018). VCI involves multiple pathobiological changes. Cerebral hypoperfusion and damage to the neurovascular unit (NVU) are postulated as the main pathobiological mechanisms in VCI (Sinha et al., 2020; Song et al., 2020). In addition, oxidative stress and the inflammatory response have also been identified as important contributors. Therefore, a "multitarget" therapeutic intervention may prove to be more effective in managing this disease.

Remote ischemic conditioning (RIC) is a non-pharmacological and non-invasive intervention that can activate multiple biological signaling pathways to generate endogenous neuroprotective effects (Hao et al., 2020; Ripley et al., 2021). Recently, RIC has emerged as a promising intervention for the prevention and treatment of cognitive impairment due to cerebrovascular disorders (see **Table 1**). A better understanding of the mechanisms in this process will assist clinicians in translating this simple and efficient therapy into clinical benefits.

In this review, we focus on the evidence of the protective effects of RIC on VCI from basic and clinical research. It includes the modifiable effect of RIC on the risk factors and pathobiologies of VCI, such as recurrent stroke, hypertension, cerebral blood flow (CBF), white matter (WM) injury, NVU, oxidative stress, and inflammation response.

VASCULAR COGNITIVE IMPAIRMENT

Vascular cognitive impairment refers to a decline in cognitive function attributable to vascular risk factors or cerebrovascular diseases as well as mixed pathologies such as AD (Gorelick et al., 2011; Skrobot et al., 2017), ranging from mild VCI to major VCI (VaD). Major VCI can be further divided into four subtypes: post-stroke dementia, subcortical ischemic vascular dementia, multi-infarct dementia (MID), and mixed dementia (Skrobot et al., 2017).

Brief History of VCI

The evolution of the terminology used to characterize cognitive impairment with a vascular origin reflects our improvement in understanding this disease. Approximately 40 years ago, the term MID was introduced to describe cognitive deficits due to large or small cerebral infarctions (Hachinski et al., 1974). It was considered as one of the two main pathologies of dementia, the other being "Alzheimer like changes" (e.g., senile plaques, neurofibrillary tangles, and granulovacuolar degeneration). Later, a variety of vascular pathologies have been shown to be associated with cognitive impairment, and thus the term "VaD" was used for a more accurate generalization of this disease (Roman et al., 1993). However, cognitive decline is a consecutive process, and patients with MCI may be ignored using this term. VCI has been proposed to cover the full spectrum of cognitive impairment. Other terminologies are also widely used, such as vascular cognitive impairment and dementia. Here, we use "VCI" to refer to the full range of severity of this disease.

Risk Factors of VCI

Over the past decades, a substantial drop in the incidence and prevalence of dementia has been observed in high-income countries (Hachinski et al., 2019). A combination of improved management of vascular risk factors and increased cognitive reserve (such as a higher level of education) may explain the trend (Wu et al., 2017).

Stroke is a strong modifiable risk factor for VCI (Portegies et al., 2016). It has been reported that 40% of stroke patients exhibit MCI, 1 year after stroke onset (Sexton et al., 2019). Moreover, 10% of patients with first-ever stroke develop new-onset dementia, and about 40% of patients with recurrent stroke have dementia (Pendlebury and Rothwell, 2009). Compared with stroke-free individuals, stroke increases the risk of dementia by almost twofold and the risk of recurrent stroke by threefold (Kuzma et al., 2018).

Hypertension is another important modifiable risk factor for VCI. There is consistent evidence that hypertension, especially midlife (45–55 years of age) hypertension is associated with late-life cognitive decline and VCI (Launer et al., 2000; Yamada et al., 2009; Gottesman et al., 2014). For individuals with prehypertension [systolic blood pressure (SBP) of 120–139 mmHg or diastolic blood pressure (DBP) of 80–89 mmHg], the risk of dementia was almost as high as that of patients with hypertension, and approximately 30% higher than that of patients with normotension (Gottesman et al., 2017).

Other modifiable risk factors include hypercholesterolemia, diabetes mellitus, alcohol consumption, obesity, depression, and smoking, where diabetes mellitus is a well-established risk factor for all causes of dementia (Gorelick et al., 2011; Iadecola et al., 2019). As for non-modifiable risk factors, there is a strong link between aging and VCI. Aging is associated with VaD, post-stroke dementia, unspecified dementia, and Alzheimer's dementia (Yang T. et al., 2017; Iadecola et al., 2019). In contrast, higher education and physical activities are thought to have a protective role in VCI (Najar et al., 2019; Jia et al., 2020).

Pathobiologies of VCI

The underlying pathobiologies of VCI have been attributed to multiple mechanisms. Altered vascular structures or functions, such as cerebral hypoperfusion, blood brain barrier (BBB) disruption and CBF dysregulation, have long been implicated in the pathogenesis of VCI (Iadecola, 2013). Disruption of the structural and functional integrity of the NVU is increasingly recognized as a key contributor to VCI, which provides a conceptual framework for studying the pathogenesis of VCI (Li et al., 2021). Additionally, other mechanisms have also been suggested to be involved in this process, including but not limited to oxidative stress, neuroinflammation, and impairment of glymphatic system clearance (Joutel and Chabriat, 2017; Zlokovic et al., 2020). It seems that these mechanisms interact with each other, and it is difficult to identify a temporal and causal relationship between them.

Cerebral Hypoperfusion and CBF Dysregulation

There is a general consensus that cerebral hypoperfusion is an etiological factor for VCI. Upon an acute or chronic reduction in CBF, the oxygen and nutrient supply to the brain is compromised, leading to tissue damages both in the gray matter and/or WM (van der Flier et al., 2018). These pathological changes can affect brain network integrity and impair cognitive function (Cremers et al., 2020). The causal relationship between cerebral hypoperfusion and VCI is supported by both human and animal studies (Duncombe et al., 2017; Wolters et al., 2017; Moonen et al., 2021). However, there are contrary observations of decreased CBF being a result of WMHs or aging of the brain instead of a casual mechanism

TABLE 1 | Clinical and animal evidence of RIC in VCI.

Clinical study

Patients	Location of RIC	Timing of RIC	Cycles	Protocol	Cognitive function	Physiological/molecular mechanisms	References
Patients with first-time non-cardiac ischemic stroke (n = 104)	Upper limb	Within 14 days after an ischemic stroke	5 min × 5 min	Daily for 6 months	↑MoCA	↑Cerebral hemodynamics ↓ICAM-1,ET-1	Feng et al., 2019
Patients with post-stroke cognitive impairment (n = 48)	Upper limb	Not mentioned	4 min × 5 min	Daily for 7 days	↑MoCA ↑ADAS-cog	Not mentioned	Li et al., 2020
Patients with subcortical ischemic vascular dementia (n = 37)	Bilateral upper limb	Cognitive impairment lasting for at least 3 months	5 min × 5 min	Daily for 6 months	↑JLO	↓WMLV ↓Hs-CRP	Liao et al., 2019
Patients with intracranial arterial stenosis (n = 58)	Bilateral upper arm	Within 7 days after an ischemic stroke or TIA	5 min × 5 min	Twice daily for 300 days	↑MoCA,MMSE	↓WMHs	Zhou et al., 2019
Patients with cerebral small-vessel disease-related mild cognitive impairment (n = 30)	Bilateral upper limb	Cognitive impairment lasting for at least 3 months	5 min × 5 min	Twice daily for 1 year	↑Visuospatial and executive ability	↓WMHs ↓Pulsation indices in MCA ↓Plasma triglyceride, total cholesterol, low-density lipoprotein, and homocysteine	Wang Y. et al., 2017
Patients with cerebral small vessel disease (n = 17)	Bilateral upper arm	Not mentioned	5 min × 5 min	Twice daily for 1 year	No effect	↓WMHs ↑Flow velocity in MCA	Mi et al., 2016

Animal study

Animal model	Location of RIC	Timing of RIC	Cycles	Protocol	Cognitive function	Physiological/molecular mechanisms	References
SD rats; BCAO	Bilateral hind limbs	Three days after the hypoperfusion	3 min × 10 min	Daily for 28 days	↑Morris water maze	↑CBF, angiogenesis ↑NOS/NO	Ren et al., 2018a
C57BL/6J mice; BCAS	Bilateral hind limbs	One week after the surgery	4 min × 5 min	Daily for 1 month or 4 months	↑NOR test, Y-maze test	↑WM integrity ↑CBF, angiogenesis	Khan et al., 2018
C57BL/6J mice; BCAS	Bilateral hind limbs	One week after the surgery	4 min × 5 min	Daily for 2 weeks	↑NOR test	↑WM integrity	Khan et al., 2015

(Continued)

TABLE 1 | Continued

Animal study							
Animal model	**Location of RIC**	**Timing of RIC**	**Cycles**	**Protocol**	**Cognitive function**	**Physiological/molecular mechanisms**	**References**
						↓neuroinflammation ↓Aβ aggregation	
SD rats; BCAO	Bilateral hind limbs	Three days after the hypoperfusion	3 min × 10 min	Daily for 28 days	↑Morris water maze	↑WM integrity ↑PTEN/Akt/mTOR	Li et al., 2017
Wistar rats; occluding both carotid arteries for 20 min, followed by reperfusion	Bilateral hind limbs	After the surgery	3 min × 10 min	Once	↑Morris water maze, passive avoidance task, Y-maze	↓Oxidative stress, inflammation ↑HO-1,BDNF	Ramagiri and Taliyan, 2017b
Wistar rats; occluding both carotid arteries for 20 min, followed by reperfusion	Bilateral hind limbs	Immediately following reperfusion	3 min × 10 min	Once	↑Morris water maze, passive avoidance task, Y-maze	↓Oxidative stress, inflammation ↑GSK-3β/CREB/BDNF	Ramagiri and Taliyan, 2017a
Wistar mice; BCAS	Bilateral hind limbs	Two weeks after surgery	4 min × 5 min	Daily for 2 weeks	↑ NOR test	↑Autophage	Wang H. et al., 2017
Swiss albino mice; occluding both carotid arteries for 20 min, followed by reperfusion	Hind limb	Before surgery	4 min × 5 min	Once	↑Morris water maze	↓Oxidative stress	He et al., 2019
SD rats; tMCAO for 60 min	Right hind-limb	Before surgery	3 min × 5 min	Once	↑Morris water maze	↑Cholinergic neurons in CA1 region	Hu et al., 2013
SD rats; occluding both carotid arteries for 20 min, followed by reperfusion	Right hind-limb	Before surgery	3 min × 10 min	Once	↑Morris water maze	↑Bcl-2	Xu et al., 2011
BULB/C mice; occluding both carotid arteries for 20 min, followed by reperfusion	Renal artery	24 h before surgery	3 min × 5 min	Once	↑Passive avoidance test	↑mTOR, SOD	Zare Mehrjerdi et al., 2013

MoCA, montreal cognitive assessment scale; ADAS-cog, Alzheimer's disease assessment scale-cognitive; JLO, judgment of line orientation; MMSE, mini-mental state examination; WM, white matter; WMLV, white matter lesion volume; WMHs, white matter hyperintensities; ICAM-1, intercellular adhesion molecule-1; ET-1, endothelin-1; Hs-CRP, high-sensitivity C-reactive protein; MCA, middle cerebral artery; NOR test, novel object recognition test; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; PTEN, phosphatase and tensin homolog; mTOR, mechanistic target of rapamycin; HO-1, hemeoxygenase -1; BDNF, brain derived neurotrophic factor; GSK-3β, glycogen synthase kinase 3 beta; CREB, cAMP response element-binding protein; CA1, cornu ammonis; Bcl-2, B-cell lymphoma 2; SOD, superoxide dismutase.

of VCI pathology (van der Veen et al., 2015; Shi et al., 2016), especially when accounting for genetic diseases, such as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, where WMHs may appear before the decline of CBF (Joutel and Chabriat, 2017). One possible explanation for this may be the disruption of the NVU (see section "Disruption of the Neurovascular Unit"). Thus, the casual role of cerebral hypoperfusion in VCI remains inconclusive and should be confirmed in future studies.

With a high metabolic rate but low energy storage, the brain needs a rapid and constant supply of CBF to maintain normal function. Cerebral autoregulation (CA) is one of the intrinsic mechanisms of regulating global CBF, and another mechanism of regional CBF regulation is neurovascular coupling (NVC) (see next part). CA enables the brain to maintain a stable CBF while BP fluctuates within a certain range, normally between and 60–150 mmHg (Lassen, 1964). When CA is impaired, cerebral perfusion passively follows BP fluctuation, which may lead to critical consequences in the microcirculation (Shekhar et al., 2017) and to cognitive impairment. It has been demonstrated that CA is often disturbed in patients with cerebrovascular diseases and is strongly associated with WMHs in the elderly (Immink et al., 2005; Purkayastha et al., 2014). However, it is unclear whether impaired CA is a cause or a consequence of VCI.

Disruption of the Neurovascular Unit

The NVU is an interconnected functional and anatomical structure composed of neurons, interneurons, glia, vascular cells, and the extracellular matrix (Levit et al., 2020). Components within the NVU act in concert to meet the energy demands of neuronal activity through the regulation of CBF. Meanwhile, they also play an important role in maintaining BBB integrity, trophic support, and waste clearance (Iadecola, 2017). It is not surprising that any damage to these cells will lead to dysfunction of the NVU, compromising cerebral hemodynamics and homeostasis, which in turn affect cognitive function. For instance, hypertension, a major risk factor of VCI, has a detrimental effect on the cerebrovascular system, from the large arteries to the capillaries, leading to cerebral hypoperfusion and NVU damage (de Montgolfier et al., 2019b; Presa et al., 2020). Moreover, elevated intravascular pressure can also directly cause BBB leakage and NVU disruption by targeting endothelial cells (Arlier et al., 2015), activating the glia (Eldahshan et al., 2019), and increasing ROS production (Faraco et al., 2016).

Neurovascular coupling is one of the most studied functions of the NVU, which emphasizes the active regulation of regional CBF in response to neural activity. The cellular and molecular bases of NVC have been previously well elucidated (Iadecola, 2017; Hosford and Gourine, 2019). In brief, neurons act as an initiator in the vascular response, which activates the downstream cells (interneurons, astrocytes, and endothelial cells), and finally leads to relaxation of vascular smooth muscle cells (VSMCs) or pericytes at different vascular levels (arteries or capillaries). This finely tuned regulation of CBF provides sufficient oxygen and nutrients for synaptic activities. Disruption of NVC will lead to cerebral hypoperfusion and contributes to the development of VCI. Neurovascular uncoupling has been demonstrated in various VCI animal models, such as of aging, hypertension, and cerebral hypoperfusion, indicating the important role of NVC in the pathogenesis of VCI (de Montgolfier et al., 2019a; Tarantini et al., 2019b; Chandran et al., 2020).

Pericytes have increasingly become a focus in VCI research. They comprise a pluripotent mural cell type mainly located in the capillaries, precapillary arterioles, and postcapillary venules (Uemura et al., 2020). Recently, a post-mortem study showed that there is substantial loss of pericytes in the WM of patients with VCI compared with controls without dementia (Ding R. et al., 2020). Several studies have reported that chronic or acute loss of pericytes can cause neurovascular uncoupling, followed by WM damage and cognitive impairment, indicating the important role of pericytes in CBF regulation (Montagne et al., 2018; Kisler et al., 2020). However, it remains unclear whether pericytes affect NVC directly via their contractility or indirectly through signal transmission. In contrast, there is evidence against the involvement of pericytes in the regulation of CBF (Hill et al., 2015). Moreover, pericyte loss was reported to cause BBB leakage via endothelial transcytosis in a rat model of CCH, which preceded neuroinflammation and WM damage (Sun et al., 2021). Furthermore, dysfunction of pericytes may also instigate BBB disruption through the increased secretion of collagen *in vitro* (Ozkan et al., 2021). Taken together, loss or dysfunction of pericytes can result in neurovascular uncoupling, BBB disruption, and cerebral hypoperfusion, which, in turn, lead to WM damage and cognitive impairment.

Endothelial dysfunction is a common feature of VCI caused by either large or small cerebrovascular diseases, which is tightly linked to CBF dysregulation, neuroinflammation, NVU dysfunction and loss of trophic support to glial cells (Pi et al., 2018). Abnormalities in NO production and endothelial nitric oxide synthase (eNOS) activity are important markers of endothelial dysfunction (Vanhoutte et al., 2016). Reduced NO bioavailability leads to vasoconstriction and compromised the regulation of CBF, which may exacerbate cerebral hypoperfusion and cognitive decline (Matin et al., 2018; Tarantini et al., 2018, 2019b). In support of this perspective, previous autopsy studies have reported a reduced endothelial-mediated vasodilation in WM lesions in VCI patients (Bagi et al., 2018). Furthermore, in the setting of inflammation, activated ECs can upregulate the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). These molecules promote the adhesion and migration of neutrophils and monocytes through the vascular wall and evoke neuroinflammation by stimulating glial cells. Activated endothelial cells have also been proved to increase vascular permeability by opening the intercellular junctions and may allow toxic proteins to leak into the central nerve system (CNS) (Donato et al., 2018). Moreover, ECs play a vital role in supporting the oligodendrocyte lineage cells within the oligovascular niche (Ohtomo and Arai, 2020). For instance, hypoxia-ischemia induces oligogenesis around vessels in a short duration, while it was inhibited with an injury of ECs occurring at the early stage (Wang X. et al., 2020). Thus, endothelium is a crucial component within the NVU, which provides a therapeutic target in the management of VCI.

Oxidative Stress

Oxidative stress refers to the imbalance between pro-oxidant and antioxidant systems, which results in the excessive production of reactive oxygen species (ROS) (Wojsiat et al., 2018). Patients with VCI have been reported to have higher levels of malondialdehyde (MDA) (Gustaw-Rothenberg et al., 2010), the end product and the hallmark of lipid peroxidation, and an altered antioxidant defense system, such as decreased superoxide dismutase and glutathione reductase (GR) (Casado et al., 2008). Nicotinamide adenine dinucleotide phosphate oxidases (NOXs) and the mitochondrial respiratory chain are the main sources of ROS (Choi et al., 2014; He et al., 2020). In VCI animal models, CCH and cerebral ischemia/reperfusion (I/R) injury lead to mitochondrial dysfunction and increased NOX expression, followed by an overload of ROS, lipid peroxidation, DNA fragmentation, protein oxidation, and phosphorylation. Finally, these pathological changes cause BBB disruption, endothelial dysfunction, neuronal damage, and cognitive decline (Choi and Lee, 2017; Han et al., 2020). As a matter of fact, oxidative stress is not only associated with VCI, but also plays a detrimental role in the pathogenesis of the risk factors for VCI, which contribute to the progression of cognitive impairment (Liyanagamage and Martinus, 2020; Touyz et al., 2020). Thus, targeting oxidative stress represents a potential therapy for the prevention and management of VCI.

Inflammation

Neuroinflammation, which is defined as inflammation involved in the CNS, is a common feature of VCI models. In bilateral common carotid artery occlusion (BCCAO) models, endothelial activation is likely to be an early event under chronic hypoperfusion. Activated ECs facilitate the attachment and extravasation of leukocytes across the BBB. These leukocytes, inflammatory cytokines, and blood products initiate the neuroinflammation by activating glia cells, which in turn aggravate WM damage and BBB injury (Duncombe et al., 2017). Furthermore, neuroinflammation can be enhanced by elevated systemic inflammation through the disrupted BBB. Aging, as the major risk factor for VCI, induces a shift in pro-inflammatory gene expression in ECs and VSMCs in the peripheral, including the induction of inflammatory cytokines, chemokines and adhesion molecules. This pro-inflammatory environment activates and recruits immune cells and ultimately induces a systemic inflammatory response. The resulting vascular inflammation leads to vascular dysfunction, BBB disruption, and consequentially exacerbated the development of VCI (Ungvari et al., 2018).

REMOTE ISCHEMIC CONDITIONING

Remote ischemic conditioning is a safe, simple, and cost-efficient approach to alleviate tissue damage in remote organs, such as the brain, the heart, and the kidney. By pressuring the limb repeatedly at a distance from the target organ using a blood pressure cuff, RIC can induce a non-lethal ischemic condition, which in turn stimulates the body and triggers endogenous protective mechanisms (Hess et al., 2015).

RIC was initially used as an once-occasion treatment to mitigate ischemic/reperfusion injury. Later, it was found that RIC has additional pleiotropic effects, such as improved endothelial function, anti-inflammation, and inhibition of platelet aggregation (Hausenloy et al., 2016). However, the protection period of single RIC is relatively short [generally for 72 h (Ren et al., 2008)], which may prevent its application in chronic conditions. To this end, repetitive daily RIC, termed chronic RIC, has gained much attention. It is able to confer a continuous and long-lasting effect, and thus, is more beneficial for the treatment of chronic diseases (Ding J. Y. et al., 2020). Although many studies on RIC have been carried out, the underlying mechanism still needs to be elucidated. Interestingly, it has been proposed that chronic RIC shares many common mechanisms with physical activities and is considered equivalent to exercise. The latter is currently recognized as an effective treatment for cognitive impairment (Zhao et al., 2018; Geng et al., 2021).

ROLE OF RIC IN MITIGATING VCI

Evidence has been shown that RIC can modify VCI-related risk factors and decrease its pathobiological alterations, which protect against cognitive decline and even improve cognitive function.

RIC Modifies the Risk Factors of VCI

Several risk factors that contribute to vascular injury and brain network disruption are modifiable. RIC may prevent VCI by reducing recurrent stroke and controlling high blood pressure (see **Figure 1**).

Patients with symptomatic intracranial atherosclerosis (sICAS), referred to as luminal stenosis >50%, are at a high risk of recurrent stroke (Hurford et al., 2020), even with standard single antiplatelet therapy (Chimowitz et al., 2005; Mazighi et al., 2006; Liu et al., 2015). Recently, the THALES trial showed that the use of dual antiplatelet therapy (DAPT) for 30 days may lead to a further reduction in the risk of early recurrence in patients with large artery atherosclerosis (Johnston et al., 2020). However, the long-term effects and safety of DAPT remain unknown.

Reduced Recurrent Stroke

Remote ischemic conditioning is a safe treatment and may be used as an adjuvant approach to the current standard therapy to reduce recurrent stroke in patients with sICAS. In a randomized RIC study, 68 sICAS patients (<80 years) who had a stroke or transient ischemic attack (TIA) within 30 days were consecutively enrolled, and all patients received standard medical treatment (Meng et al., 2012). RIC was performed using an electric device with blood pressure cuffs inflated to 200 mmHg for 5 min and deflated for 5 min for five cycles on bilateral arms twice daily. The rate of stroke recurrence in the RIC group was 5 and 7.9% at 90 and 300 days, respectively, compared to 23.3 and 26.7% in the control group. An improvement in cerebral

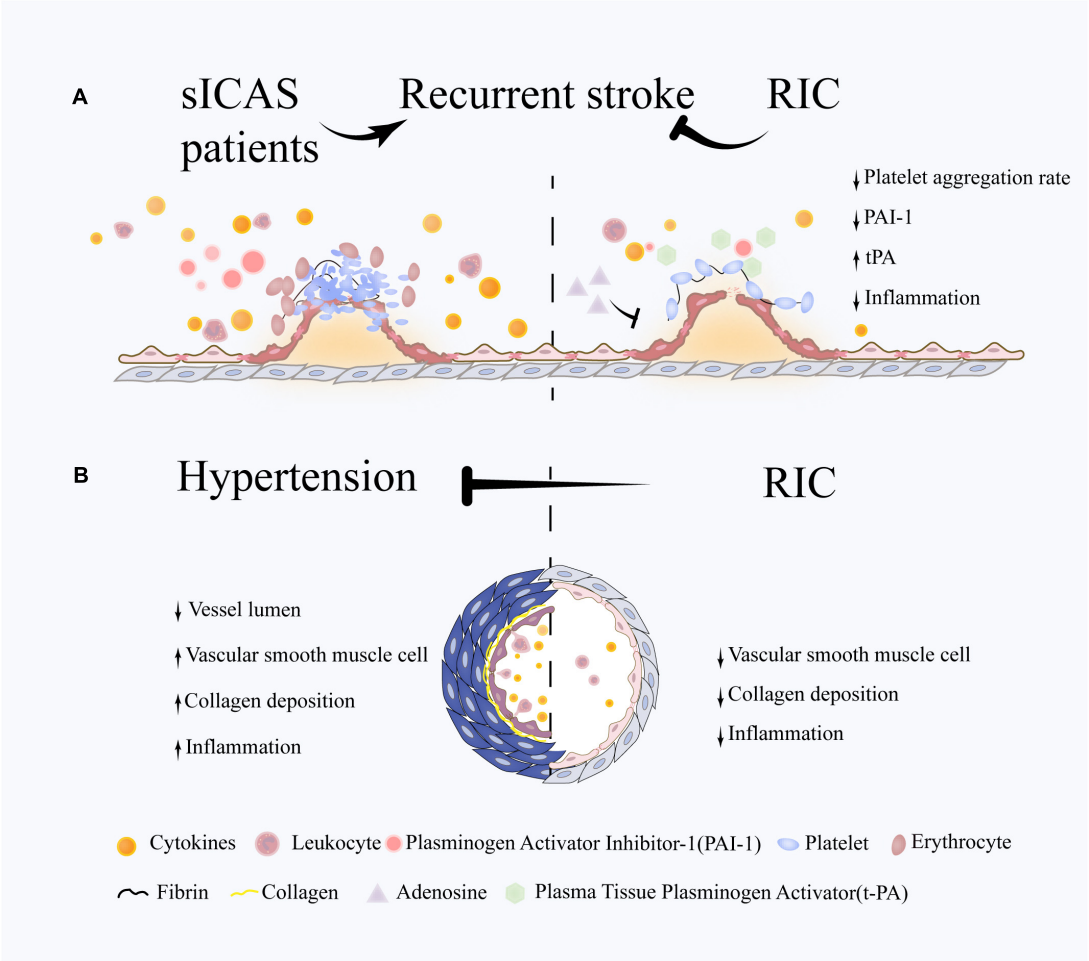

FIGURE 1 | Potential role of RIC on VCI-related risk factors. **(A)** Following an abrupt rupture of an atherosclerotic plaque, platelets respond rapidly through adhesion and aggregation, then thrombins generate a fibrin network to reinforce platelet aggregation and trap a large number of red blood cells, leading to the formation of thrombosis and vascular occlusion. The immune system are also involved in this process through activating the leukocytes and releasing large amount of cytokines. RIC is proven to reduce stroke recurrence in sICAS patients. The underlying mechanisms may involve the attenuation of excessive platelet aggregation and inflammation and the promotion of coagulation system. **(B)** Hypertension is characterized by and increased vascular resistance, which plays an important role in VCI. Vascular remodeling occurs at the early stage in hypertension and is associated with chronic inflammation response, hypertrophy of vascular smooth muscle cells (VSMCs) and deposition of extracellular matrix. RIC is efficient in blood pressure lowering in both hypertensive and prehypertensive patients. One of underlying mechanisms is the suppression of pathological vascular remodeling and downregulation of inflammation response.

hemodynamics was also observed at the 300 days follow-up. Later, the same study group demonstrated the efficiency of this protocol in patients with sICAS above 80 years of age (Meng et al., 2015). Compared with the control group, RIC showed no difference in terms of safety at the 30 days follow-up, such as local skin integrity and cerebral hemorrhage, while it significantly reduced the incidence of cerebrovascular events (2 infarctions and 7 TIA vs. 8 infarctions and 11 TIA, respectively) after 180 days of treatment. Additionally, the plasma levels of platelet aggregation rate and plasminogen activator inhibitor-1 were downregulated, while plasma tissue plasminogen activator were elevated in the RIC group for the initial 30 days, together with a reduction in hsCRP, interleukin-6 (IL-6), and leukocyte count. The inhibitory effect of RIC on coagulation has also been observed in other clinical and experimental studies (Ropcke et al., 2012; Lanza et al., 2016; Gorog et al., 2021). It was speculated that adenosine may play an important role in this process (Przyklenk and Whittaker, 2017). During the ischemic period of RIC, the concentration of adenosine is elevated, which is a potent inhibitor of platelet function by binding to the adenosine A2 receptor on the platelet surface. Thus, the beneficial effect of RIC on reducing recurrent stroke may be partially attributed to the inhibition of platelet activity.

Decreased Blood Pressure

A self-experimentation study on a 72-year-old man with normotension/prehypertension revealed that repeated RIC (twice daily for 10 days) decreased BP and had a persisting effect even 5 days after cessation with no rebound (Madias, 2015). Subsequently, two small self-controlled studies were designed

to verify the BP-lowering effect of RIC for a longer period in hypertension/prehypertension patients. The first clinical trial included patients who were newly diagnosed with essential hypertension and did not undergo any medical treatment. The patients received three cycles of RIC on the upper arm for 5 min per cycle for 30 days (Tong et al., 2019). Chronic RIC significantly reduced SBP by 8 mmHg and DBP by 6 mmHg and improved microvascular endothelial function, as measured by the finger reactive hyperemia index. The second study was conducted in elderly patients with prehypertension or grade one hypertension (Gao et al., 2021). None of the patients received any antihypertensive agents. Chronic RIC consists of five cycles of ischemia and reperfusion interval for 5 min per cycle on bilateral upper arms twice daily for 4 weeks. Compared with the baseline, the SBP and DBP were decreased by 10.2 and 5.4 mmHg, respectively, and the arterial stiffness is also improved, as measured by pulse wave velocity. Furthermore, the authors performed RIC in spontaneously hypertensive rats (SHRs), which are the most commonly used genetic animal models to study hypertension and can exhibit cognitive deficits and WMHs similar to those in humans (Lerman et al., 2019). Hypertension results in occlusive vascular remodeling and promotes arterial stiffness in the large cerebral arteries, as well as penetrating arterioles. These pathological changes lead to reduced vessel lumen and compromised cerebrovascular function (Iadecola and Gottesman, 2019). RIC for 6 weeks significantly decreased the BP, reduced the inflammatory response, and suppressed excessive vascular remodeling in the conduit arteries and small resistance arteries of the brain.

Thus, RIC is effective in lowering BP in individuals with hypertension/prehypertension, which may alter cerebral vasculature, improve vascular function, and protect against cognitive decline. Given that most patients with hypertension will receive antihypertensive medication, further investigation is needed to determine whether chronic RIC has synergistic effects with antihypertensive drugs.

Evidence of RIC on other VCI-related risk factors is not currently available. However, one clinical study explored the effect of RIC on vascular and neuronal function in patients with peripheral arterial diseases and type 2 diabetes mellitus (Hansen et al., 2019). The authors reported negative results and found no improvement in tissue oxygenation or other outcomes, indicating that diabetes may have implications for RIC. In contrast, a study has shown that RIC attenuated cerebral I/R injury, even in the presence of diabetes in a transient middle cerebral artery occlusion (tMCAO) animal model (Liu et al., 2020). The underlying mechanisms may be the suppression of excessive inflammation and activation of the brain ERK signaling pathways. However, it is difficult to draw a conclusion based on these limited and inconsistent evidence, but this indicates that diabetes may need to be considered in future studies.

In summary, chronic RIC is efficient in the management of risk factors for VCI. It can be implemented synergistically with antiplatelet therapy to reduce recurrent stroke in patients with sICAS and as an alternative intervention to treat hypertension partially by regulating the pathological process to the vasculature.

Potential Effects of RIC on VCI Pathobiologies

Pathobiological changes in VCI exhibit reciprocal causation, which aggravates the progression of this disease. Risk factors, such as hypertension and aging have been linked to inflammation, especially chronic inflammation. It promotes oxidative stress and leads to NVU disruption and vascular injury, resulting in compromised CBF and WM integrity, which influences higher-level cognitive process. The protective effects of RIC on VCI occur at different biological levels, encompassing a series of molecular changes (see **Figure 2**).

RIC Improves the Cerebral Blood Flow

Increasing evidence indicates that RIC can improve CBF in acute artery occlusion or chronic cerebral hypoperfusion. It has been reported that a single RIC treatment in preclinical studies significantly enhanced regional CBF after acute ischemic stroke (Hoda et al., 2012, 2014). Meanwhile, repeated daily RIC is efficient in improving global CBF in chronic ischemia conditions, such as sICAS and Moyamoya disease (MMD) (Meng et al., 2012; Ding J. Y. et al., 2020).

The promotion of vascular remodeling, including arteriogenesis and angiogenesis, may underlie the potential mechanisms of RIC on CBF. Arteriogenesis and angiogenesis are critical mechanisms for the maintenance of sufficient cerebral supply after acute or chronic CBF decline and the prevention of consequential cognitive decline (Freitas-Andrade et al., 2020).

Arteriogenesis, also known as collateral formation, leads to dilation and remodeling of preexisting capillaries into larger conductance vessels as a restoration of normal blood flow (Deindl and Quax, 2020). In a tMCAO mouse model, which is the most commonly used animal model of ischemic stroke, repeated daily RIC for 14 days showed a significant increase in CBF and promotion of arteriogenesis, as indicated by an increase in arterial diameter and vascular smooth muscle cell proliferation. And this was associated with elevated expression of Notch1 and Notch intracellular domain in the arteries of the peri-infarct area (Ren et al., 2018b). Notch proteins are a family of transmembrane proteins that can become cleaved and translocate to the nucleus. Activation of Notch1 could promote arteriogenesis in a hindlimb ischemic model, which was suppressed by inhibition or knockdown of NOTCH1 (Kikuchi et al., 2011). This was also confirmed in developmental and other diseases (Tirziu et al., 2012; Cristofaro et al., 2013). Thereby, the Notch signaling pathway plays a key role in regulating arteriogenesis (Limbourg et al., 2007).

Angiogenesis refers to the formation and differentiation of new blood vessels (Cooke and Meng, 2020). It has been reported that RIC facilitates angiogenesis in VCI animal models by virtue of the activation of eNOS/NO/nitrite system (Ren et al., 2018a). NO is an important signaling molecule in the regulation of vascular remodeling, which is mainly produced by ECs via eNOS under ischemic conditions (Jin et al., 2021). Because of its highly reactive nature, NO can be oxidized to nitrite during circulation and reduced to NO in a hypoxic environment (Farah et al., 2018). Thus, nitrite is considered as

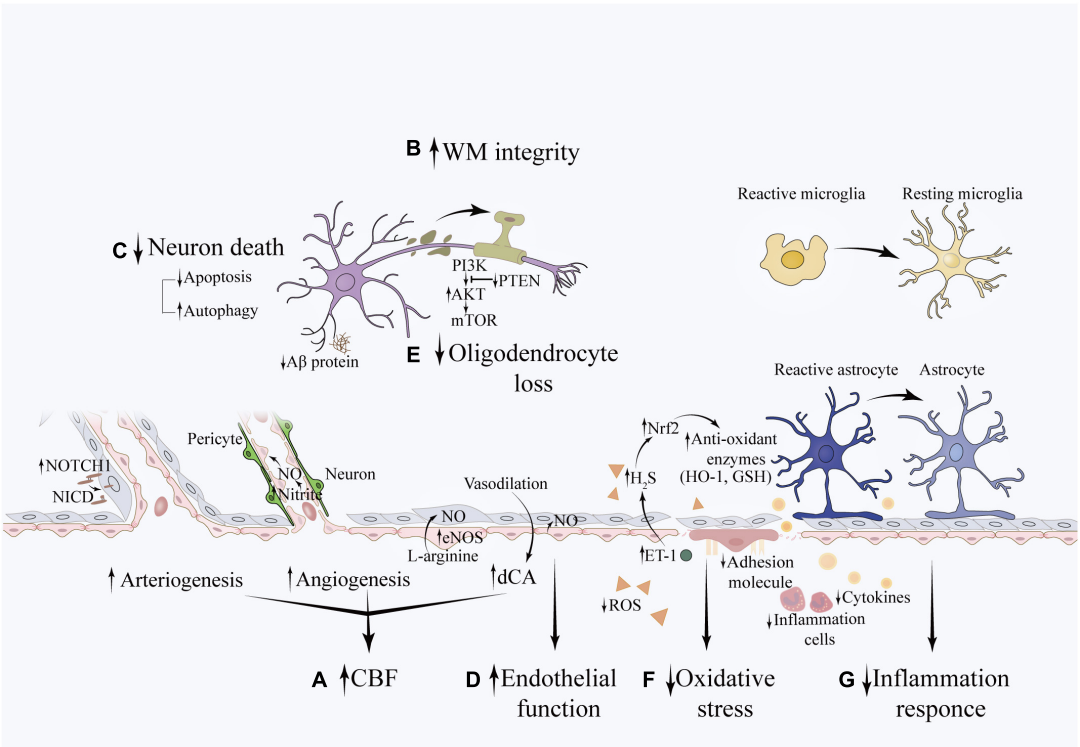

FIGURE 2 | Potential effects of RIC on VCI pathobiologies. The beneficial effects of RIC on VCI is associated with **(A)** increased CFB: the NOTCH1 signaling pathway may be involved in angiogenesis, which increases vascular diameter and promotes collateral formation. The eNOS/Nitrite/NO system plays an important role in the process of angiogenesis. The sheer stress induced by RIC upregulates the synthesis of eNOS in triggered organ and increases the concentration of nitrite in the circulation (not shown in this illustration). Nitrite reduces to NO under hypoxia condition and promotes new capillary formation. In addition, NO is an essential vasoactive agent in the regulation of vascular tension and cerebral autoregulation. **(B)** improved WM integrity **(C)** decreased neuronal death **(D)** restoration of endothelial function **(E)** reduced oligodendrocyte loss: the potential underlying mechanism is associated with decreased apoptosis of oligodendrocytes through activation of the PTEN/Akt/mTOR pathway. **(F)** attenuated oxidative stress: RIC can upregulate the expression of Nrf2 and attenuate the oxidative stress induced by hypoperfusion through promoting the expression of anti-oxidant enzymes, such as GSH and HO-1. This may partially mediated by the elevation of ET-1 in the plasma, which further upregulates the concentration of H_2S within the brain. **(G)** decreased inflammation response: RIC can decrease the systemic inflammation by downregulating the level of inflammatory cytokines and leukocyte count. Meanwhile, RIC can mitigate neuroinflammation as evidenced by decreased expression of IBA-1 and GFAP, which are the markers of reactive microglial and reactive astrocytes, respectively. The expression of ICAM-1 and VCAM-1, markers of endothelial activation, are also downregulated. In addition, this was accompanied with less deposition of Ab protein.

a "storage" pool of NO and a potential endocrine mediator in the plasma. Several clinical and preclinical studies have consistently reported that the concentration of plasma nitrite is upregulated after RIC treatment (Farah et al., 2018; Ikonomidis et al., 2021). The augmentation of nitrite was dependent on reactive hyperemia during the reperfusion phase of RIC, which leads to an upregulation of eNOS synthesis and NO release in the ECs (Dezfulian et al., 2017). In a VCI mouse model induced by bilateral common carotid artery stenosis (BCAS), chronic RIC was performed as four cycles of altering ischemia and reperfusion to 200 mmHg for 5 min per cycle with a remodeled rodent BP instrument on bilateral hind limbs (56). Compared with the control group, 1 month of RIC significantly increased the plasma nitrite level, enhanced angiogenesis, improved CBF and preserved cognitive function. What is more, the effect of RLIC on angiogenesis maintained for up to 6 months. Consistently, this protective effect of RIC was also confirmed in a rat model of VCI caused by BCCAO (Ren et al., 2018a). In this study, the expression of p-eNOS in the brain was significantly elevated from week 2 and persisted until week 4. Furthermore, intraperitoneal administration of L-NAME, a non-selective NOS inhibitor, abolished the protective effect, indicating an important role of the eNOS/NO/nitrite system in mediating the beneficial effects of RIC.

In addition to vasculature integrity, regulation of CBF is important to ensure proper brain function (Popa-Wagner et al., 2015). In our previous study, we found that a single RIC treatment significantly improved dynamic cerebral autoregulation (dCA), and the effect lasted for at least 24 h after the RIC procedure in healthy individuals (Guo et al., 2019). The effect of chronic RIC on dCA and other CBF regulation mechanisms is worth investigating in patients with VCI.

In conclusion, RIC exerts beneficial effects on CBF by facilitating cerebrovascular angioarchitecture and improving the autoregulation of CBF, which may in turn enhance cognitive function.

RIC Promote the White Matter Integrity

Several clinical trials have confirmed that chronic RIC is a promising intervention for WMHs and cognitive decline. In a randomized trial of RIC, 58 patients above 80 years of age with ICAS were randomly assigned to the RIC or sham RIC group (Zhou et al., 2019). Patients in the RIC group received five cycles of ischemia and reperfusion for 5 min per cycle on bilateral upper arms twice daily for 300 days. The severity of WMHs was measured using the Fazekas scale and the Scheltens scale on T2 fluid-attenuated inversion recovery imaging, and global cognitive function was assessed using the Mini-Mental State Examination (MMSE) and the Montreal Cognitive Assessment scale (MoCA). At baseline, cognitive dysfunction was present in approximately 90% of the participants (MoCA < 26). Compared with the sham RIC group, RIC significantly decreased WMHs and improved cognitive function at 180 and 300 days, respectively. Using the same protocol, another randomized controlled trial was performed in 30 patients with cognitive impairment associated with cerebral small vascular disease (CSVD) (Wang Y. et al., 2017). After 1 year of treatment, there was a significant reduction in the volume of WMHs and a better performance in visuospatial and executive function in the RIC group. This may be partially modified by a reduction in plasma lipid levels (such as low-density lipoprotein), along with pulsation indices of the middle cerebral arteries. Moreover, in patients with subcortical ischemic vascular disease, compared with sham RIC, chronic RIC (5 min $\times$ 5 min, twice daily for 6 months) significantly improved visuospatial function (Liao et al., 2019). Although no significant difference was found between the two groups, a more pronounced reduction in WMH volume was observed in the RIC group (pre-treatment 84.00 vs. post-treatment 69.87, $P = 0.060$). In contrast, one small pilot randomized clinical study on 17 patients with CSVD reported that RIC failed to improve global cognitive function at a 1-year follow-up, although it significantly reduced the WMH volume (Mi et al., 2016). A possible explanation for this result is that the cognitive function of most individuals in both groups was not impaired at baseline (with a median MMSE score of 30 and MoCA score of 26 in the RIC group, and 28.5 and 27.5 in the sham group, respectively). This may cause a "ceiling effect" for the RIC. It is noteworthy that subjects in the sham group showed a tendency of cognitive decline, while it was preserved in the RIC group, indicating that RIC may slow down cognitive decline. However, further clinical studies are needed to confirm this finding. Damage of oligodendrocytes is a major pathological change in WM lesions (Alber et al., 2019), which is later discussed in detail (see section "Oligodendrocyte").

Taken together, chronic RIC confers a protective effect toward VCI by reducing WM damage. It can improve cognitive function in patients who have been diagnosed with VCI and may prevent cognitive decline in patients with cerebrovascular diseases.

RIC Protects the Neurovascular Unit

Neurons

Neurons play a central role in the NVU. Neural dysfunction or death, especially in the hippocampus and prefrontal lobe, which are engaged in the learning and memory processes, is considered as a major cause of VCI (Deramecourt et al., 2012; Bartsch and Wulff, 2015; Meftahi et al., 2021). It has been shown that RIC is capable of reducing neural death and improve cognitive function in VCI animal models (Khan et al., 2015, 2018; Ren et al., 2018a). In a tMCAO rat model, for instance, a substantial loss of cholinergic neurons in the hippocampal CA1 region was observed 3 days after surgery, while RIC with three cycles of ischemia and reperfusion for 5 min each significantly protected against cell death and preserved cognitive function, as evaluated using the Morris water maze test (Hu et al., 2013).

Numerous mechanisms underly the protective effect of RIC on neurons, and the suppression of apoptosis might be one of them. Neuronal apoptosis is a crucial pathological change in cognitive function associated with acute or chronic cerebral hypoperfusion (Poh et al., 2020; Wan et al., 2020), which is characterized by downregulation of anti-apoptotic Bcl-2, upregulation of pro-apoptotic Bax, and activation of caspases (Wang X. X. et al., 2020). Thereby, anti-apoptosis is considered a target for VCI. RIC has been shown to preserve the cognitive function of rats after BCAO, and the upregulation of Bcl-2 in the hippocampus may at least partially contribute to this protective effect (Xu et al., 2011). The underlying mechanism is likely to be related to the activation of the JAK2–STAT3 pathway and the PI3K–pAkt pathway by RIC (You et al., 2019).

It appears that regulation of the autophagic process may also be involved in the protective effect of RIC. Autophagy is a lysosome-dependent cellular catabolic pathway, which enables cells to degrade damaged organelles and aggregated proteins for cellular and energy homeostasis (Wang X. X. et al., 2020). Accumulating evidence has indicated that autophagy is widely activated upon ischemic insult (Jiang et al., 2017; Deng et al., 2020). However, the role of autophagy in VCI is controversial. Several studies have indicated a protective role of autophagy (Yang C. et al., 2017; Wang F. et al., 2019; Wang N. et al., 2019; Thammisetty et al., 2021). In contrast, multiple studies have reported a detrimental role of autophagy in VCI, which aggregated neuronal death (Jia et al., 2015; Hu et al., 2017; Qin et al., 2018; Su et al., 2018; Xia et al., 2019; Zhang et al., 2019; Tian et al., 2020; Yang et al., 2020). Furthermore, others have indicated that impaired autophagy flux, characterized by elevated autophagosome formation and/or suppressed autophagy degradation, is tightly linked to cell death and cognitive impairment (Xu et al., 2020; Chen et al., 2021). RIC has been shown to alleviate neuronal death in the hippocampus and preserve WM integrity through the promotion of autophagy. In a BACS rat model, RIC for 2 weeks significantly upregulated the expression of Beclin1, LC3, Atg5-Atg12, and Atg 7, while downregulating the expression of p62 in the hippocampus and WM (Wang H. et al., 2017). The former proteins are important in the initiation and formation of the autophagosome and the latter, p62, can bind to the ubiquitined protein and LC3, serving as a marker of autophagic degradation, and decreased p62 levels are generally related to autophagy activation (Klionsky et al., 2016).

Taken together, RIC decreases neuronal death and preserves cognitive function at least partially via the suppression of apoptosis and promotion of autophagy.

Endothelial cells

It has been well established that RIC preserves endothelial function in healthy individuals (Jones et al., 2015; Rytter et al., 2020). Recently, the protective effect of RIC on endothelial function has also been proved in subjects with cerebrovascular diseases (Hyngstrom et al., 2020). This trial was conducted on 24 chronic stroke survivors (>6 months post-stroke), and RIC was performed on the unilateral thigh of the participants with five cycles of ischemia and reperfusion intervals for 5 min per cycle every other day for 2 weeks. Endothelial function, measured by brachial artery flow-mediated dilation (FMD), was significantly elevated in the RIC group after the treatment; however, no significant changes were observed in the sham RIC group. Although cerebral endothelial function was not evaluated in this study, FMD may be a reliable proxy for its determination as a marker of peripheral endothelial function (Pretnar-Oblak et al., 2006). Thus, it was postulated that RIC may improve cerebral endothelial function in stroke survivors, which is beneficial for cognitive performance.

The mechanism underlying the effect of RIC on endothelial function remains unknown. Nonetheless, previous studies in healthy subjects have addressed the participation of glucagon-like peptide (GLP)-1 receptor (Verouhis et al., 2019). In this study, twelve participants were enrolled, and endothelial dysfunction was induced by forearm ischemia for 20 min. The RIC consisted of four cycles of cuff inflation and deflation for 5 min per cycle was applied to the left thigh, which showed significant protection against endothelial dysfunction following I/R injury. This beneficial effect of RIC was abolished when the GLP-1 receptor antagonist exendin (9–39) was administered to the participants. Of note, the plasma concentration of GLP-1 did not change after RIC. A possible explanation is that GLP-1 may act locally, without being released into the circulation and relaying the protective effect of RIC by activating other pathways.

In conclusion, RIC can improve the endothelial function in healthy individuals as well as in chronic stroke survivors, which may be mediated by the activation of GLP-1 receptor.

Oligodendrocytes

Oligodendrocytes are extensively distributed in the WM and are important for axonal myelination. In rats subjected to BCCAO, RIC for 4 weeks significantly inhibited oligodendrocyte loss, promoted myelination in the corpus callosum, and preserved cognitive function compared to rats in the control group (Li et al., 2017). The underlying mechanism may involve the downregulation of Phosphatase and tensin homolog (PTEN) and upregulation of the phosphatidylinositol-3-kinase (PI3K)/Akt and the mammalian target of rapamycin (mTOR) signaling pathway (Li et al., 2017). The serine/threonine kinase Akt is involved in a variety of biological processes such as cell survival, growth, proliferation, and differentiation (Manning and Toker, 2017). A key kinase downstream of Akt is the mTOR, which gives rise to two distinct complexes: mTOR complex 1 (mTORC1) and complex 2 (mTORC2) (Saxton and Sabatini, 2017; Figlia et al., 2018). It has been reported that RIC upregulate the expression of mTOR and improved the cognitive function in a global brain ischemia mice model (Zare Mehrjerdi et al., 2013). The hyperactivation of the PI3K/Akt/mTOR pathway in transgenic mice leads to hypermyelination (Narayanan et al., 2009; Goebbels et al., 2010), while ablation of mTOR results in a deficit in oligodendrocyte differentiation and myelin loss (Ishii et al., 2019). PTEN is a negative regulator of the PI3K/Akt/mTOR signaling pathway through dephosphorylating PI3K to phosphatidylinositol 4,5-bisphosphate (Yehia et al., 2019). Thus, downregulation of PTEN may active the PI3K/Akt/mTOR signaling pathway, which in turn promotes myelination, reduces WM damage and improved cognitive function.

RIC Attenuates Oxidative Stress

There are evidence suggest that RIC is a viable antioxidant therapy for VCI. In a rat model of global cerebral ischemia (GCI) induced by the occlusion of the bilateral carotid arteries for 20 min followed by reperfusion, the level of ROS were elevated, while the level of antioxidants were consumed in the brain (Ramagiri and Taliyan, 2017b), indicating a state of oxidative stress. And this was accompanied by neuronal damage in the hippocampus and cognitive dysfunction. RIC (three cycles of 10 min ischemia and reperfusion) applied immediately following reperfusion significantly reduced ROS, such as MDA, and prevented the reduction of the defensive enzyme glutathione (GSH).

By using the same animal model, others have also observed the antioxidant properties of RIC by elevating heme oxygenase-1(HO-1) (Ramagiri and Taliyan, 2017a). HO-1 is an important antioxidant that can degrade pro-oxidant heme to cytoprotective carbon monoxide, iron, and biliverdin (Bauer and Raupach, 2019). Following reperfusion, RIC significantly improved cognitive function, increased the expression of HO-1 and GSH, decreased the expression of MDA, and reduced neuroinflammation and CA1 neuronal death. In the presence of the HO-1 inhibitor, tin protoporphyrin IX (SnPP), the protective effect of RIC against I/R injury-induced cognitive decline was abolished, suggesting that HO-1 plays an important role in RIC-induced neuroprotection.

One mechanism of RIC protection against oxidative stress is the activation of Nuclear factor-like 2(Nrf2). Nrf2 is a key regulator of the antioxidative stress defense system that maintains the equilibrium of redox responses. Under pathological conditions, Nrf2 dissociates from Keap-1, translocates to the nucleus, and binds to the antioxidant response element, which regulates the expression of many antioxidant genes (Liu et al., 2019). Nrf2-knockout mice are more susceptible to cerebral I/R injury (Yang et al., 2019) and exhibit exacerbated WM damage under chronic hypoperfusion (Sigfridsson et al., 2020). Upregulation of Nrf2 signaling through pharmacological or genetic approaches protects against WM injury, neuronal death, and cognitive impairment in VCI models (Sigfridsson et al., 2018; Mao et al., 2019). In a GCI mouse model, RIC was performed as four cycles of ischemia and reperfusion for 5 min per cycle before surgery (He et al., 2019). Compared with the sham group, animals in the experimental group showed a significant restoration in cognitive function, assessed using the Morris Water Maze, 7 days after ischemia, along with an upregulation

of Nrf2 and GR in the brain. This beneficial effect of RIC may be attributed to an increase in endothelin-1 (ET-1) in the plasma, which may act as an upstream mediator of hydrogen sulfide in the brain, and in turn upregulate Nrf2 expression.

In summary, the above findings indicate that RIC is able to attenuate the oxidative stress by decreasing the ROS expression and improving the antioxidant system. The activation of Nrf2 may play an important role in this process, which induces the expression of antioxidant enzymes.

RIC Mitigates Inflammation Responses

Numerical studies have reported the effect of RIC in ameliorating systemic inflammation and neuroinflammation. In a randomized controlled prospective study enrolled 58 patients with sICAS aged 80–95 years, repeated RIC for 30 days safely reduced inflammation biomarkers (Meng et al., 2015). In this trial, 30 patients in the RIC group received 5 cycles of ischemia and reperfusion for 5 min per cycle, twice daily on bilateral arms, and 28 patients in the control group received sham RIC (inflation to a pressure of 30 mmHg). All patients received standard medical care. Although cognitive function was not assessed, the levels of plasma hsCRP, IL-6, leukocyte count, and platelet aggregation rates were significantly decreased in the RIC group compared to those in the control group, suggesting that the underlying mechanisms of RIC involve the relief of systemic inflammatory response.

Another clinical study examined the efficacy of RIC on post-stroke cognitive impairment in patients with first-time non-cardiac ischemic stroke (Feng et al., 2019). During the acute phase (<14 days after onset), 104 patients were enrolled and randomized into control or RIC groups. Both groups received standard treatment. RIC were performed with 5 cycles of unilateral arm ischemia and reperfusion for 5 min per cycle for 6 months. Participants in the RIC group showed a lower ICAM-1 level and a higher MoCA scores compared with the control group, indicating a better global cognitive function. Specifically, participants in the RIC group performed better on the cognitive domains of visuospatial and executive functioning and attention.

In consistent with the clinical studies, in a VCI mouse model, 2 weeks of RIC with four cycles of ischemia and reperfusion for 5 min per cycle significantly downregulated the expression of ICAM-1, VCAM-1, glial fibrillary acidic protein, and ionized calcium binding adaptor molecule-1 (IBA-1) in the brain parenchyma, accompanied by improved cognitive function, blood flow, and decreased WM damage and amyloid-β (Aβ) aggregation (Khan et al., 2015). ICAM-1 and VCAM-1, derived from ECs, are important adhesion molecules that regulate leukocytes crossing the BBB (Haarmann et al., 2015; Muller, 2019). Opening of the BBB initiates local inflammation and compromises cerebral vascular function, which in turn may lead to impairment of Aβ clearance, WM integrity, and cognitive function. Thus, downregulation of these adhesion molecules may mitigate neuroinflammation and help maintain cognitive function.

Overall, RIC can attenuate the systemic inflammation and neuroinflammation response, including reduced hsCRP, IL-6, leukocyte counts, adherence molecules, reactive microglial and reactive astrocytes. It is therefore important in maintaining the brain health and normal cognitive function.

DISCUSSION

Over the past decades, substantial progress has been made in clarifying the pathobiologies of VCI. However, no pharmaceutical therapies targeting these mechanisms have been applied clinically. RIC is a potential intervention to prevent the decline of cognitive function and even reverse cognitive impairment in patients with VCI, particularly visuospatial ability. It can be safely applied in daily life as an adjuvant treatment for alleviating risk factors, such as recurrent stroke and prehypertension. Meanwhile, RIC can act on multiple pathobiological changes in VCI through various signaling pathways to exert neuroprotective effects. This might render it even more efficient for VCI than single target drugs.

However, RIC research on VCI is still in its early stages. The efficacy of RIC on VCI has mainly been proven in small-scale clinical trials, which makes it difficult to avoid selective bias. Thus, large-scale trials are needed to confirm these findings. WMHs have been frequently used as biomarkers for the assessment of treatment efficacy. It was reported that fewer patients were needed if using visually rated WM lesion progression, instead of cognitive scoring scales, in clinical trials of CSVD (Schmidt et al., 2012). This may be important for proof-of-concept studies.

In animal studies, BCCAO and BCAS are the most commonly used methods to induce typical pathogenic changes in VCI. However, it can only mimic VCI of large vessel origin. Using animal models of small vessel diseases, such as SHR, may help to fully evaluate the effectiveness and to elucidate the mechanisms of RIC (Hainsworth et al., 2017). In addition, most animal studies have focused on the protective effect of RIC within the brain and did not account for the initiation or signal transduction of RIC in the periphery, although this has been extensively described in other animal models. It seems that the eNOS/NO/nitrite system at least partially mediates the protective effect of RIC from triggered organs to the brain. Considering the important role of ECs in this process, endothelial function, as measured by FMD, may be a simple, straightforward, and efficient parameter to evaluate RIC response in clinical settings; however, this hypothesis remains to be verified.

From a methodological viewpoint, the optimal algorithm for RIC on VCI has not been scientifically proven, while most of the clinical studies in this field used a protocol of 5 min × 5 min cycles; however, 3 min × 10 min was commonly used in animal studies. It has been reported that the effect of RIC on cardioprotection presented a U-type tendency in a mouse model (Johnsen et al., 2016). Four to six cycles of RIC provided significant protection, with no further gain after eight cycles. Two minutes and 5 min of ischemia interval offered similar protection, while 10 min abrogated this effect. However, ischemia intervals lasting for 10 min provided cerebral protection in the VCI animal model. It seems that the optimal protocol for the brain is different from that for the heart; this should be explored in future research.

Despite these limitations and challenges, RIC has therapeutic advantages owing to its multitarget characteristics, which could provide a synergistic effect in VCI. Importantly, the effect of RIC on each pathobiology of VCI has been confirmed in both animal and human studies. Therefore, RIC appears to be a promising intervention to prevent the occurrence and development of VCI.

CONCLUSION

To date, there is no effective pharmacotherapy for VCI to slow down or even reverse cognitive decline, especially in patients with mild VCI. Cerebrovascular risk factors, such as stroke and hypertension, play a detrimental role in VCI, which results in large or small vessel injuries and various pathological changes within the brain. RIC can act as a multitarget therapy to combat VCI by reducing recurrent stroke, lowering high blood pressure, enhancing CBF, alleviating WM damage, protecting components with in the NVU and attenuating oxidative stress and inflammatory response. However, this non-invasive and cost-effective intervention warrants further larger clinical studies and in-depth animal studies to support its beneficial effects.

AUTHOR CONTRIBUTIONS

RX and Z-NG conceived the perspective of the work. RX searched the literature and drafted the manuscript. QH and YW critically revised the article. YY and Z-NG was responsible for checking the whole manuscript. All authors contributed to and approved to the final manuscript.

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Cerebral Edema Formation After Stroke: Emphasis on Blood–Brain Barrier and the Lymphatic Drainage System of the Brain

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Brain edema is a severe stroke complication that is associated with prolonged hospitalization and poor outcomes. Swollen tissues in the brain compromise cerebral perfusion and may also result in transtentorial herniation. As a physical and biochemical barrier between the peripheral circulation and the central nervous system (CNS), the blood–brain barrier (BBB) plays a vital role in maintaining the stable microenvironment of the CNS. Under pathological conditions, such as ischemic stroke, the dysfunction of the BBB results in increased paracellular permeability, directly contributing to the extravasation of blood components into the brain and causing cerebral vasogenic edema. Recent studies have led to the discovery of the glymphatic system and meningeal lymphatic vessels, which provide a channel for cerebrospinal fluid (CSF) to enter the brain and drain to nearby lymph nodes and communicate with the peripheral immune system, modulating immune surveillance and brain responses. A deeper understanding of the function of the cerebral lymphatic system calls into question the known mechanisms of cerebral edema after stroke. In this review, we first discuss how BBB disruption after stroke can cause or contribute to cerebral edema from the perspective of molecular and cellular pathophysiology. Finally, we discuss how the cerebral lymphatic system participates in the formation of cerebral edema after stroke and summarize the pathophysiological process of cerebral edema formation after stroke from the two directions of the BBB and cerebral lymphatic system.

Keywords: cerebral edema, blood-brain barrier, glymphatic system, meningeal lymphatic system, ischemic stroke

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INTRODUCTION

Worldwide, stroke is the leading cause of adult disability and the second main cause of death after coronary heart disease, affecting more than 13.7 million patients every year (GBD 2015 Mortality and Causes of Death Collaborators, 2016; Phipps and Cronin, 2020). Stroke includes ischemic stroke, subarachnoid hemorrhage (SAH), and cerebral hemorrhage, of which ischemic stroke accounts for 80% of all stroke cases (GBD 2015 Mortality and Causes of Death Collaborators, 2016). They are all accompanied by cerebral edema, and swollen tissues in a fixed volume of the skull caused by edema that exert a mechanical force on adjacent tissues and capillaries, leading to decreased blood perfusion, aggravated ischemia and edema, and tissue damage (Simard et al., 2007; Rungta et al., 2015;

Leinonen et al., 2017). Malignant cerebral edema is a devastating complication of ischemic infarction, which accounts for 10% to 78% of patients with all types of ischemic stroke (Wu et al., 2018). It can result in massive cerebral swelling, subsequent raised intracranial pressure (ICP), rapid neurological deterioration, and transtentorial herniation (Huttner and Schwab, 2009). The mortality rate in patients with malignant cerebral edema is close to 80% (Huttner and Schwab, 2009). However, treatment options for cerebral edema remain limited, and available treatments are suboptimal. Therefore, understanding the underlying molecular and cellular mechanisms of edema formation is critical.

Cerebral edema occurs in three distinct phases that mature separately over time and space: the early cytotoxic edema phase, the subsequent ionic edema phase, and the most severe vasogenic edema phase (Simard et al., 2007; Stokum et al., 2016; Clément et al., 2020). Cytotoxic edema occurs within minutes after ischemic insult without BBB disruption, which is usually the consequence of ATP depletion and is characterized by the swelling of astrocytes and neuronal dendrites (Liang et al., 2007; Risher et al., 2009; Badaut et al., 2011b). Although cytotoxic edema does not generate tissue swelling, the ionic gradient between the vascular compartment and interstitial fluid (ISF) it causes provides the driving force for the subsequent ionic and vasogenic edema, which do cause swelling (Mori et al., 2002; Simard et al., 2007; Stokum et al., 2016). The term ionic edema (interstitial edema) was introduced to explain the form of cerebral edema in the early hours of ischemic stroke, with barrier breakdown not occurring until 4–6 h after the onset of ischemia (Gotoh et al., 1985; Young et al., 1987; Hatashita and Hoff, 1990; Schielke et al., 1991; Simard et al., 2007). The ionic edema is followed by BBB breakdown: vasogenic edema, which is characterized by allowing water and plasma proteins, such as albumin and IgG, to leak into the brain interstitial compartment (Stokum et al., 2016; Zhang et al., 2020). The stepwise recruitment of transcellular and paracellular pathways contributes to the breakdown of the BBB (Dreier et al., 2018). As early as 6 h after stroke, a rise in the number of caveolae and an increased transcytosis rate disturb the transcellular pathway, whereas structural abnormalities in tight junctions (TJs) activate the paracellular pathway after 2 days (Kang et al., 2013; Knowland et al., 2014).

The water source of ionic brain edema can only come from blood and CSF (Simard et al., 2007). The hypothesis that local blood perfusion acts as a water source for ionic cerebral edema has been confirmed in numerous experiments. For example, the post-ischemic degree of reperfusion is positively correlated to edema (Bell et al., 1985). Furthermore, edema fluid is first found and located mostly in peri-infarct regions that are actively perfused (Quast et al., 1993; Simard et al., 2006). Recent studies describe that a brain-wide paravascular pathway provides a conduit for CSF influx prompted us to ponder whether CSF serves as the immediate source of ions and water for edema (Iliff et al., 2012). The hypothesis that CSF influx can drive ionic brain edema formation is supported by some indirect evidence (Thrane et al., 2014). For example, the increased paravascular space following pericyte constriction and microvascular collapse can reduce resistance to CSF influx (Hall et al., 2014). Furthermore, the impairment of glymphatic pathway function after injury or infarction is likely to trigger a reduced clearance of interstitial solutes and exacerbate edema (Ren et al., 2013; Iliff et al., 2014). This circumstantial evidence is not convincing. However, Humberto Mestre and his colleagues directly described that the influx of CSF into the brain tissue drives acute tissue swelling within minutes of ischemic stroke (Mestre et al., 2020). The discovery of the classical lymphatic drainage system in the dura mater of the brain, which can absorb CSF from the adjacent subarachnoid space and provides the pathway for the entrance and exit of immune cells from the CNS, calls for a reassessment of cerebral edema formation and sheds new light on the etiology of the neuroinflammatory mechanisms of BBB damage in ischemic stroke (Aspelund et al., 2015; Louveau et al., 2015).

Several fundamental pathophysiologic processes contribute to cerebral edema development after stroke, including the disruption of TJs, the loss of homeostatic ionic gradients, inflammatory responses, and the activated glymphatic system. After the disruption of TJs, inflammatory responses and the activation of ion channels can be considered to promote the occurrence of cerebral edema by exerting an influence on the permeability of BBB. We summarize these aspects into only one part: the increase of BBB permeability to promote cerebral edema. However, the influence of the CNS lymphatic system on the occurrence and progression of cerebral edema induced by stroke has not been reviewed. Therefore, this article summarizes the various pathophysiologic processes that affect the permeability of the BBB to promote the occurrence of cerebral edema and focuses on the effect of the CNS lymphatic system on the development of cerebral edema after stroke.

INCREASED BBB PERMEABILITY CONTRIBUTES TO EDEMA

BBB dysfunction that occurs during cerebral ischemia enables considerable vascular fluid to pass through microvascular endothelium into the brain interstitial compartment and eventually leads to vasogenic edema formation (Sandoval and Witt, 2008; Prakash and Carmichael, 2015; Stokum et al., 2016). Any disorder of the factors that maintain the functional integrity of the BBB will lead to an increase in the permeability of the BBB. Here, we discuss and summarize the mechanisms that can increase the permeability of the BBB. In theory, these mechanisms will eventually lead to the aggravation of cerebral edema after stroke.

Anatomical Considerations of BBB

The concept of BBB was first proposed in the early 20th century (Zlokovic, 2008). Today, the concept of the BBB as an impermeable barrier has evolved into a dynamic and metabolic interface that maintains the fragile homeostasis of the brain through regulating the trafficking of fluid and solutes bi-directionally and metabolizing potentially neurotoxic compounds (Jiang et al., 2018). Although the BBB is formed primarily by the brain microvascular endothelium, the complete function of the BBB requires the harmonious functional interplay

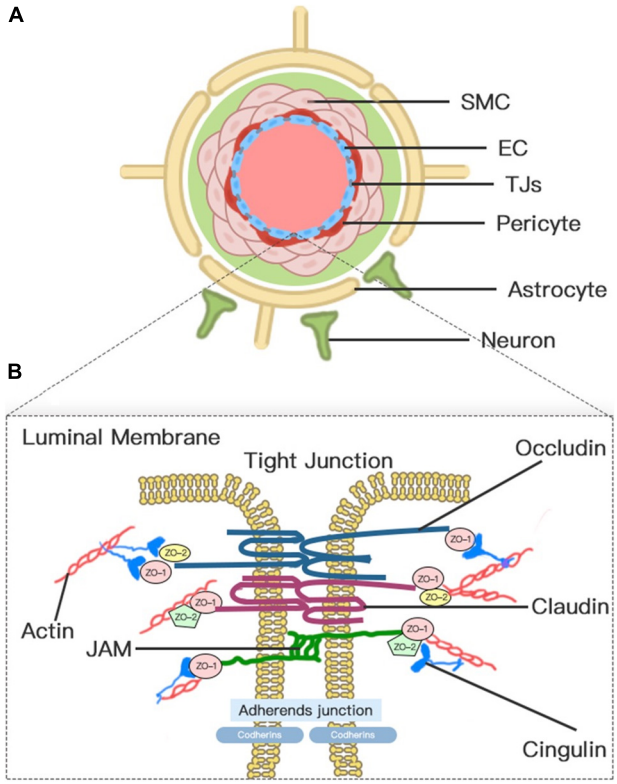

FIGURE 1 | Neurovascular unit (NVU) and blood–brain barrier (BBB). **(A)** Transverse-section representation of the NVU. The concept of the NVU highlights the importance of the intimate interactions between components of the BBB and cells in brain parenchyma, including pericytes, astrocytes, microglia, and neurons. The BBB is centrally positioned within the NVU, which is formed by a monolayer of endothelial cells sealed by tight junctions. **(B)** A schematic blow-up of the tight junctions (TJs) and adherens junctions (AJs) at the BBB as defined in the text.

of multiple cells (**Figure 1**), including astrocytes, pericytes, microglia, neurons, vascular smooth muscle cells (SMCs), and extracellular matrix (ECM) components. Therefore, to emphasize further the cellular interplay in maintaining the function of the BBB, we introduce the concept of the neurovascular unit (NVU).

Blood–brain barrier endothelial cells (ECs), which act as the first line of defense in the innermost layer of the BBB, are differentiated from common ECs by a lack of fenestrations, minimal pinocytotic activity, and the presence of extensive TJs and numerous mitochondria (Abdullahi et al., 2018). Notably, the brain endothelium plasmalemma is divided into luminal and abluminal membrane faces by extensive inter-endothelial TJs. The different expression of transport proteins and metabolic enzymes between luminal and abluminal membrane faces leads to the polarization of ECs and finally restricts the flux of blood-to-brain substances across the microvascular endothelium (Sanchez-Covarrubias et al., 2014). The constitutive and *de novo* expression of ion transporters serve as drivers of ionic edema after stroke (Stokum et al., 2016). Several factors that are produced by ECs, such as platelet-activating factor, superoxide radicals, endothelins, and eicosanoids, impair perfusion, increase BBB permeability, and induce cell damage when overexpressed (Spatz, 2010).

Pericyte is a mesenchymal cell type located in the endothelial basement membrane of the capillaries and microvessels (Spatz, 2010). Astrocytes, whose endfeet are almost surrounding the abluminal ECs surface, act as intermediaries in the NVU responding to neuronal synaptic activity (Koehler et al., 2006). Pericytes and astrocytes all play important roles in the formation, maturation, and maintenance of the BBB and the regulation of capillary blood flow (Alvarez et al., 2013; Stokum et al., 2016; Jiang et al., 2018). Pericyte-deficient mice are identified with endothelial hyperplasia, increased capillary diameter, an abnormal cellular distribution of junctional proteins, and increased transendothelial permeability (Hellstrom et al., 2001). Chemical factors produced by astrocytes, such as Sonic Hh, vascular endothelial growth factor (VEGF), angiopoietins-1, Src suppressed C kinase substrate, and TGF-β, can promote vascular growth and BBB differentiation and maturation and support BBB integrity (Alvarez et al., 2011, 2013). In addition, a high density of aquaporin-4 (AQP4) water channels highly polarized to perivascular astrocytic endfeet act as an indispensable component of the glymphatic system, which facilitates the circulation of CSF through the brain interstitial space (Iliff et al., 2012).

Endothelial cell junctions include TJs and adherens junctions (AJs) (**Figure 1**). TJs between adjacent ECs are responsible for the formation of a continuous and impermeable barrier. AJs are likely to play an auxiliary role to help the localization and stabilization of TJs that are formed by cadherins and associated proteins that are directly linked to actin filaments (Dejana et al., 2008; Redzic, 2011). Three integral transmembrane proteins, namely, claudins, occludin, and junctional adhesion molecules (JAMs), are involved in the assembly of TJs (**Figure 1**; Stamatovic et al., 2016). The stability of TJs can be maintained by cytoplasmic accessory molecules comprising zonula occludens (ZO)-1, ZO-2, and ZO-3, and cingulin, which fasten these transmembrane proteins to the actin cytoskeleton (Ballabh et al., 2004). In addition to these cellular components, the luminal membrane and basement membrane also participate in maintaining vascular permeability (Spatz, 2010). The glycocalyx coating the luminal EC membrane is composed of proteoglycans, glycosaminoglycan, and absorbed plasma proteins, and glycocalyx damaged by ischemia or injury permits the attachment of leukocytes (Spatz, 2010). The basement membrane separates the endothelium from the astrocyte and prevents the vascular leakage of plasma protein through the cooperation of multiple ECM proteins, including collagens, laminins, heparin sulfate proteoglycans, fibronectin, vitronectin, nidogens, perlecan, and agrin (Spatz, 2010; Yao and Tsirka, 2014).

Pathways Involved in BBB Permeability and Edema

TJ Disruption

Vasogenic edema is characterized by increased paracellular permeability of the BBB, which is mainly caused by TJ disruption (**Figure 2**; Wolburg and Lippoldt, 2002; Stokum et al., 2016). Therefore, the pathological mechanism of TJ

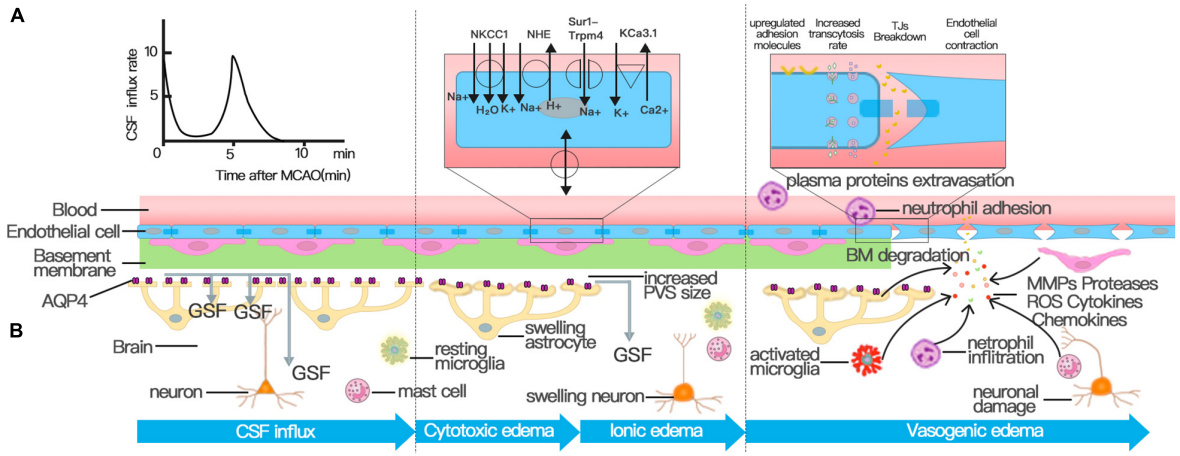

FIGURE 2 | Three distinct phases of cerebral edema. **(A)** CSF influx occurred and peaked at 11.4 ± 1.8 s and 5.24 ± 0.48 min after MCAO. The first peak of CSF influx is the hydrostatic pressure gradient due to vascular obstruction. The increased PVS size triggered by spreading depolarizations (SDs) drives the second wave of perivascular CSF influx, which facilitates the swelling of astrocytic endfeet. **(B)** Cerebral edema can be classified into three phases: cytotoxic, ionic, and vasogenic edema phases. The cytotoxic edema phase is characterized by swelling of astrocytes and happens simultaneously with the second peak of CSF entry. The second stage of cerebral edema formation, the ionic edema stage, is mainly driven by endothelial ion channels and transporters in the context of an intact BBB, such as NKCC, NHE, KCa3.1, and Sur1-Trpm4 channel. The breakdown of BBB causes vasogenic edema. Successive alterations to the transcellular and paracellular pathway of the BBB contribute to the breakdown of BBB following stroke. First, the increase in the number of endothelial caveolae and the rate of transcytosis impairs BBB function by disturbing the transcellular pathway. Then the destruction of TJs activates the paracellular pathway. In addition to NVU cells involved in the regulation of BBB permeability, various immune cells and inflammatory factors play an important role in the destruction of TJs.

destruction is particularly important. Progressive TJ dysfunction can be organized into three phases: protein modification, protein translocation, and protein degradation; each of the phases can increase BBB permeability and promote the formation of edema (Jiang et al., 2018). In the first phase, inflammatory factors and cytokines released during ischemic brain injury, such as VEGF, chemokine monocyte chemoattractant protein-1 (CCL2), tumor necrosis factor (TNF)-α, and IL-6, can induce the phosphorylation of TJs, leading to BBB hyperpermeability (Stamatovic et al., 2006; Murakami et al., 2009; Murakami et al., 2012; Rochfort and Cummins, 2015). Attenuating TJ phosphorylation can also inhibit the leakage of BBB after transient focal cerebral ischemia (Kago et al., 2006; Takenaga et al., 2009). In the second phase, TJ translocation, which is largely mediated by endocytosis and actin polymerization, also compromises BBB integrity (Jiang et al., 2018). For example, occludin, claudin-5, and JAM-A redistribute from the cytoskeleton or interendothelial cell cleft after ECs are treated with CCL2 or cultured in an environment with oxygen glucose deprivation (OGD) (Stamatovic et al., 2009, 2012; Liu et al., 2012). Experiments have also identified the redistribution of occludin and cadherin from the membrane fraction to the actin cytoskeleton fraction due to robust actin polymerization and stress fiber formation (Shi et al., 2016). The degradation of TJ protein is the last step of TJ disruption and the most critical step to destroy the integrity of BBB, which causes increased paracellular leakage and the infiltration of peripheral immune cells at the BBB. The activation of matrix metalloproteinases (MMPs) is one of the most significant contributors to TJ degradation in stroke (Abdullahi et al., 2018). MMP-2 and MMP-9 are the most studied and main MMPs that are increased following stroke (Turner and Sharp, 2016). Although ECs, microglia, and astrocytes overexpress MMPs, infiltrating neutrophils have proved to be a major source of MMP-9 (Romanic et al., 1998; Tang et al., 2006; Turner and Sharp, 2016). Upregulated MMP-9 and MMP-2 after stroke degrade TJs, such as occludin and Claudin-5, and microvascular basal lamina (Asahi et al., 2001; Liu et al., 2012; Qi et al., 2016). By selectively inhibiting MMP-2/9, the impairment of BBB integrity and the volume of edema in cerebral ischemic mice can be significantly reduced (Cui et al., 2012; Liu et al., 2012; Qi et al., 2016). Therefore, the destruction of TJs, which leads to the decrease of BBB integrity, is one of the key factors leading to the formation of cerebral edema after stroke.

Actived NVU Cells

The NVU, which emphasizes cell–cell and cell–matrix interactions in the brain, provides a more integrative answer to BBB disruption after stroke (Lo et al., 2003). The permeability of the BBB is constantly regulated by different cell types in the NVU. Therefore, the occurrence of cerebral edema after stroke is also closely related to various cellular components in the NVU.

As a first-line defense located between the blood plasma and interstitium, the continuous endothelium is essential for physiologic homeostasis and directly reacts to harmful substances from the periphery. Under the pressure of ischemia and hypoxia after stroke, actin polymerization elicits stress fibers and concomitant endothelial cell contraction mediated by zipper-interacting protein kinase (ZIPK) through the phosphorylation of the myosin light chain (Vandenbroucke et al., 2008; Komarova and Malik, 2010; Shi et al., 2016; Zhang et al., 2019). Following endothelial cell contraction, the formed

paracellular gap improves paracellular permeability and allows macromolecules and inflammatory cells to enter brain parenchyma (Komarova and Malik, 2010; Zhang et al., 2019). The global deletion of ZIPK in an animal model of middle cerebral artery occlusion (MCAO) significantly attenuates BBB dysfunction by inhibiting EC contraction, as proven by reduced infarct and edema volume (Zhang et al., 2019). Oxidative stress after stroke can also induce endothelial cell apoptosis, which is suggested to have detrimental consequences on BBB integrity, subsequently leading to brain edema (Rizzo and Leaver, 2010; Al Ahmad et al., 2012). Protecting ECs from apoptosis after stroke is beneficial to the integrity of BBB and the reduction of brain edema (Park et al., 2004; Zhang et al., 2016; Yang et al., 2017). The increased number of endothelial caveolae and transcytosis rate account for the BBB disruption that occurs in the early phase of stroke (Knowland et al., 2014; Nahirney et al., 2016; Haley and Lawrence, 2017).

Activated pericytes and astrocytes also contribute to the breakdown of BBB and promote cerebral edema formation after stroke. Pericytes migrate from the brain microvascular wall in a rat MCAO model, and the detachment increases the permeability of the BBB to water and tracers (Gonul et al., 2002; Duz et al., 2007; Armulik et al., 2010). Stimulation by some factors, such as TNF-α and thrombin, makes pericytes and astrocytes important sources of MMP-9, which can cause BBB dysfunction through the degradation of TJs and the basal lamina and the enhancement of pericyte migration (Takata et al., 2012; Machida et al., 2015; Turner and Sharp, 2016). Activated astrocytes in stroke can also facilitate the destruction of BBB by increasing VEGF (Li et al., 2014). In EC–astrocyte co-cultures, microvesicles released from ECs cultured in OGD conditions promote the apoptosis of astrocytes, increase the permeability of BBB, and downregulate TJ proteins (Pan et al., 2016).

Inflammation Responses

After stroke onset, circulating leukocytes adhere, migrate, and eventually accumulate in the lesion site and then release inflammatory factors to cause secondary BBB disruption. Neutrophils are the earliest leukocyte subtype that infiltrates an ischemic brain and contribute to the breakdown of BBB by secreting MMP-9, neutrophil elastase, and reactive oxygen species (ROS) (**Figure 2**; Jickling et al., 2015). In a study of a mouse model of ischemic stroke, we proved that MMP9, which is derived mainly from neutrophils rather than brain parenchymal cells, causes BBB disruption (Gidday et al., 2005; Tang et al., 2006; Wang et al., 2009). In an experimental intracerebral hemorrhage, neutrophil depletion by anti-polymorphonuclear leukocyte antibodies reduces the production of MMP-9, infiltration of activated microglia/macrophages, and leakage of the BBB (Moxon-Emre and Schlichter, 2011). Neutrophil elastase released from neutrophils is another harmful inflammatory reaction that contributes to BBB disruption and vasogenic edema (Stowe et al., 2009; Ikegame et al., 2010). Neutrophils exacerbate BBB breakdown by producing neutrophil extracellular traps (Kang et al., 2020), which damage ECs by releasing many cytotoxic proteases, such as histone, elastase, and myeloperoxidase (Villanueva et al., 2011). In addition to that of neutrophils, the recruitment of monocytes and lymphocytes is also involved in the regulation of BBB function. T cells and B cells have both protective and damaging roles in cerebral ischemia; however, the role of each type of lymphocyte in stroke and the effect on BBB permeability after ischemic stroke should be further clarified (Jian et al., 2019; Yang et al., 2019).

Mast cells, as resident cells in the brain and meninges, also promote BBB damage and edema formation by releasing their granule contents, such as histamine, TNF-α, proteases, heparin, and various chemoattractants (**Figure 2**; Lindsberg et al., 2010; Arac et al., 2014; Dong et al., 2014). Rats treated with a mast cell stabilizer (cromoglycate) after MCAO show significantly reduced ischemic brain swelling by 40%, BBB leakage by 50%, and less postischemic neutrophil infiltration by 37% (Strbian et al., 2006).

Macrophages, which can be transformed from brain-inhabited microglia or differentiated from peripheral monocyte, also promote neuroinflammation and blood vessel disintegration after ischemic stroke (da Fonseca et al., 2014; Benakis et al., 2015; Fumagalli et al., 2015; Jian et al., 2019). Both microglia- and monocyte-derived macrophages have a phagocytic function, express the same phenotypic markers, and can transform to pro-inflammatory/anti-inflammatory (M1/M2) phenotype. The number of monocytes infiltrating the ischemic brain is lower than that of activated microglia (Kokovay et al., 2006; Denes et al., 2007). Therefore, we mainly discuss the damaging effect of microglia on BBB. Recently, the presence of CD31-positive particles (blood vessels) in the intracellular vesicles of perivascular microglia indicates the phagocytosis of blood vessels by perivascular microglia and finally contributes to the breakdown of the BBB (Jolivel et al., 2015). Ischemia can also induce the generation of NOX-dependent ROS in brain microglia and inflict damage on BBB by activating transcription factors or ion channels, such as JNK, p38 MAPK, JAK-STAT, NF-kB, and Hv1 (Kacimi et al., 2011; Wu et al., 2012). The expression of a large array of inflammatory mediators, such as IL-1, IL-6, matrix metalloproteases, MMPs, and TNF-α, by microglia after ischemic stroke also enhances vascular permeability in the brain (Zhou et al., 2013; Lee et al., 2014; Thurgur and Pinteaux, 2019). The inhibition of microglial activation by pretreatment with minocycline can suppress vasogenic edema and infarct formation in ischemic stroke (Tanaka et al., 2018).

Various chemical factors, such as cytokines, chemokines, ROS, MMPs, and VEGF, secreted from the periphery and resident immune cells and glial cells play a key role in the regulation of BBB disruption in ischemic stroke (Yang et al., 2019). Among the most extensively studied cytokines in the context of stroke, TNF-α, IL-1, and IL-6 have been shown to disrupt the BBB (Blamire et al., 2000; Pradillo et al., 2012; Cohen et al., 2013; Rochfort et al., 2014; Kangwantas et al., 2016; Pradillo et al., 2017). The most notorious upregulated chemokines in response to hypoxia/ischemia are CCL2, macrophage inflammatory protein-1α, and stromal-derived factor-1, which play an important role in leukocyte recruitment and promote BBB destruction (Eugenin and Berman, 2003; Stamatovic et al., 2005; Dimitrijevic et al., 2006; Chui and Dorovini-Zis, 2010; Takata et al., 2012). Oxidative stress caused by ROS and nitric oxide (NO) plays a critical role in MMP activation and BBB breakdown after

stroke (Yang et al., 2019). Leukocytes, glial cells, and vascular ECs are important sources of ROS and NO (Iadecola et al., 1995; Lassegue and Clempus, 2003). Furthermore, VEGF secreted by neurons, astrocytes, and macrophages is also involved in the activation of MMPs and BBB disruption following ischemia (Kovacs et al., 1996; Valable et al., 2005; Lee et al., 2007). The upregulation of cell adhesion molecules, such as selectins, immunoglobulin superfamily, and integrins, promotes the infiltration of leukocytes, especially neutrophils, to the CNS and leads to BBB damage (Yang et al., 2019). In general, the neuroinflammatory mechanism of BBB damage in ischemic stroke is very complex but is a promising target to reduce BBB damage, edema, and brain injury after stroke.

Ion Transporter Dysfunction

The depletion of intracellular ATP leads to the cytotoxic edema of all CNS cell types after ischemic stroke, of which astrocytes are particularly prominent; cytotoxic edema ultimately provides the driving force for ionic edema, vasogenic edema, and complete hemorrhagic conversion (Liang et al., 2007; Stokum et al., 2016). The mechanism behind cytotoxic edema and ionic edema is ion transporter dysfunction at the NVU rather than the breakdown of the BBB (**Figure 2**; Brillault et al., 2008; Stokum et al., 2016; Sifat et al., 2019).

Na + –K + –2Cl -cotransporter (NKCC) is expressed the astrocytes, neurons, and ECs of the brain (Kahle et al., 2008; Jayakumar and Norenberg, 2010), and reside predominantly in the luminal membrane of BBB ECs (O'Donnell et al., 2004). NKCC is activated via phosphorylation in response to hypoxia, aglycemia, and arginine vasopressin and contributes to edema formation during cerebral ischemia (Yan et al., 2001; Foroutan et al., 2005; Brillault et al., 2008; Yuen et al., 2014). The inhibitory effect of bumetanide on NKCC activities can reduce brain Na absorption and edema formation in rat MCAO stroke models (Yuen et al., 2014, 2019). The sodium–hydrogen antiporter (NHE) family member, NHE1, is ubiquitously expressed in all cell types in the brain; is stimulated by hypoxia, aglycemia, and arginine vasopressin as with NKCC; and contributes to astrocyte swelling, ionic edema formation, microglial activation, and BBB breakdown (Lam et al., 2009; Stokum et al., 2016; Begum et al., 2018; Song et al., 2018). The inhibition of NHE activities by the intravenous delivery of Na/H exchange inhibitor HOE642 decreases brain edema in an ischemic stroke model (Lam et al., 2009; Yuen et al., 2014, 2019). Mice with the selective ablation of the NHE1 gene in astrocytes exhibit less edema, reduced BBB breakdown, and alleviated disruption of TJ protein after transient MCAO (tMCAO) (Begum et al., 2018). Furthermore, ischemia also induces the *de novo* expression of the sulfonylurea receptor 1–transient receptor potential 4 channel (SUR1-TRPM4) in all cells of the NVU and contributes to the formation of ionic edema (Simard et al., 2014; Stokum et al., 2016). The blockage of the SUR1-TRPM4 channel results in a significant reduction in infarct volume, cerebral edema, and hemispheric swelling in rodent models of ischemic stroke (Simard et al., 2006, 2009; Wali et al., 2012). In the mouse edema model, the up-regulated SUR1-TRPM4 in astrocytes can synergize with APQ4 to promote the influx of water and the swelling of astrocytes (Stokum et al., 2018). The recently discovered KCa3.1, a calcium-activated potassium channel expressed by ECs, is also involved in the formation of cytotoxic edema after ischemic stroke (Chen et al., 2015).

Ion channels can not only cause edema by transporting ions but also participate in the activation of resident cells in the brain, such as astrocytes and microglia, and lead to the destruction of the BBB. The stimulation of NHE1 in astrocytes causes a robust release of glutamate and the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, TNF-α, and MMP-9 (Cengiz et al., 2014; Begum et al., 2018). The pharmacological inhibition or genetic knockout of astrocytic NHE1 protein significantly reduces cerebral microvessel damage, BBB breakdown, and loss of the TJ protein occludin in ischemic brain (Cengiz et al., 2014; Begum et al., 2018). The Na^+–Ca^{2+} exchanger (NCX) in astrocytes is also involved in Ca2 + induced ROS production, DNA ladder formation, and nuclear condensation (Matsuda et al., 2001). Microglial NCX, Kv1.3, and NHE1 channels all contribute to proinflammatory microglial activation (Rangaraju et al., 2017; Song S. et al., 2020). By contributing to excessive hydrogen ion extrusion and sustained NOX activation, activated NHE1 causes the production of ROS and the expression of cytokines in microglia after lipopolysaccharide or hypoxia stimulation (Liu et al., 2010; Wu et al., 2012; Lam et al., 2013). The pharmacological inhibition or genetic knockout of microglial NHE1 and Kv1.3 reduces the secretion of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, and iNOS (Liu et al., 2010; Shi et al., 2011; Nguyen et al., 2017; Rangaraju et al., 2017; Di Lucente et al., 2018; Song et al., 2018). NCX1-mediated Ca2 + influx plays a critical role in microglial phagocytic activity through Ca2 + -mediated purinergic receptors (Sunkaria et al., 2016). Calcium overload increases brain ROS levels in type 5 NOX-dependent manner, which contributes to BBB breakdown (Casas et al., 2019).

PATHWAYS INVOLVED IN THE GLYMPHATIC SYSTEM AND EDEMA

At the periphery, impaired lymphatic system function is one of the common causes of edema and leads to the pathologic accumulation of protein-rich lymphatic fluid in the intercellular interstitium (Cho and Atwood, 2002). Acquired lymphedema caused by axillary lymph node dissection and filariasis is the most common cause of clinical lymphedema (Cho and Atwood, 2002). For a long time, due to the delayed discovery of the lymphatic drainage system of the brain, research on the mechanism of cerebral edema has mainly focused on BBB. However, discoveries of recent studies, including the brain pseudolymphatic system—glymphatic system and meningeal lymphatic system—have brought light to us to clarify the mechanism of cerebral edema.

Anatomical Considerations of the Glymphatic System

Solutes in CSF have been thought to recycle from the subarachnoid space into brain parenchyma by the convective bulk flow rather than via an anatomically discrete structure

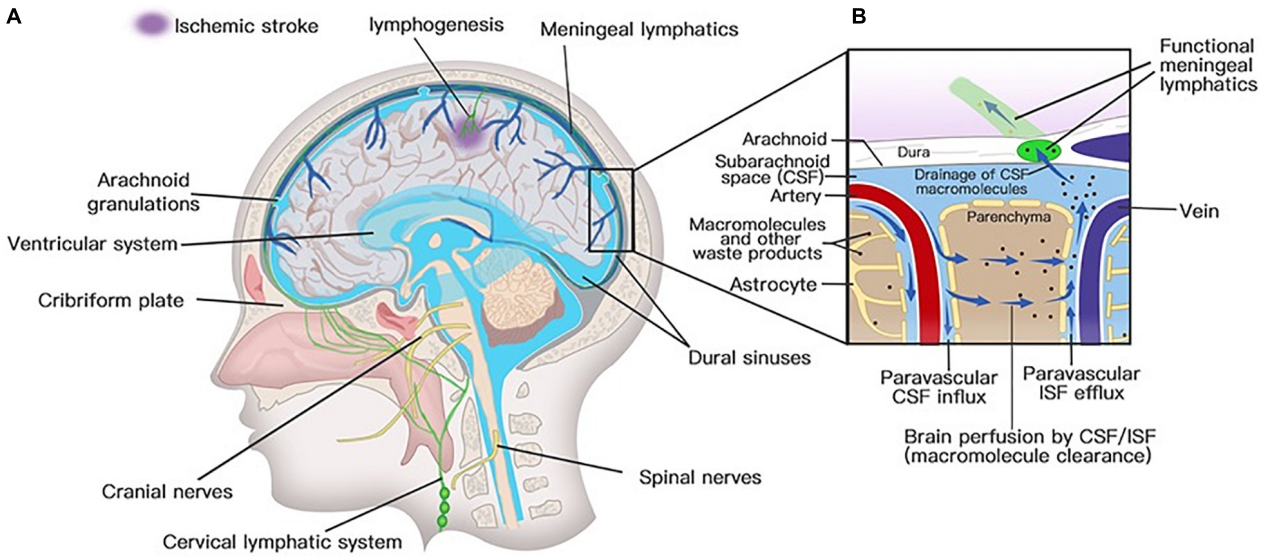

FIGURE 3 | Lymphatic drainage system in the brain. **(A)** Meningeal lymphatic vessels run down toward the base of the skull along the sinus, the vein, and the meningeal arteries and drain out of the skull via the foramina of the base of the skull alongside arteries, veins, and cranial nerves. Meningeal lymphatic cells grow into the injured brain parenchyma induced by photochemical thrombosis. **(B)** Cerebrospinal fluid (CSF) enters the parenchyma by bulk flow along paravascular spaces, and ISF is cleared along paravenous drainage pathways. Meningeal lymphatic vessels absorb CSF from the adjacent subarachnoid space and ISF from the glymphatic system and transport fluid into deep cervical LNs (dcLNs) via foramina at the base of the skull.

(Abbott, 2004; Sykova and Nicholson, 2008). The precise anatomical or functional structures for the clearance of metabolic waste products from the ISF to the CSF were first described by Iliff and his colleagues (Iliff et al., 2012). Via *in vivo* two-photon imaging and other techniques, the movement of a fluorescent tracer injected in the subarachnoid compartment flowing into and through the brain interstitium was depicted to represent the exchange of CSF and ISF. In the initial segments of the pathway, flux fluid and macromolecules from the subarachnoid space rapidly enter the brain by bulk flow through paravascular spaces called Virchow Robinson spaces, which exist around vascular SMCs and perivascular astrocytic endfeet (**Figure 3**; Iliff et al., 2012). Then, fluids and macromolecules accumulate around along capillaries and parenchymal venules and are eventually cleared along paravenous drainage pathways (**Figure 3**; Iliff et al., 2012). The phenomenon of larger tracer from the subarachnoid space being confined in paravascular spaces of the brain is also consistent with a recent study demonstrating that narrow clefts between overlapping endfeet may serve a sieving function to control the exchange of water and solutes between blood and brain (Mathiisen et al., 2010). In addition, paravascular AQP4 channels, which are highly polarized to paravascular astrocytic endfeet (Mathiisen et al., 2010), facilitate bulk ISF solute clearance from the parenchyma (Iliff et al., 2012). The putative glymphatic transports have also been successfully demonstrated in humans with BBB disruption using non-invasive, high-resolution 3D isotropic contrast-enhanced T2 fluid-attenuated inversion recovery imaging (Wu et al., 2020).

Perivascular drainage pathways refer to a route responsible for the solutes diffuse across brain ECs through basement membranes between the SMCs in the tunica media of capillaries and arteries (Carare et al., 2008; Morris et al., 2016). The perivascular drainage of ISF and solutes is in the reverse direction of blood flow, which only occurs after some form of attachment of solutes or valve-like mechanism to prevent backflow during the pulse wave (Schley et al., 2006; Carare et al., 2008; Morris et al., 2016). However, the latest research has constructed a novel multiscale model of arteries to prove that the arterial pulsations from the heart are not strong enough to be the motive force for perivascular drainage, whereas the vasomotion of cerebrovascular SMCs acts as the drivers of perivascular drainage (Aldea et al., 2019). Perivascular drainage pathways should be further elaborated because the relatively dense pericapillary basement membrane is usually considered a physical obstacle to solute movements, and the non-specific binding of the fluorescent dextran tracer to the capillary basement membrane may cause the illusion of the abovementioned results (Barber and Lieth, 1997; Brinker et al., 2014). As the study on the effect of post-stroke perivascular drainage of edema formation is very limited, this review focuses on the effects of paravascular drainage on edema formation.

Paravascular Pathway Is Involved in Edema Formation

To test whether CSF is the source of edema fluid, Humberto Mestre and his colleagues deduced a subtle way to study changes in glymphatic function after MCAO; the approach involved the use of *in vivo* magnetic resonance and multimodal optical imaging to map the influx of CSF tagged with a fluorescent tracer (Mestre et al., 2020). They found that the influx of CSF along paravascular spaces occurred and peaked at 11.4 ± 1.8 s

and 5.24 ± 0.48 min after MCAO and built a new notion that CSF is the primary source of early edema fluid after ischemic stroke (**Figure 2**; Mestre et al., 2020), which broke the traditional concept that CSF is not a source of edema fluid (Stokum et al., 2016). The hydrostatic pressure gradient caused by a loss in blood flow after the MCAO was used to explain the first peak in the CSF influx. Experiments also proved that the pressure gradient in distal paravascular spaces caused by spreading depolarization drove CSF influx and caused the spreading edema, which also depends on AQP4 expression (Mestre et al., 2020). Therefore, the influx of CSF in the glymphatic system is increased after ischemic stroke (Mestre et al., 2020). However, before this article was published, most articles demonstrated a decreased paravascular CSF influx after ischemic stroke (Iliff et al., 2013; Gaberel et al., 2014; Ji et al., 2021). The possible reasons for these conflicting conclusions are the different time windows of observation in each experiment and the different models constructed. For example, this study observed the change of glymphatic perfusion at 3 and 24 h after MCAO but did not study the glymphatic influx rate within 20 min (Gaberel et al., 2014). The establishment of a model of internal carotid artery ligation may also lead to different conclusions from the model of MCAO (Iliff et al., 2013). The mechanism of cerebral edema should be explored by conducting more studies to observe the change of CSF influx in the glymphatic system in each time window after stroke.

Solute clearance along paravascular spaces is also markedly impaired after ischemic stroke (Arbel-Ornath et al., 2013; Pu et al., 2019). The glymphatic system may also play a positive effect in clearing edema fluid in days and weeks after stroke (Lempriere, 2020). Therefore, future works should investigate how the function of the lymphatic system can be adjusted to optimize edema recovery.

A recent article points out that the pressure gradient caused by vasoconstriction draws the influx of CSF into the brain parenchyma, driving acute ischemic tissue swelling (Mestre et al., 2020). As such, cerebral vasospasm, which is common in SAH, may also promote early edema formation after SAH in a manner similar to ischemic stroke (Rowland et al., 2012). However, this hypothesis needs more experimental proof.

Studies have also shown severely impaired glymphatic system perfusion and a reduced glymphatic system waste clearance function from the brain parenchyma after SAH (Gaberel et al., 2014; Goulay et al., 2017; Golanov et al., 2018). The main reason for glymphatic inhibition is the occlusion of perivascular spaces by fibrin/fibrinogen clots, which can be removed through the intraventricular injection of fibrinolytic tissue-type plasminogen activator (Gaberel et al., 2014; Goulay et al., 2017). Although these trials did not provide evidence that intravascular thrombus aggravates edema after SAH by compressing the paravascular space, a recent study showed that brain edema can be alleviated by preserving the function of the glymphatic system after SAH (Fang et al., 2020). Therefore, the impaired function of glymphatic system waste clearance may be a factor leading to the formation of cerebral edema after SAH. In general, the function of the glymphatic system may play an important role in the cerebral edema of SAH. However, the specific mechanism still requires confirmation by more experimental studies.

AQP4 Is Involved in Edema Formation

The AQP family contains 13 different members, of which the expression levels of AQP1, AQP4, and AQP9 in rodent models and humans are upregulated after stroke (Gonen and Walz, 2006; Ribeiro Mde et al., 2006; Yatsushige et al., 2007; Vella et al., 2015; Stokum et al., 2018). Since studies have determined the involvement of AQP4 in the process of cerebral edema in early 2000, AQP4 has become a hot research (Manley et al., 2000; Vella et al., 2015). However, the exact mechanism of AQP4 regulating edema is still controversial and unclear. Now, the discovery of the glymphatic system and the special status of AQP4 in the glymphatic system have brought a new understanding of these controversies (Rasmussen et al., 2018). Therefore, the role of AQP4 regulation on edema is discussed in the section on the glymphatic system.

Aquaporin, a water channel highly polarized to paravascular endfeet, provides support for paravascular CSF–ISF exchange and drives the clearance of bulk interstitial solutes from brain parenchyma (Iliff et al., 2012). Some brain disorders, such as cerebral infarction, SAH, and traumatic brain injury, reduce polarized localization at the endfeet of reactive astrocytes (Wang et al., 2012; Ren et al., 2013; Fang et al., 2020). The abnormal distribution of AQP4 impairs the clearance of solute from cerebral parenchyma (Pu et al., 2019). AQP4 contributes to the spreading of edema after MCAO by facilitating the transport of CSF into the brain (Mestre et al., 2020). This pathophysiological process is in line with evidence demonstrating that AQP4-deficient mice and wild types treated with AQP4 inhibitors progress with less cerebral edema after ischemic stroke (Manley et al., 2000; Igarashi et al., 2011; Yao et al., 2015; Hirt et al., 2017; Pirici et al., 2018). Furthermore, the accelerated influx of water into the brain and elevated ICP in AQP4-overexpressing mice induced by intraperitoneal water injection confirm that the water channel protein promotes the occurrence of cerebral edema by increasing the permeability of the BBB (Yang et al., 2008). The function of AQP4 to control water uptake across BBB also reflects that up-regulated AQP4 can promote the formation of cerebral edema (Haj-Yasein et al., 2011). However, the function of astroglial AQP4 to drive the clearance of interstitial solutes from the brain parenchyma also illustrates that AQP4 may facilitate the absorption of excess fluid in brain edema (Iliff et al., 2012). Notably, much experimental evidence demonstrates the role of AQP4 in the resolution of brain edema (Papadopoulos et al., 2004; Finnie et al., 2008; Tourdias et al., 2009; Badaut et al., 2011a). In a model of vasogenic brain edema, AQP4-deficient mice have a significantly higher increase in ICP and brain water content compared with wild-type mice (Papadopoulos et al., 2004).

Interestingly, in contrast with animal brain models of ischemic stroke, we did not find similar results of cerebral edema in SAH (Luo et al., 2016; Liu et al., 2020). On the one hand, AQP4-deficient mice did not develop better neurological function and less neuroinflammation at day 7 after SAH (Luo et al., 2016). On the other hand, AQP4 deletion in mice significantly increased the water content in the whole brain and aggravated the neurological deficit following SAH; the possible mechanism involves AQP4 knockout impairing the glymphatic system

function of facilitating ISF drainage to eliminate toxic factors in the brain (Tait et al., 2010; Liu et al., 2020). The increased vasogenic edema caused by AQP4 deletion is also found in other models of BBB disruption, including status epilepticus,brain tumor, and brain abscess (Bloch et al., 2005; Lee et al., 2012; Vella et al., 2015).

In conclusion, AQP4 plays a dual role in the process of cerebral edema after stroke with a harmful role in the early stages of edema formation and plays a beneficial role during edema subsidence (Badaut et al., 2011b; Vella et al., 2015; Clément et al., 2020). We believe that the role of AQP4 is closely related to its location in astrocytes and its supporting role in the glymphatic system. Therefore, controlling the function of AQP4 is a potential effective target for treating post-stroke cerebral edema, although there is still a lot of research work to be done.

MENINGEAL LYMPHATICS

We have discovered that tracers injected into the brain parenchyma and ISF pass into the CSF and further into deep cervical lymph nodes (CLNs) (Koh et al., 2005; Iliff et al., 2012; Plog et al., 2015). However, it was not until 2015 that we found the basic structure of the metastatic pathway through the discovery of meningeal lymphatic vessels in mice (Aspelund et al., 2015). In the human brain, we also provided *in vivo* evidence of CSF tracer drainage to CLNs via meningeal vessels and that tracer enhancement within lymph nodes parallels glymphatic enhancement (Eide et al., 2018). Therefore, the discovery of the classical lymphatic drainage system in meninges also promotes us to think about its role in cerebral edema.

Anatomical Considerations of the Meningeal Lymphatic System

Meningeal lymphatic vessels were discovered by chance after whole-mount mouse brain meninges stained by immunohistochemistry for different cells were used to determine the gateways responsible for T cells into and out of the meninges (Louveau et al., 2015). They found that high concentrations of T lymphocytes were abluminal and aligned linearly in CD31-expressing structures along sinuses (Louveau et al., 2015). These structures express markers of the lymphatic system, such as lymphatic vessel endothelial hyaluronan receptor-1, podoplanin, Prospero homeobox protein 1, VEGF receptor 3, CD31, and chemokine (C-C motif) ligand 21 (CCL21) (Aspelund et al., 2015; Louveau et al., 2015). Structurally, the meningeal lymphatic vessels are close to initial lymphatic capillaries but are devoid of SMCs, positive for the immune-cell chemoattractant protein CCL21, the punctate expression pattern of Claudin-5 and vascular endothelial cadherin, and the lack of integrin-a9 expression (Louveau et al., 2015). The lymphatic vessels accompany arteries and veins in the meninges, including the transverse sinus, sigmoid sinus, retroglenoid vein, superior sagittal sinus, rostral rhinal vein, and the major branches of the middle, anterior meningeal arteries (Aspelund et al., 2015), and meningeal septae penetrating the cerebral cortex (Lohrberg and Wilting, 2016). Lymphatic vessels can also be seen followed by the olfactory (CN I), optic (CN II), trigeminal (CN V), glossopharyngeal (CN IX), vagus (CN X), and accessory (CN XI) nerve sheaths (Aspelund et al., 2015; Absinta et al., 2017; Antila et al., 2017) and exit the skull along with CN IX, X, and XI (Aspelund et al., 2015). Except for CN IX, X, and XI, lymphatic vessels are also observed to exit the skull along the meningeal portions of the pterygopalatine artery, sigmoid sinus, and retroglenoid vein (**Figure 3**; Aspelund et al., 2015). The mechanism for the emergence of lymphatic vessels from the skull has yet to be discovered. The newly discovered meningeal lymphatic vessels constitute the second part of the current relatively complete CNS lymphatic drainage system. First, GSF flows into the brain interstitium along arterial perivascular spaces and is then cleared along paravenous drainage pathways to the CSF (Rasmussen et al., 2018). The last step is draining the CSF along the meningeal lymphatic vessels into the CLNs and communicating with the periphery. Therefore, the meningeal lymphatic system and the glymphatic system constitute a relatively complete cerebral lymphatic drainage system.

Meningeal Lymphatic Involved in Edema Formation

More than two decades ago, some researchers have systematically studied the effects of cervical lymphatic blockade (CLB) in conditions such as ischemic stroke and SAH; the specific mechanisms by which CLB exerts an influence on stroke lesions remain unclear (Xing et al., 1994; Xia et al., 2003; Sun et al., 2000, 2006, 2009, 2011; Li et al., 2005, 2007; Si et al., 2006; Zheng et al., 2008). Before meningeal lymphatic vessels were discovered, cellular and soluble constituents of CSF were thought to enter the lymphatic vessels in brain mucosa through the cribriform lamina to elicit immune responses in CLNs (Weller et al., 2010; Laman and Weller, 2013; Mathieu et al., 2013). Now, studies have confirmed that meningeal lymphatic vessels, in addition to taking up and draining CSF, also directly communicate with CLNs to regulate intracranial inflammatory processes (Louveau et al., 2015; Dave et al., 2018; Ahn et al., 2019). Therefore, CLB not only directly leads to the disruption of meningeal lymphatic drainage, leading to intracranial hypertension and cerebral edema, but may also affect neuroinflammation by affecting the connection between the brain and the peripheral immune system (Sun et al., 2018). We can indirectly evaluate the role of meningeal lymphatic vessels in the entire process of edema occurrence and resolution by observing the effect of CLB on post-stroke edema.

In an ischemic stroke model, Sun et al. (2000) and Si et al. (2006) randomly assigned mice to the MCAO group and MCAO plus CLB group to determine the major effects of CLB (Sun et al., 2000; Si et al., 2006). In their experiments, they all observed that CLB aggravates brain edema caused by MCAO, which can be indicated by the content of water, sodium, calcium, and glutamate (Sun et al., 2000; Si et al., 2006). Compared with the MCAO only group, CLB + MCAO mice show decreased superoxide dismutase activity and a more markedly increased malondialdehyde content, which may indicate that CLB can deteriorate ischemic brain damage by promoting oxidative stress damage (Sun et al., 2000). Furthermore, the cerebral infarction

volume and mRNA expression levels of N-methyl-D-aspartame receptor 1 in the ischemic hemisphere are markedly higher in rats with MCAO + CLB than in those with only MCAO at different time points (Si et al., 2006). CLB was also found to aggravate cerebral edema in a SAH model. After infusing arterial blood into the cisterna magna of mice to establish an experimental model of SAH with and without CLB, investigators found that regional CBF drops more obviously, and the increased ICP and brain water content were more serious in SAH plus CLB groups (Sun et al., 2006).

Many experiments have directly expounded the important role of the meningeal lymphatic system in the post-stroke activation of the processes of brain drainage and edema clearing (Chen et al., 2019; Semyachkina-Glushkovskaya et al., 2020; Yanev et al., 2020). The increase in the diameter of meningeal lymphatic vessels has been observed in a variety of experimental models, including cerebral hemorrhage, SAH, and the opening of the BBB (Semyachkina-Glushkovskaya et al., 2017, 2018, 2019, 2020). For example, only during the opening of the BBB can optical coherence tomography allow the observation of meningeal lymphatic vessels with increased diameter (Semyachkina-Glushkovskaya et al., 2017). Furthermore, the increased diameter of meningeal lymphatic vessels suggests the activation of meningeal lymphatic drainage function after stroke (Semyachkina-Glushkovskaya et al., 2020). Compared with the slow and non-remarkable accumulation of gold nanorods in the deep CLNs of normal mice, the extensive accumulation of gold nanorods in cavities of deep CLNs within three hours after SAH indicated the activation of lymphatic clearance as SAH progressed (Semyachkina-Glushkovskaya et al., 2019). Recent work also demonstrates that the increased outflow rate of meningeal lymphatics participates in the clearance of extravasated erythrocytes from CSF into CLNs after SAH (Chen et al., 2020). One week after SAH, long-term meningeal lymphatic clearance was proven to be dysfunctional (Pu et al., 2019).

In addition to cerebral hemorrhage and SAH, meningeal lymphatic drainage also plays a role in the pathophysiology of ischemic stroke (Chen et al., 2019; Yanev et al., 2020). Pavel Yanev and his colleagues found that meningeal lymphatic vessels sprouted from an adjacent sinus into the anatomical area corresponding to stroke in a mouse ischemic stroke model induced by photothrombosis; however, they detected no lymphangiogenesis in the tMCAO model (Yanev et al., 2020). Coincidentally, in a zebrafish ischemic stroke model induced by photochemical thrombosis, meningeal lymphatic cells rapidly grew into the injured brain parenchyma (**Figure 3**; Chen et al., 2019). These ingrown meningeal lymphatic vessels played a role in resolving cerebral edema and guiding and supporting the growth of nascent blood vessels (Chen et al., 2019). The role of lymphangiogenesis in promoting the regression of edema has also been confirmed in myocardial infarction (Vieira et al., 2018). Meningeal lymphatic hypoplasia was found to exacerbate stroke severity by increasing infarct size and causing sustained motor deficits in the tMCAo model (Yanev et al., 2020). Meningeal lymphatic dysfunction slows the efflux of macromolecules from the brain parenchyma (Da Mesquita et al., 2018). Furthermore, preexisting meningeal lymphatic dysfunction leads to aggravated neuroinflammation and cognitive outcomes following traumatic brain injury (Bolte et al., 2020).

In general, the meningeal lymphatic system plays a neuroprotective function after stroke and promotes the resolution of edema. Its function may mainly depend on two aspects. On the one hand, in the early stage of stroke, the activation of meningeal lymphatic drainage function can remove excess fluid in the skull; on the other hand, meningeal lymphatic vessels directly invade the injured brain parenchyma to resolve edema. The augmentation of lymphogenesis by treatment with VEGF-C improves heart function following myocardial infarct in mice (Klotz et al., 2015). VEGF-C also stimulates the drainage of meningeal lymphatic vessels in aged mice, resulting in improved cognitive function (Da Mesquita et al., 2018). Therefore, promoting the growth of meningeal lymphatic vessels seems to be beneficial for cerebral edema resolution and tissue repair.

Meningeal Immunity Is Involved in Edema Formation

The discovery of meningeal lymphatic vessels has led to a collapse of dogma that the brain was an "immune-privileged" site, and its function to carry both fluid and immune cells from the CSF to the deep CLNs sets off an upsurge of how lymph nodes participate in the CNS immune response (Aspelund et al., 2015). Neuroinflammation following ischemic stroke plays a pivotal role in the breakdown of BBB, leading to vasogenic edema formation, hemorrhagic transformation, and aggravated patient prognosis (Rosenberg and Yang, 2007; Khatri et al., 2012). After focal cerebral ischemia in rats, the elevation of VEGF-C in CSF can increase pro-inflammatory macrophages by activating the lymphatic endothelium. By blocking VEGF-C/VEGFR3 signaling in lymphatic ECs, cytokine/chemokine expressions in superficial CLNs and pro-inflammatory macrophages in the ischemic area are significantly decreased, and the final effect is an obvious reduction in cerebral infarction volume (Esposito et al., 2019). This article focused on the aggregation and activation of macrophages during stroke, which is regulated by VEGF-C/VEGFR3 signaling in lymphatic ECs. However, the lymphocytes in the CLNs involved in the immune damage of stroke also include T and B cells, and many factors, such as neuropilin-2 (Xu et al., 2010), angiopoietins (Alitalo, 2006), BMP9-ALK1 (Yoshimatsu et al., 2013), DAMPs (Shichita et al., 2017), may participate in the activation of lymphatic endothelium after stroke. The function of meningeal lymphatic network in controlling immune responses in the CNS was also proved in a mouse model of glioblastoma (Song E. et al., 2020). The increased meningeal lymphatic drainage via VEGF-C can promote the priming of CD8 T cells in the draining CLNs and antitumor T cell responses (Song E. et al., 2020). Furthermore, the surgical and pharmacological blockade of meningeal lymphatic function diminishes the migration of activated encephalitogenic T cells into the CNS in an animal model of multiple sclerosis (Louveau et al., 2018). The presence of meningeal lymphatic vessels provides a link between the CNS and the peripheral immune system and provides a new therapeutic target for reducing neuroinflammation after stroke. However, the mechanism by

which CLNs and meningeal lymphatic vessels participate in the immune response after stroke remains to be discovered.

CONCLUSION

Traditionally, the occurrence of cerebral edema can be divided into three distinct phases: an early cytotoxic phase, a middle ionic phase, and a later vasogenic phase. Cytotoxic edema occurs within minutes after ischemic insult and ionic edema is the form of cerebral edema which forms immediately following cytotoxic edema and before barrier breakdown not occurring until 4–6 h after the onset of ischemia. Vasogenic edema, which is characterized by the breakdown of the BBB, manifests hours after the initial insult. However, the discovery of the glymphatic system and meningeal lymphatic vessels adds new content to each phase. Ion transporter dysfunction at the NVU lays the foundation for cytotoxic edema and ionic edema, and GSF, which flows rapidly into brain parenchyma through the glymphatic system within minutes after insult, also acts as the source of edema fluid. As the primary initial event driving tissue swelling, the glymphatic inflow of CSF may provide a basis for the treatment of cerebral edema after stroke. The breakdown of the BBB, which results in vasogenic edema formation, involves many joint effects, including the destruction of TJs, imbalance of the NVU, damage of inflammatory response, and activation of ion channels. Inflammation is a key element among many factors that lead to the progression of BBB damage in stroke. The glymphatic system plays a dual role in the process of cerebral edema after stroke with a harmful role in the early stage of edema formation and plays a beneficial role during edema subsidence. The function of glymphatic system is supported by astrocytic AQP4. AQP4 can not only control water flux across the BBB, but also facilitate the circulation of CSF, and drive the clearance of bulk interstitial solutes from the brain parenchyma. Therefore, in the earlier phases of cerebral edema (cytotoxic and ionic edema), the up-regulation of AQP4 can aggravate brain edema formation, while in the vasogenic edema phases, AQP4 may play a key role in the elimination of water of vasogenic origin. The activation of meningeal lymphatic drainage function and the ingrown of meningeal lymphatic vessels into the injured brain parenchyma promote the resolution of edema after stroke. Consequently, the rigorous dissection of the pathophysiology of the cerebral lymphatic system may eventually lead us to novel mechanisms and targets for cerebral edema after stroke.

AUTHOR CONTRIBUTIONS

SC and LM collected information and drafted and revised the manuscript. LS contributed to collecting information and editing the manuscript. LM directed the work and finalized the manuscript. All authors agreed to be accountable for the content of the work.

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Mast Cells Mediate Inflammatory Injury and Aggravate Neurological Impairment in Experimental Subarachnoid Hemorrhage Through Microglial PAR-2 Pathway

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Subarachnoid hemorrhage (SAH) is a devastating cerebrovascular disease with high mortality and disability. Aberrant neuroinflammation has been identified as a critical factor accounting for the poor prognosis of SAH patients. Mast cells (MCs), the sentinel cells of the immune system, play a critical in the early immune reactions and participate in multiple pathophysiological process. However, the exact role of MCs on the pathophysiological process after SAH has not been fully understood. The current study was conducted to determine the role of MCs and MC stabilization in the context of SAH. Mouse SAH model was established by endovascular perforation process. Mice received saline or cromolyn (MC stabilizer) or compound 48/80 (MCs degranulator). Post-SAH evaluation included neurobehavioral test, western blot, immunofluorescence, and toluidine blue staining. We demonstrated that SAH induced MCs activation/degranulation. Administration of MC stabilizer cromolyn conferred a better neurologic outcome and decreased brain edema when compared with SAH+vehicle group. Furthermore, cromolyn significantly inhibited neuroinflammatory response and alleviated neuronal damage after SAH. However, pharmacological activation of MCs with compound 48/80 dramatically aggravated SAH-induced brain injury and exacerbated neurologic outcomes. Notably, pharmacological inhibition of microglial PAR-2 significantly reversed MCs-induced inflammatory response and neurological impairment. Additionally, the effect of MCs-derived tryptase in mediating neuroinflammation was also abolished by the microglial PAR-2 blockage *in vitro*. Taken together, MCs yielded inflammatory injury through activating microglia-related neuroinflammation after SAH. These data shed light on the notion that MCs might be a novel and promising therapeutic target for SAH.

Keywords: subarachnoid hemorrhage, mast cells, neuroinflammation, microglia, PAR-2

INTRODUCTION

Subarachnoid hemorrhage (SAH), mainly caused by ruptured intracranial aneurysm, is a serious cerebrovascular disease with high mortality and chronic disability worldwide (Macdonald and Schweizer, 2017; Hostettler et al., 2020). Although studies on SAH have been carried out for decades, the prognosis of patients with SAH remains unsatisfactory (Rautalin et al., 2020; Savarraj et al., 2020). Among the multiple pathological events that occur after SAH, prolonged inflammation has been identified to be the critical factor accounting for the poor prognosis of SAH. Accumulating studies, including ours, suggested that inhibition of inflammatory response affords a robust neuroprotection against SAH (Gris et al., 2019; Khey et al., 2019; Peng et al., 2020). Thus, it is important to explore the pathophysiological process of neuroinflammation and further develop novel therapeutic strategies to improve the outcome of SAH.

Mast cells (MCs), one of the granulocytes derived from the myeloid stem cell that widely distributed in tissues surrounding blood vessels, nerves, smooth muscle cells, and synovial membranes, are an important regulator participating in both innate immunity and adaptive immunity (El Ansari et al., 2020). Upon activation, MCs could rapidly migrate to the site of injury and undergo degranulation by releasing the preformed mediators, including tryptase, chymase, histamine, heparin, serotonin, and prostaglandins into extracellular, which further recruit immune cells and amplify the inflammatory response (Chelombitko et al., 2020). For years, MCs have been mainly studied due to their pathogenic role in allergic responses during the progression of urticaria and asthma (Falduto et al., 2020). However, recent studies revealed the critical effect of MCs in central nervous system (CNS) disease. In the pathological process of ischemic stroke and acute stress, MCs are believed to be the "first responder" and contribute to blood-brain barrier (BBB) disruption via mediating glial dysfunction (Ribatti, 2015; D'amico et al., 2020). In addition, MCs are associated with the progression of several neurodegenerative and neuropsychiatric diseases, including amyotrophic lateral sclerosis, frontotemporal dementia, and multiple sclerosis (Brown and Weinberg, 2018; Novellino et al., 2020). Furthermore, MCs were recently reported to aggravate the neurological impairment after hemorrhagic stroke (Kuwabara et al., 2017; Furukawa et al., 2020; Zhang et al., 2021). Although the pathophysiological role of MCs in human diseases has been studied for years, little is known about the role of MCs in the pathological process after SAH.

Therefore, the current study was designed to investigate the role of MCs under SAH condition and explore the potential mechanism. Here, for the first time, we reported that MCs contribute to the inflammatory injury after SAH through interacting with microglia in a mouse model of SAH.

MATERIALS AND METHODS

Animals and Subarachnoid Hemorrhage Model

Male C57BL/6J mice (body weight 20–25 g, purchased from Slac Laboratory Animal Co., Ltd., Shanghai, China) were kept at room temperature (22 ± 1°C) with a 12 h day/night cycle (humidity: 60 ± 5%). Mice are free to water and food. All procedures involving animals conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Zhejiang University.

The SAH model was established via endovascular perforation as previously described (Nishikawa et al., 2018). Briefly, mice were anesthetized with 1% pentobarbital (50 mg/kg, i.p.). Subsequently, the left carotid artery and its branches were exposed, and a sharped nylon suture was inserted from the external carotid artery and further went along into the internal carotid artery, and ultimately perforated the bifurcation of the anterior and middle cerebral arteries. Mice in sham group underwent the same procedure except the suture was withdrawn without puncture.

Study Design

This study was completed in three separate experiments. A total of 234 mice, including the dead ones, was used in the current study as shown in **Supplementary Table 1**.

Experiment 1

To determine the activation of MCs after SAH, mice were randomly divided into sham group and SAH groups with different time points (6, 12, 24, 48, and 72 h). The ipsilateral cerebral cortex was harvested for western blotting and immunofluorescence staining.

Experiment 2

To determine the role of MCs in the pathological process of SAH following, the selective MCs stabilizer cromolyn and MCs degranulator compound 48/80 (C48/80) was used. Mice were randomly divided into sham group, SAH + vehicle group, SAH + cromolyn group, and SAH + C48/80 group. Toluidine blue stain, neurological test, brain water content, western blot, and immunofluorescence staining was performed at 24 h post-modeling.

Experiment 3

To determine the mechanism of MCs-mediated brain injury, mice were randomly divided into SAH + vehicle group, SAH + C48/80 group, SAH + ENMD-1068 (PAR-2 antagonist) group, and SAH + C48/80 + ENMD-1068 group. Neurological test, brain water content, western blot, and immunofluorescence staining were assessed at 24 h after SAH.

Drug Administration

Mast cell stabilizer cromolyn (purchased from Med-ChemExpress, NJ, United States), MCs degranulator compound 48/80 (purchased from Cayman Chemicals, MI, United States), and PAR2 antagonist ENMD-1068 (purchased from Med-ChemExpress, NJ, United States) were dissolved in saline with a final concentration of 100 mg/kg, 0.5 mg/mL, and 0.5 mg/kg, separately. Mice received 0.2 ml cromolyn or compound 48/80 intravenously at 5 min before the induction of SAH, while ENMD-1068 was administrated intravenously at 1 h before modeling. The deliver route and concentration of

agents were obtained as previously reported (Strbian et al., 2007; de Almeida et al., 2020). Animals in the sham-operated group and SAH + vehicle group received the same volume of saline.

Subarachnoid Hemorrhage Grade

The severity of SAH was evaluated according to the SAH grading scale as previously reported (Sugawara et al., 2008). Briefly, the basal cistern was divided into six segments, and each segment was scored from 0 to 3 based on the amount of bleeding. A total score ranging from 0 to 18 was obtained by adding the scores together. All the tests and SAH grades were evaluated by an independent researcher.

Neurological Behavioral Analysis

Two neurological tests were introduced at 24 h after modeling as previously reported (Matsumura et al., 2019; Peng et al., 2020). The modified Garcia scoring system consists of six tests: spontaneous activity, symmetry in limb movement, forepaw outstretching, climbing, body proprioception, and the response to vibrissae touch. Each test was scored as either 0–3 or 1–3, and the total scores ranged from 3 to 18. A higher indicated a better neurological function. Another neurological test was conducted through three activity examinations of appetite, activity, and deficits. Grading of neurologic deficits was as follows: severe neurologic deficit (score = 4–6), moderate neurologic deficit (score = 2–3), mild neurologic deficit (score = 1), and no neurologic deficit (score = 0). A lower score indicated a better neurological function. A higher score suggested a worse neurological deficit. All the neurological performance was evaluated by a blinded investigator.

Brain Water Content

Brain water content was tested to evaluate the severity of brain edema. Mice were sacrificed at 24 h after modeling and left hemispheres were immediately collected and weighed to get the wet weight. Then, the samples were dried at 105°C for 72 h to get the dry weight. The brain water content was calculated as [(wet weight − dry weight)/(wet weight)] *100%.

Western Blot

Western blot was performed as previously described (Xu et al., 2017). Briefly, mice were sacrificed and left hemispheres were harvested. Equal amounts of protein (50 μg) were loaded onto sodium dodecyl sulfate–polyacrylamide gels and electrophoresed. Subsequently, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were incubated overnight at 4°C with primary antibodies: TNF-α (1:5000, Abcam ab6671), IL-1β (1:2000, Santa Cruz SC-23459), IL-6 (1:1000, Santa Cruz SC-1265), PAR-2 (1:500, Abcam, ab180953), and anti-β-actin (1:5000, Proteintech, Cat. No. 60008-1-Ig). The membranes were processed with horseradish-peroxidase conjugated secondary antibodies at room temperature for 1 h. Bands were visualized using the ECL Plus chemiluminescence reagent kit (Millipore, MA, United States). The band densities were quantified with the Image J software (NIH). The results were expressed as the relative density of the band as compared β-actin.

Brain Tissue Sections

Mice were anesthetized and transcardially perfused with 0.1M PBS followed by 4% paraformaldehyde (pH 7.4). Then, brains were immersed in 4% formaldehyde and then dehydrated in 30% sucrose solution. Coronal frozen sections (8 μm) were placed onto slides for toluidine blue staining and immunofluorescence staining.

Toluidine Blue Staining

In order to evaluate mast cell activation and degranulation, brain tissue sections were stained with toluidine blue solution (purchased from Bioss biotechnology Co., Ltd, Beijing, China) according to manufacturer's protocol. The mast cell count was performed on each slide through a microscope by a blinded investigator.

Immunofluorescence Staining

Brain slices were washed with 0.1M PBS and incubated with 5% BSA containing 0.3% Triton X-100 for 1 h at room temperature. Then, sections were incubated overnight at 4°C with primary antibodies: Iba-1 (1:500, Abcam, ab5076), NeuN (1:500, Abcam, ab104224), PAR-2 (1:500, Abcam, ab180953). After undergoing washes with PBS, the sections were incubated with secondary antibodies for 2 h at 4°C in the dark. Terminal deoxynucleotide transferasedeoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) staining was performed to detect apoptotic cell death according to the manufacturer's protocol (Roche Inc., Basel, Switzerland). Each mouse had 4 brain slides examined, and each slide was examined under three fields of vision to acquire the mean number of target cells using a fluorescence microscope (Olympus, Tokyo, Japan) by a blinded investigator. The data were expressed as cells per square millimeter.

Microglia Culture and Quantitative Real-Time Polymerase Chain Reaction

Murine BV2 cells were cultured in the DEME medium with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco, United States) under the atmosphere of 5% carbon dioxide at 37°C. A total of 250 nM recombinant mouse tryptase (synthesize by GL Biochem [Shanghai] Ltd, China) was introduced into the medium for 6 h to simulate MCs degranulation insult *in vitro* as previously described. After incubation, the medium was removed and BV2 cells were collected for Quantitative Real-Time PCR (RT-PCR) assay. To further determine the potential molecular mechanism, BV2 cells were pretreated with 500 μM PAR-2 antagonist ENMD-1068 (purchased from Med-ChemExpress, NJ, United States) for 4 h before tryptase incubation. The administration of tryptase and ENMD-1068 were obtained base on the previous study (Zhang et al., 2019).

Total RNA of cultured BV2 cell was extracted from using TRIzol reagent (Invitrogen, United States). RNA was reverse transcribed into cDNA with a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, United States). The RT-PCR was conducted with UltraSYBR Mixture (CWBio, China), specific primers (as shown in **Supplementary Table 2**),

and cDNA on a Mx3000P real-time PCR system (Agilent Technologies, United States). GAPDH was set as internal control, and results were presented as fold changes compared with the control group.

Statistical Analysis

The data were shown as the mean ± standard deviation (SD). Differences among the groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. All statistical analyses were performed on SPSS (version 22.0), and statistical significance was determined as $P < 0.05$.

RESULTS

Mast Cells Rapidly Actives in the Brain After Subarachnoid Hemorrhage Insult

To determine whether MCs could response to SAH in brain, western blot was performed to evaluate the expression of tryptase and chymase, two kinds of critical proteases generated by MCs after activation. The result indicated that the expression of tryptase significantly increased at 6 h and peaked at 24 h after SAH (**Figure 1A**). A similar tendency was observed in the expression of chymase, which started to increase at 6 h and peaked at 24 h after SAH (**Figure 1B**). Additionally, immunostaining further confirmed the increased expression of tryptase and chymase post-modeling. More importantly, both tryptase and chymase demonstrated a tendency of degranulation with spread from intracellular to extracellular after SAH (**Figures 1C,D**).

Mast Cells Activation Aggravated Brain Edema and Neurological Deficits After Subarachnoid Hemorrhage

To investigate the effect of MCs on the pathological process following SAH, the selective MCs stabilizer cromolyn and MCs degranulator compound 48/80 were introduced. Toluidine blue staining suggested that there was an increased MCs infiltration and degranulation after SAH compared to sham group (**Figure 2**), which was consistent with increased level of tryptase and chymase (as shown in **Figure 1**). Furthermore, when compared with SAH + vehicle group, the MCs infiltration and degranulation was significantly inhibited by the administration of cromolyn, while compound 48/80 remarkably promoted MCs activation (**Figure 2**), suggesting the efficacy of the mentioned stabilizer and degranulator in brain under SAH condition.

There was a significant loss of body weight in modeling groups. And compared with SAH + vehicle group, mice in SAH + cromolyn group demonstrated a faster weight gain after SAH (**Figure 3A**). When the animals were sacrificed, subarachnoid blood clots were observed around the circle of Willis and the ventral brainstem. No significant difference in SAH grade among modeling groups was noted (**Figures 3B,C**). Moreover, remarkable neurobehavioral dysfunction and increased brain water content were observed in the modeling groups when compared with the sham group. Post-SAH treatment with MCs stabilizer cromolyn significantly decreased brain water content and alleviated neurological deficits, while mice administrated with MCs degranulator compound 48/80 suffered a worse brain edema and neurological impairment (**Figures 3D–F**).

Mast Cells Mediated Neuroinflammation and Microglial Activation

Given the critical role of inflammatory response on determining the outcome of SAH, the next part was conducted to determine the adverse effects of MCs in the pathological process following SAH were exerted through mediating neuroinflammation. Data from western blot indicated that the expression of pro-inflammatory mediators, including TNF-α, IL-1β, and IL-6, were significantly increased after SAH (**Figures 4A–D**). And treatment with cromolyn significantly downregulated the expression of the mentioned cytokines, while compound 48/80 administration further upregulated the level of these cytokines (**Figures 4A–D**). Accordingly, the number of microglia, one of the most important immune cells in mediating neuroinflammation, was increased after SAH (**Figures 4E,F**). Compared with SAH + vehicle group, cromolyn treatment significantly decreased the activation of microglia, while MCs degranulator compound 48/80 further promoted the microglial activation (**Figures 4E,F**).

Mast Cells Activation Mediated Neuronal Injury After Subarachnoid Hemorrhage

TUNEL staining was conducted to evaluate the effect of MCs on neuronal injury. As shown in **Figure 5**, the percentage of TUNEL-positive neurons significantly increased in SAH groups when compared with sham group, suggesting a significant neuronal injury after SAH (**Figure 5**). And treatment with cromolyn significantly reversed these processes, while administration of compound 48/80 further upregulated the percentage of TUNEL-positive neurons (**Figure 5**).

Blockade of Microglial Protease-Activated Receptor-2 Alleviated Mast Cells-Mediated Inflammatory Injury

Protease-activated receptor-2 (PAR-2) is a MC-tryptase receptor located in microglia. Consistent with the increased expression of tryptase, immunostaining and western blot demonstrated an upregulated level of PAR-2 after SAH (**Figures 6A,B**). To further investigate the mechanism of MCs-mediated neuroinflammation under SAH condition, the selective PAR-2 antagonist ENMD-1068 was introduced. Compared with SAH + vehicle group, MCs degranulator compound 48/80 conferred a robust inflammatory response, which was evidenced by the increased expression of tryptase, PAR-2, TNF-α, IL-1β, and IL-6. However, although it did not affect the level of tryptase, PAR-2 antagonist ENMD-1068 significantly reversed the pro-inflammatory effect of compound 48/80, evidenced by decreased level of inflammatory cytokines (**Figures 6B–G**). More importantly, the adverse effects of

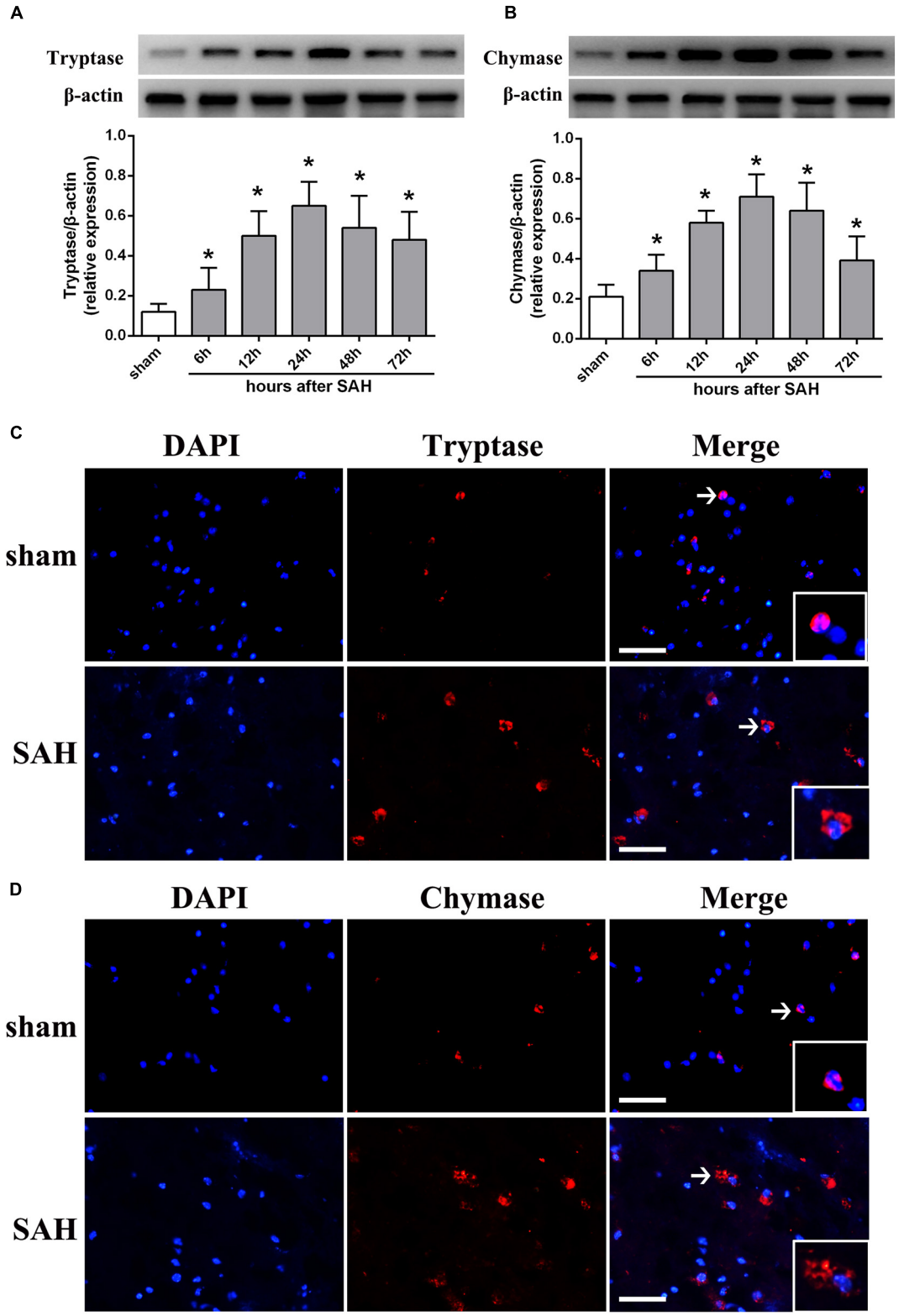

FIGURE 1 | The expression pattern of MCs-derived tryptase and chymase after SAH. **(A,B)** Representative western blotting images and quantitative analyses of tryptase and chymase expression in ipsilateral basal cortex after SAH. $n = 6$. The bars represent the mean ± SD. $^*P < 0.05$ versus sham. **(C,D)** Representative microphotographs of immunofluorescence double staining showing the expression and distribution of tryptase and chymase in sham group and SAH 24 h group. Scale bar = 50 μm.

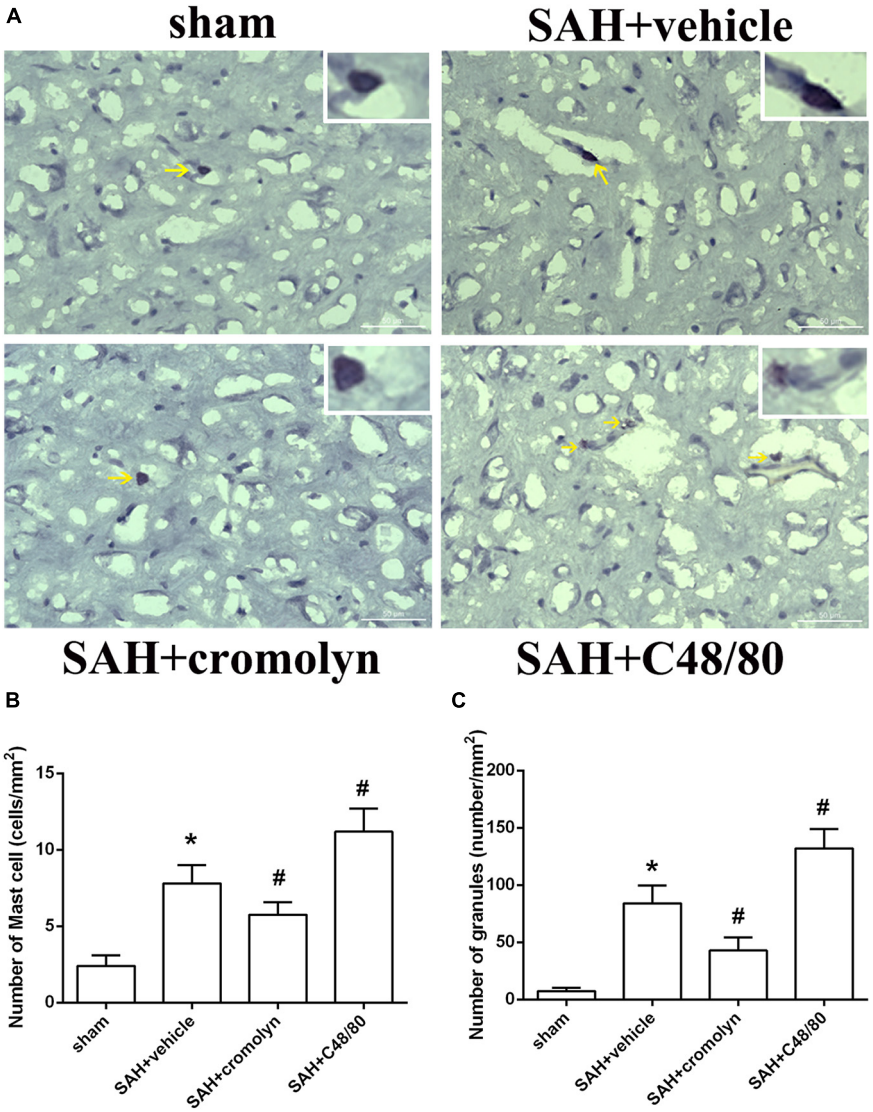

FIGURE 2 | Effect of cromolyn and compound 48/80 on MCs infiltration and degranulation at 24 h after SAH. **(A)** Toluidine blue staining of mast cells in cortex region (yellow arrow: infiltrated MCs). **(B,C)** Quantitative analyses of MCs infiltration and degranulation. $n = 6$. Data are represented as mean ± SD. $^*P < 0.05$ versus sham group. $\#P < 0.05$ versus SAH + vehicle group.

compound 48/80 on aggravating brain edema and neurological impairment were significantly reversed by ENMD-1068 (**Figures 6H–J**).

Effect and Mechanism Studies *in vitro*

The cultured BV2 cells were incubated with mast cell derived tryptase and the level of inflammatory cytokines was analyzed by RT-PCR. Consistent with the results *in vivo*, data from RT-PCR indicated that stimulate microglia with mast cell derived tryptase significantly increased the level of TNF-α, IL-1β, and IL-6, accompanied by the upregulation of microglial M1 marker CD16 and iNOS (**Figure 7**). More importantly, blockage of microglial PAR-2 with ENMD-1068 remarkably alleviated tryptase-induced upregulation of mentioned inflammatory mediators and microglial M1 markers (**Figure 7**), suggesting that mast cell-derived tryptase promoted microglia polarized into pro-inflammatory M1 phenotype and aggravated microglia-associated neuroinflammation, at least partly, by interacting with microglial PAR-2.

DISCUSSION

In the current study, we focused on the role of brain MCs in the pathological process following SAH and explored the potential mechanism. The major findings of the study are listed as follows: (1) MCs rapidly activated in response to SAH; (2) MCs stabilizer cromolyn significantly alleviated SAH-induced brain edema and neurological deficits, as well as attenuated neuroinflammation

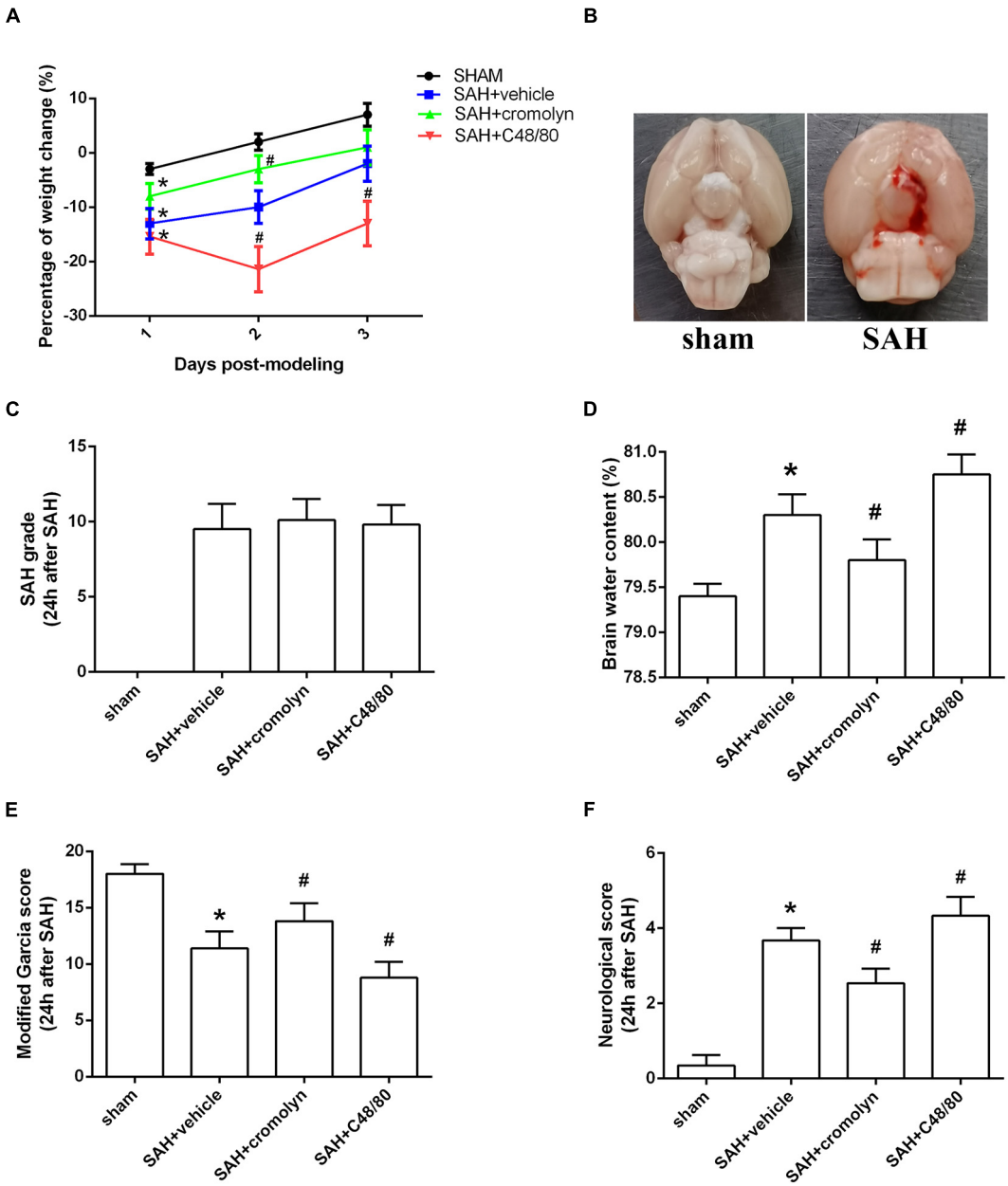

FIGURE 3 | Effect of MCs on brain edema and neurological outcome at 24 h post-modeling. **(A)** The change of body weight in animals among different groups. **(B)** Typical brains from sham-operated and SAH mice. **(C)** The quantification of SAH severity. **(D)** The quantification of brain water content. **(E,F)** The quantification of neurobehavioral test. $n = 6$. Data are represented as mean ± SD. *$P < 0.05$ versus sham group. #$P < 0.05$ versus SAH + vehicle group.

and neuronal damage, while MCs degranulator compound 48/80 exacerbated brain injury following SAH; (3) Pharmacological inhibition of microglial PAR-2 reversed MCs-mediated inflammatory response and neurological impairment after SAH. Based on the evidence above, MCs contribute to brain injury through mediating microglia-related neuroinflammation under SAH condition (**Figure 8**).

Subarachnoid hemorrhage accounts for 5% of all the stroke cases and mortality could be over 67% in the first few months. And the survivors could suffer from chronic neurological disorders, such as cognitive and/or motor impairments (Muehlschlegel, 2018). Although the pathophysiological event that occurs after SAH is quite complex, recent studies increasingly implicate that excessive inflammation is the critical factor in determining the prognosis of SAH (Lucke-Wold et al., 2016). Neuroinflammation is a complex mechanism involving different immune and inflammatory cells and different inflammatory mediators. Previous studies indicated that inflammatory response peaks at 24–48 h post-bleeding. And the elevated neuroinflammation is associated with higher clinical

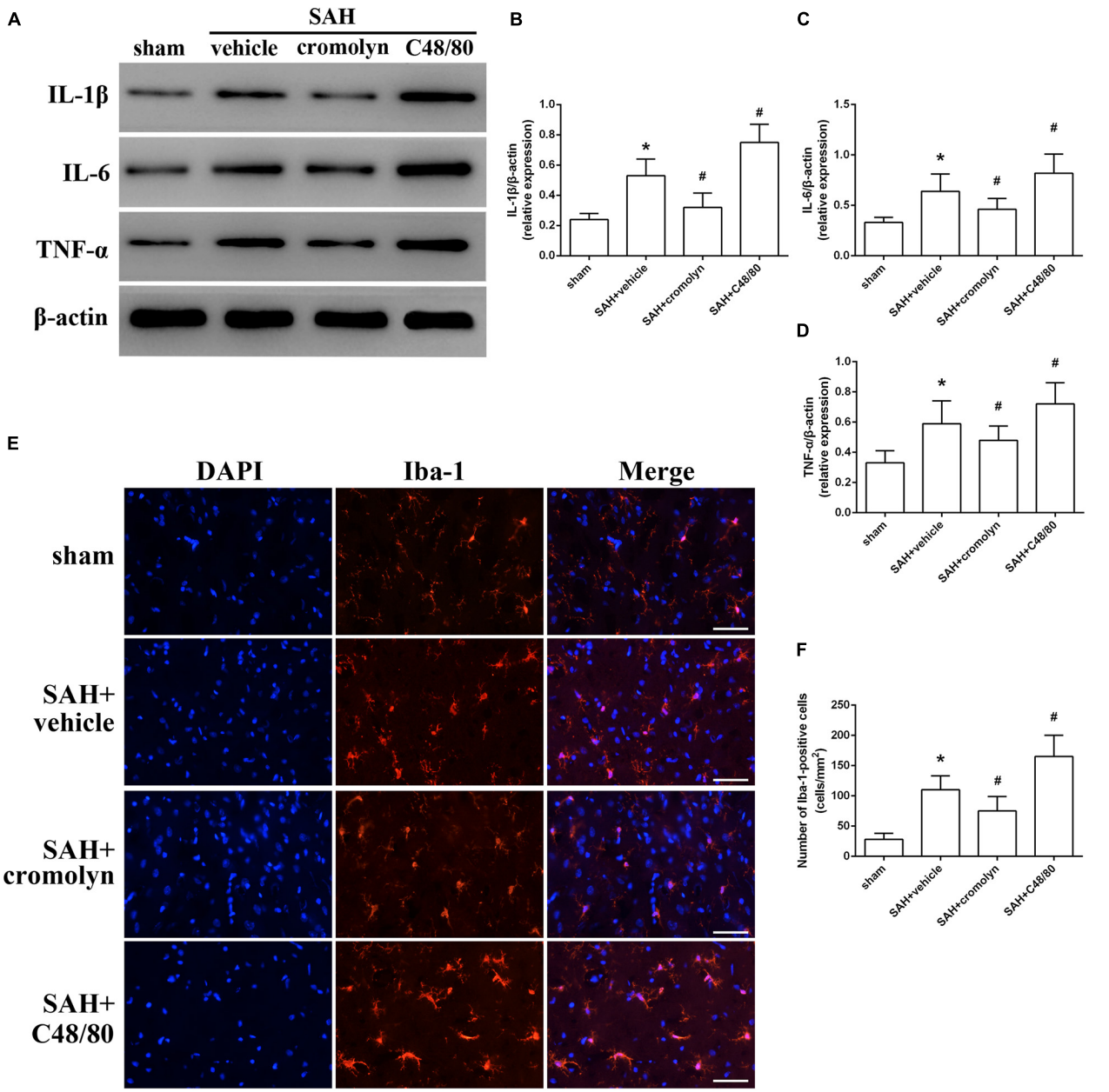

FIGURE 4 | Effect of MCs on SAH-induced neuroinflammation at 24 h post-modeling. **(A–D)** Representative picture and quantitative analysis of expression of TNF-α, IL-1β, and IL-6 in different groups. **(E,F)** Representative picture and quantitative analysis of microglia activation in ipsilateral basal cortex after SAH. scale bar = 50 μm. $n = 6$. Data are represented as mean ± SD. *$P < 0.05$ versus sham group. #$P < 0.05$ versus SAH + vehicle group.

severity, delayed cerebral ischemia, mortality, and functional outcome after SAH (Ahn et al., 2019; Bevers et al., 2019). In addition, recent studies suggested that inhibition of Enhancer of Zeste Homolog 2 (EZH2) or thromboxane-prostaglandin (TP) receptors could afford a robust neuroprotective effect against SAH via inhibiting neuroinflammation in a rat model of SAH (Lagier et al., 2019; Luo et al., 2020). Similarly, our previous studies have also confirmed that inhibition of neuroinflammation confers a robust neuroprotection against SAH in murine models (Xu et al., 2017; Peng et al., 2020). Based on the evidence above, further investigations to discover new therapeutic strategies targeting SAH-induced neuroinflammation remain a high priority.

Mast cells are the immune cell widely located in regions in contact with the external environment, including respiratory tract, skin, and gut (Metz et al., 2007). Due to the peculiar anatomical location, MCs act as the first line immune sentinel cells in responding to allergens, pathogen, or environmental stimulation such as cold. Once activated, MCs rapidly respond to signals from the surrounding microenvironment and attendant

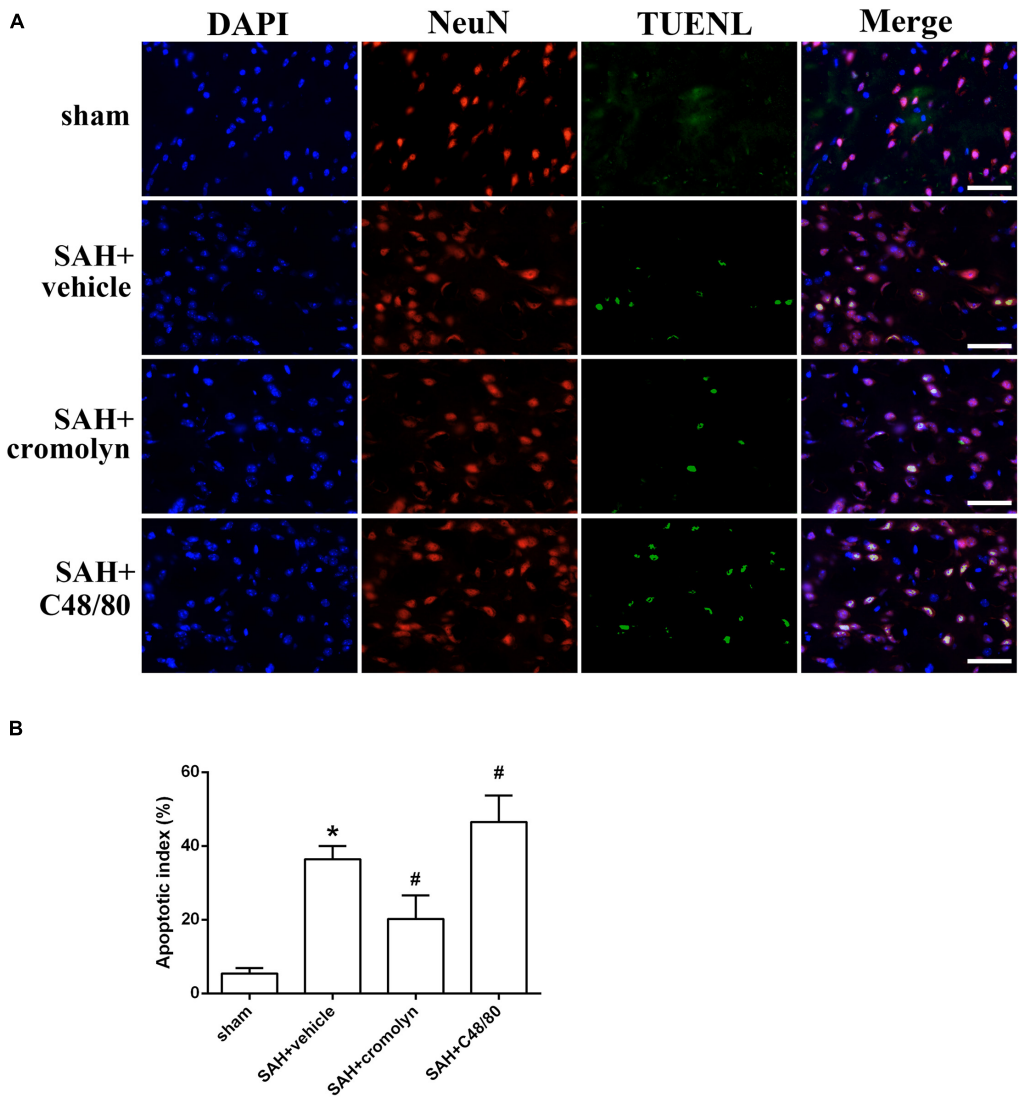

FIGURE 5 | Effect of MCs on SAH-induced neuronal injury. **(A)** Representative microphotographs showing the co-localization of TUNEL positive cells (green) with NeuN (red) in cortex. **(B)** The quantitative analysis of TUNEL-positive neurons. Scale bar = 50 μm. $n = 6$. Data are represented as mean ± SD. *$P < 0.05$ versus sham group. #$P < 0.05$ versus SAH + vehicle group.

pathological conditions via migrating to an injury site and further mediating tissue damage and/or repair through releasing the granule into surroundings (El Ansari et al., 2020). On the one hand, the activated MCs could facilitate wound healing by recognizing antigens through pattern recognition receptors and the high-affinity immunoglobulin E receptor, resulting in the releasing of granules, which further mediates cell recruitment, fibrosis, angiogenesis, and extracellular matrix deposition (Ozpinar et al., 2020). One the other hand, the cytoplasmic granules from MCs also contribute to multiple inflammatory and allergic pathogenesis diseases, including asthma, allergy, arthritis, interstitial cystitis, irritable bowel syndrome (IBD), ulcers, and prostatitis (Chelombitko et al., 2020; Fu et al., 2020). Notably, accumulating studies implicate the role of MCs in CNS. Similar to the effects in peripheral tissue, MCs in the brain confer a complex role in maintaining CNS homeostasis, both beneficial and adverse (da Silva et al., 2014). MCs are necessary for the normal neuronal development and cognitive function (Fitzpatrick and Morrow, 2017; Lenz et al., 2018). However, excessive activation of MCs has also been identified to participate in the pathological process of multiple CNS diseases. Liu et al. reported that MCs activation was closed related to the postoperative cognitive dysfunction (POCD) (Liu and Yin, 2018). Similarly, aberrant activation of MCs could mediate neuroinflammation via interaction with glial cells and neurons in Parkinson's disease (PD) and depression (Kempuraj et al., 2019; Friesen et al., 2020). Moreover, MCs activation significantly exacerbates brain edema and neuronal damage in mice subjected to both middle cerebral artery occlusion (MCAO) and intracerebral hemorrhage (ICH) through

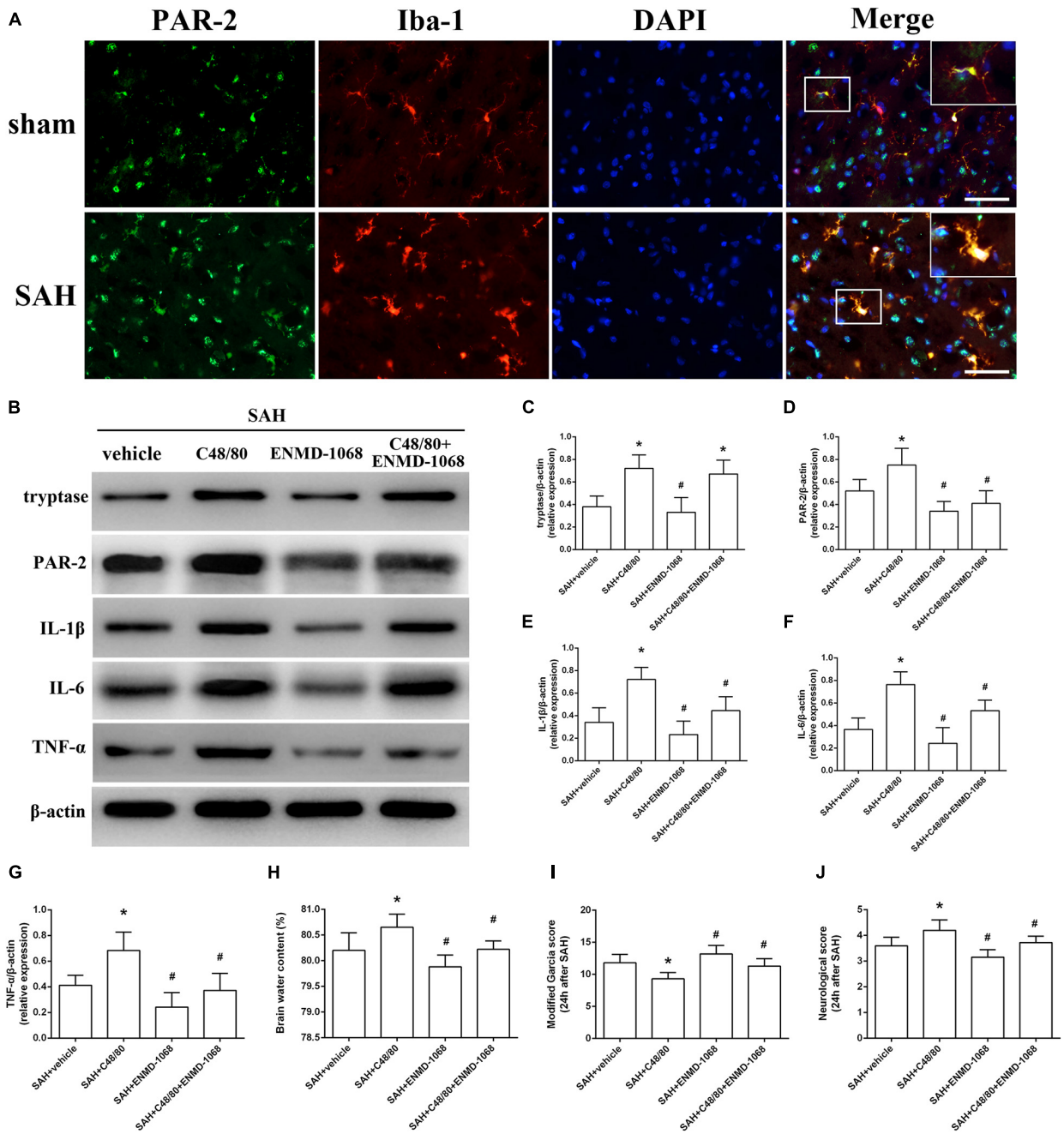

FIGURE 6 | Pharmacological inhibition of microglial PAR-2 alleviated MCs-mediated inflammatory injury at 24 h after SAH. **(A)** Immunofluorescence staining demonstrates the expression of PAR-2 in sham and SAH mice. $n = 6$. Scale bar = 50 μm. **(B–G)** Representative western blotting images and quantitative analyses of tryptase, PAR-2, TNF-α, IL-1β, and IL-6 expression in ipsilateral basal cortex after SAH. **(H)** The quantification of brain water content. **(I,J)** The quantification of neurobehavioral test. $n = 6$. Data are represented as mean ± SD. $^*P < 0.05$ versus SAH + vehicle group. $\#P < 0.05$ versus SAH + compound 48/80 group.

degrade neurovascular matrix and open tight junction, while MC-deficient rats demonstrated a reduced mortality, brain swelling, and neurological outcome (McKittrick et al., 2015; Parrella et al., 2019). Moreover, an increased number of infiltrated MCs was reported in the aneurysm tissues of intracranial aneurysm patients, and MCs stabilizer reduced the size and the thinning of aneurysm, suggesting a critical role of MCs in the development of aneurysm (Ishibashi et al., 2010; Hasan et al., 2012). Despite the fact that the activation of MCs has been determined in multiple human diseases, the potential function of MCs in the pathological process after SAH is yet to be elucidated.

Therefore, in the first part of study, we explored whether MCs are involved in the pathological process after SAH.

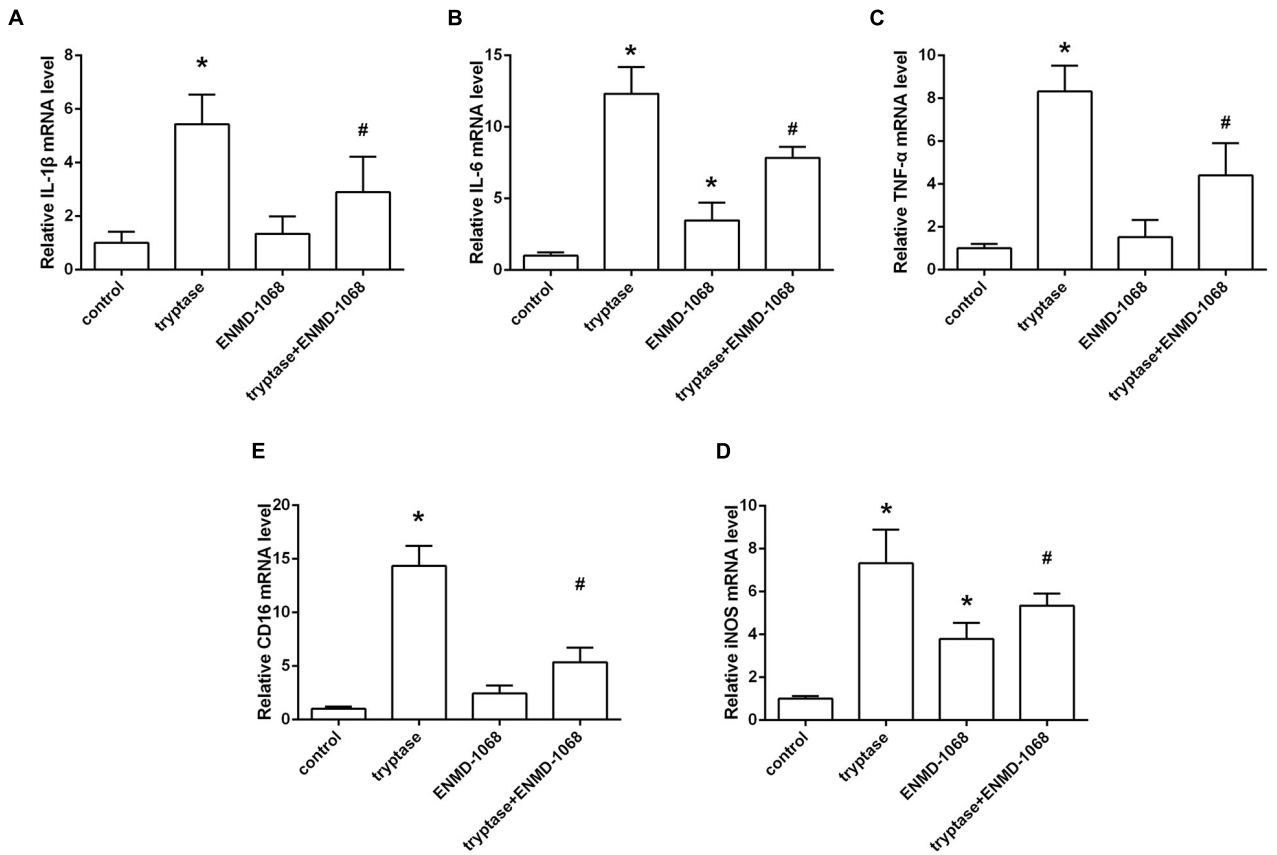

FIGURE 7 | Effect and mechanism studies using cultured BV2 cell *in vitro*. **(A–E)** Quantification of RT-PCR on the relative mRNA level of pro-inflammatory cytokines genes (IL-1β, IL-6, and TNF-α) and M1 microglia markers genes (CD16, iNOS). Data were represented as mean ± SD. *$P < 0.05$ versus control group. #$P < 0.05$ versus tryptase group.

And consistent with previous studies demonstrating the MCs activation at early stage after TBI (Kempuraj et al., 2020), we noted a significant and rapid activation of MCs under SAH, evidenced by the increased expression of cytoplasmic proteases and degranulation of mast cells in the brain. Given the critical roles of MCs in CNS homeostasis and diseases, we speculated that MCs might influence the outcome of SAH. Thus, in the next part of this study, the role of MCs activation after SAH was investigated by using cromolyn and compound 48/80. Cromolyn is one of the most widely used MCs stabilizers in the management of asthma worldwide through preventing MCs degranulation and inhibiting the release of inflammatory mediators such as histamine and tryptase, while compound 48/80 is an agent with a robust effect in modulating MCs degranulation. And consistent with previous study (Strbian et al., 2007), we noted that administration of cromolyn significantly inhibited the activation and degranulation of MCs, while compound 48/80 enhanced the activation of MCs, indicating the efficacy of both compounds in the pathological process following SAH. McKittrick et al. (2015) reported that MCs activator compound 48/80 significantly aggravated cerebral edema and neurological dysfunction in a rat ischemic stroke model via upregulating gelatinase activity, while knockdown of MCs diminished brain swelling, BBB leakage, and neutrophils infiltration. Similarly, in the current study, we noted that inhibition of MCs with cromolyn confers a robust neuroprotection via decreasing brain edema and neurological deficits, while compound 48/80 significantly aggravated SAH-induced brain injury. Moreover, given the critical role of inflammation in determining the outcome of SAH, we further explore the effect of MCs in SAH-induced neuroinflammation. As reported (Hughes et al., 2017), our data indicated that MCs stabilizer remarkably inhibited inflammatory response, evidenced by the decreased number of activated microglia and downregulated pro-inflammatory cytokines. Additionally, treatment with cromolyn significantly alleviated neuronal damage. However, activation MCs with compound 48/80 further deteriorate SAH-induced inflammation and neuronal injury. All of this evidence suggested that activation of MCs affords to brain damage after SAH via mediating inflammatory injury. However, the potential mechanism remains unclear.

Therefore, the last part of this study was aimed to determine the exact mechanism of MCs-related neuroinflammation under SAH condition. Microglia are the most important resident immune cell distributed in the CNS. Generally, microglia keep in rest status, morphology characterized by a small soma and long protrusions, and provide immune surveillance for injury

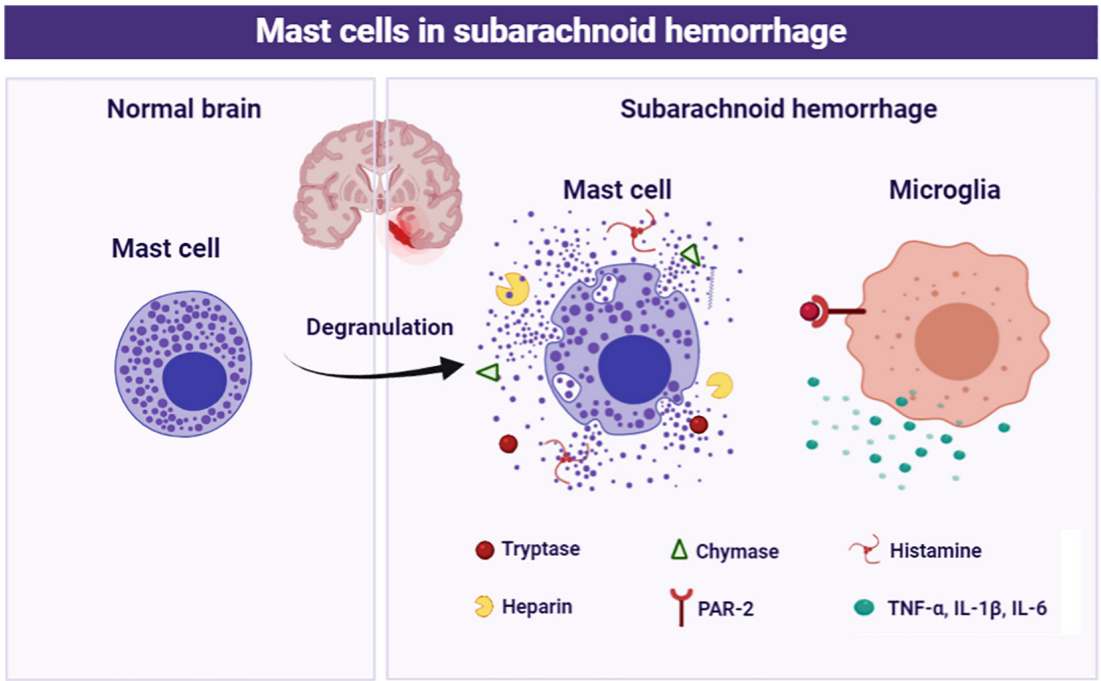

FIGURE 8 | Schematic diagram of MCs in the pathological process following SAH. MCs are rapidly activated and degranulation in response to SAH. Subsequently, MCs-derived tryptase binds to PAR-2 on microglia, resulting in microglial activation and ultimately mediate inflammatory damage.

and pathogen. Upon activation, microglia could rapidly migrate to the site of injury and transform into different phenotypes with distinct physiological functions. Among the multiple phenotypes, M1 microglia, characterized by the CD16 and iNOS, could secrete pro-inflammatory cytokines and chemokines (such as IL-1β and TNFα), ultimately elevating the immune response and exacerbating brain damage at the early stage after SAH (Xiong et al., 2016). Accumulating evidence indicated that inhibition of the microglia-related inflammation conferred a robust neuroprotective effect against SAH (Xie et al., 2018; Gris et al., 2019; Peng et al., 2020). Notably, recent studies indicated that MCs might be an important factor in modulating microglial activation. On the one hand, MCs degranulation secretes histamine and proteases, which recruit monocytes and neutrophils, resulting in an inflammatory microenvironment and microglial activation (Christy et al., 2013). On the other hand, MCs could activate microglia directly through interacting with numerous receptors distributed on the surface of microglia (Conti et al., 2020; Sandhu and Kulka, 2021). Among the multiple participants, proteinase-activated receptor 2 (PAR-2) is one of the most important MCs-microglial interaction-related receptors. PAR-2, also known as GPR11 or F2RL1, belongs to the 7-transmembrane receptors that couple to guanosine-nucleotide-binding proteins family that are encoded by F2RL1 gene (Kawaguchi et al., 2020). PAR-2 is widely expressed in mammals, especially in epidermal keratinocytes and immune cells, including neutrophils, dendritic cells, T cells, and macrophages (Sutherland et al., 2019; Shah et al., 2020). Upon its extracellular amino terminus between arginine and serine was cleaved by protease, PAR-2 is activated and further modulate multiple biological processes such as metabolism, tumorigenesis, and inflammatory response (Kim et al., 2020; Wang et al., 2020; Bang et al., 2021; Jiang et al., 2021). Recent studies indicated PAR-2 is the critical bridge between the cross-talk of MCs and microglia. Tryptase released from MCs could cleave and activate proteinase-activated receptor 2 (PAR-2) on microglial cells. Subsequently, the activated PAR-2 could further phosphorylate mitogen-activated protein kinases (MAPK), resulting in the activation of microglia and release of inflammatory cytokines (Zhang et al., 2016). Recently, Ocak et al. (2020) reported that microglia PAR-2 signal was significantly activated, accompanied with the enhanced neuroinflammation in a rat model of asphyxial cardiac arrest. Similarly, in the current study, we noted that the level of microglial PAR-2 was remarkably upregulated after SAH, accompanied by the increased level of pro-inflammatory mediators TNF-α, IL-1β, and IL-6. However, pharmacological inhibition of PAR-2 remarkably reversed MCs-mediated inflammatory response, evidenced by the decreased pro-inflammatory mediators expression and downregulation of M1 microglia markers. More important, blockage of PAR-2 significantly diminished MCs-induced brain edema and neurological impairment after SAH. All the mentioned evidence suggested a critical role of microglial PAR-2 in MCs-induced tryptase-dependent neuroinflammation in the pathological process of SAH.

Several limitations of this study should not be ignored. Firstly, we mainly focused on the role of MCs in mediating microglia-related neuroinflammation in the current study.

However, given that PAR-2 are widely expressed in the CNS, further studies to determine the potential interaction between MCs with other type of cells, such as astrocyte and endothelial cells, are required in the future. Secondly, both the delivery routes of cromolyn or PAR-2 antagonist ENMD-1068 administrated in this study were obtained from previous studies. It could be important to further explore the pharmacokinetics of these small molecule compounds after SAH *in vivo* and develop novel approaches to further promote translational potential as SAH treatment. Additionally, MCs *per se* can secrete a variety of cytokines, and it is unclear whether the effect of cromolyn or C48/80 on pro-inflammatory cytokines secretion was partly exerted by regulating MCs itself. Lastly, neuroinflammation has been considered as a double-edged sword in the pathophysiological process after acute CNS. Therefore, more efforts might be required to determine the possible effects of MCs at the subacute or chronic stages of SAH.

In summary, the current study investigated the role of MCs in the pathological process after SAH and explored the potential mechanism. Our data indicated that MCs activation contributes to brain edema and neurological impairment after SAH. Furthermore, MCs-derived tryptase exacerbates microglia-related neuroinflammation via interacting with microglial PAR-2. Taken together, the current study supports the notion that targeting MCs or PAR-2 might be a novel and promising therapeutic strategy for SAH.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang University. All procedures involving animals conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

AUTHOR CONTRIBUTIONS

BQ, SZ, CZ, YC, and HC performed the experiments. JZ, HZ, CX, and HX analyzed the data. YP, JL, GY, CG, and LW wrote and edited the manuscript. LW, YP, and GC designed and supervised the project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2021.710481/full#supplementary-material

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REVIEW
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Microglia Phenotype and Intracerebral Hemorrhage: A Balance of Yin and Yang

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Intracerebral hemorrhage (ICH) features extremely high rates of morbidity and mortality, with no specific and effective therapy. And local inflammation caused by the over-activated immune cells seriously damages the recovery of neurological function after ICH. Fortunately, immune intervention to microglia has provided new methods and ideas for ICH treatment. Microglia, as the resident immune cells in the brain, play vital roles in both tissue damage and repair processes after ICH. The perihematomal activated microglia not only arouse acute inflammatory responses, oxidative stress, excitotoxicity, and cytotoxicity to cause neuron death, but also show another phenotype that inhibit inflammation, clear hematoma and promote tissue regeneration. The proportion of microglia phenotypes determines the progression of brain tissue damage or repair after ICH. Therefore, microglia may be a promising and imperative therapeutic target for ICH. In this review, we discuss the dual functions of microglia in the brain after an ICH from immunological perspective, elaborate on the activation mechanism of perihematomal microglia, and summarize related therapeutic drugs researches.

Keywords: intracerebral hemorrhage, microglia, neuroinflammation, neuroprotective, stroke

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INTRODUCTION

Intracerebral hemorrhage (ICH) has become one of the most common and lethal diseases in the last decades (Zhou M. et al., 2019). It affects more than 2 million patients worldwide every year, with the majority in developing countries (Cordonnier et al., 2018; Zhu et al., 2019). ICH represents 10–25% of all strokes but leads to more than 50% of the deaths (Lan et al., 2017b; Cordonnier et al., 2018). 43–51% of patients with ICH die within 30 days, and only 12–39% of survivors keep living independently which imposes an enormous burden upon healthcare systems (Zhou et al., 2014; An et al., 2017). Neither internal medical managements, including hemostasis and intensive blood pressure-reduction, nor surgery methods as hematoma evacuation, has been testified efficacious by clinical randomized controlled trials (Mayer et al., 2008; Mendelow et al., 2013; Hemphill et al., 2015; Baharoglu et al., 2016; Morotti et al., 2017; Cordonnier et al., 2018). However, inspiringly, immune intervention promises a specific therapy strategy when neurologists shift attention to ICH secondary injury. Lately, fingolimod has been demonstrated signally improved neurological functional recovery in patients with ICH by means of regulating immunocytes number and activity (Fu et al., 2014; Li Y.-J. et al., 2015).

Microglia, as the resident immunocyte accounting for 5–10% of all human brain cells (Ma et al., 2017; Liu et al., 2021), take the lead in both tissue damage and repair processes after ICH. The perihematomal activated microglia not only arouse acute inflammatory responses, oxidative stress, excitotoxicity, and cytotoxicity to damage neurovascular unit (M1 phenotype), but also transform the phenotype to inhibit inflammation, clear hematoma, and promote tissue regeneration (M2 phenotype). M1 and M2 microglial phenotypes play opposite functions, but they are actually complementary, interconnected, and can be transformed into each other, work coordinately and even interdependently (Hu et al., 2015; Orihuela et al., 2016), just like yin and yang in ancient Chinese philosophy. Their balance directly determines which way the pathophysiology goes towards, brain tissue repair or excessive damage. Thus, microglia may be a promising and imperative therapeutic target for ICH.

In this review, we describe the dualistic roles of microglia in ICH from an immunological perspective, expound on the detailed mechanism of perihematomal microglial activation and polarization, and summarize the related therapeutic researches.

MICROGLIA

German neuropathologist Franz Nissl firstly discovered microglia with platinum stain in 1899 and called it "Staebchenzellen". Then, Spanish neurohistologist Del Rio-Hortega coined the term "microglia" in 1919 and described in detail its superior ability of rapid proliferation, migration, and phagocytosis, which laid the groundwork for follow-up studies (Ginhoux and Prinz, 2015; ElAli and Rivest, 2016; Smolders et al., 2019).

After a century of exploration, microglia are customarily regarded as the macrophage in the brain due to the similarity in morphology, functions, and biomarkers (Nayak et al., 2014; Ginhoux and Prinz, 2015). Microglia can be identified with classical macrophage markers, such as ionized calcium binding adapter molecule1 (Iba1), surface glycoprotein F4/80, integrin CD11b, and the epitope of keratan sulfate 5D4 (Nayak et al., 2014; Dudvarski Stankovic et al., 2016; Lan et al., 2017b). However, microglia have been demonstrated to possess different embryological origin and transcriptional profile from that of macrophage, which suggest the functions of microglia and microphage are not identical. Microglia are recognized as Tmem119-positive and CD45-low, while macrophages are Tmem119-negetive and CD45-high (Li Q. et al., 2018).

Activated microglia have been found to differentiate into two broad subtypes with distinct cellular makers and biological functions (Sica and Mantovani, 2012; Zhao H. et al., 2015; Dudvarski Stankovic et al., 2016; Lan et al., 2017b; Ma et al., 2017; Li Q. et al., 2018; Tschoe et al., 2020). According to the M1/M2 dichotomy proposed by Mills in 2000, activated microglia are categorized into pro-inflammatory M1 phenotype (classical activation) and anti-inflammatory M2 phenotype (alternative activation). The process that resting microglia differentiate into M1/M2 phenotype is referred to as polarization. Recently, M2 microglia are alternatively divided into M2a/M2b/M2c subtypes. Classical inflammatory factors such as IL-1β, IL-6, and Tumor necrosis factor-α (TNF-α) were used as the main markers of M1 microglia, while M2a microglia markers are represented by anti-inflammatory factors IL-4, IL-10, scavenger receptor CD36, and mannose receptor CD206, M2b microglia express major histocompatibility complex II (MCH-II), CD86, IL-10, and M2c microglia express phagocytic receptor CD163, insulin-like growth factor 1 (IGF-1), brain-derived neurotrophic factor (BDNF). Different markers of microglia phenotypes show different roles that they play after ICH. The particular information on microglia subtypes is summarized in **Table 1** (Lan et al., 2017b; Ma et al., 2017; Tschoe et al., 2020; Liu et al., 2021).

SPATIOTEMPORAL PATTERN OF MICROGLIAL ACTIVATION AFTER ICH

As the immune monitor in the brain, microglia become activated immediately after ICH, make morphological changes from a highly ramified phenotype to a rod, spherical, and finally an amoeba shape with contracting, thickening, and largening (more than 7.5 μm in diameter; Walker et al., 2014; Yang S. S. et al., 2016; Shtaya et al., 2019; Wei et al., 2020).

Spatially, microglia usually show different activation levels, morphologies (ameboid, branched, or intermediate), and directivities in different distances from the hematoma (Wang G. et al., 2013; Yang S. S. et al., 2016). Amoeba microglia mainly appear in close proximity to the hematoma, and partial microglia are found activated away from the hematoma, such as the ipsilateral cerebral cortex, corpus callosum, and hippocampus.

In the time course, microglia activation begins within 1–4 h, peaks in 1–3 days, declines at day 7, and returns to physiological level in 3–4 weeks after ICH (Zhou et al., 2014; Wan et al., 2016; Zhu et al., 2019). As shown in **Figure 1**, both M1 and M2 phenotypes of microglia are presented in the perihematomal area throughout the course of the disease, while the M1/M2 proportion is continually changing. It stays in an M1-dominated state for a week after ICH and deflects to an M2 preponderance within 1–2 weeks (Wan et al., 2016). In animal models, M1 makers including IL-1β, IL-6, TNF-α, including inducible nitric oxide synthase (iNOS) increase dramatically within 3 days after ICH, while interferon-γ (IFN-γ) mostly increase in the later phase. The levels of M2 makers like Arginase-1 (Arg-1), resistin-like-α (Fizz1), CD206 go up gradually within 1 week and decline in 7–14 days except for transforming growth factor-β (TGF-β), which remains relatively high at days 14 (Zhao H. et al., 2015; Dang et al., 2017; Lan et al., 2017b; Taylor et al., 2017).

Notably, despite the time point is different, almost all microglial markers increase, which makes it difficult to faultlessly describe the dynamic phenotypic changes. With regard to this fact, it is better to evaluate microglial activation with as many makers as possible at present.

TABLE 1 | Particular information on microglia subtypes.

Phenotype	Polarization agents	Makers	Roles
M1	LPS, IFN-γ, TNF-α, IL-1β, IL-17	IL-1β, IL-6, IL-12, IL-23	pro-inflammation
		TNF-α	pro-inflammation
		iNOS	oxidative damage
		MHC-II	antigen presentation
		CCL2, CCL5, CCL20	chemokine
		CXCL10	chemokine
		MMP2, MMP9	matrix decomposition
		CD16, CD32	phagocytosis, chemotaxis
M2			
M2a	IL-4, IL-13	IL-4, IL-10	anti-inflammation
		TGF-β	anti-inflammation
		CD36	phagocytosis
		CD206	phagocytosis
		CCL22	chemokine
		Arg-1	tissue regeneration
		Ym-1	stabilizing extracellular matrix
		Fizz1	tissue regeneration
M2b	TLRs agonist, IL-1R ligands, Fc receptors	MCH-II	pro-inflammation
		CD86	pro-inflammation
		IL-1RA	anti-inflammation
		IL-10	anti-inflammation
M2c	IL-10, TGF-β, glucocorticoid	CD163	phagocytosis
		IGF-1	tissue regeneration
		NGF	tissue regeneration
		BDNF	tissue regeneration
		NT3, NT4/5	tissue regeneration
		Arg-1	tissue regeneration
		YM-1	stabilize extracellular matrix
		Fizz1	tissue regeneration

LPS, lipopolysaccharide; IFN-γ, interferon γ; TNF-α-II, tumor necrosis factor α; iNOS, inducible nitric oxide synthase; MHC-II, major histocompatibility complex II; MMP, matrix metalloproteinase; Arg-1, arginine 1; Ym-1, chitinase 3-like 3; IGF-1, insulin-like growth factor 1; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT3, neurotrophin 3; NT4/5, neurotrophin 4/5; FIZZ1, resistin-like-α.

FUNCTIONS OF ACTIVATED MICROGLIA AFTER ICH

After ICH, blood swarms into the brain parenchyma causing an expanding hematoma which leads to immediate neurological impairment and microglial activation. Respectively, M1 microglia are commonly considered as the deleterious phenotype, and M2 microglia as the beneficial one (Xi et al., 2014; Zhou et al., 2014) , as shown in **Figure 2**. Microglia possess phenotypic and functional plasticity. Promoting M1-M2 phenotypic transformation has become the mainstream strategy of microglial intervention in ICH treatment.

M1 Microglia

M1 microglia secrete a large number of inflammatory factors, proteases, chemokines, prostaglandins, and other toxic substances. Since multiple damage-inducing factors overlap, brain cells die in various forms such as apoptosis, necrosis, pyroptosis, ferroptosis, which leads to the irreversible destruction of brain structure (Xi et al., 2014; Zhou et al., 2014).

In brain parenchyma, M1 microglia are the major source of inflammatory mediators, such as IL-1β, IL-6, IL-12, IL-23, and TNF-α (Jiang et al., 2020). Although inflammation is essential for innate immunity, it is the chief culprit to the sustained neurological deterioration in a sterile environment (Zhu et al., 2019). While inflammatory cytokines diffuse, functional neurons and neuroglia quickly die under the stress condition (Shen et al., 2017). The diffused inflammatory cytokines also promote polarization of surrounding microglia

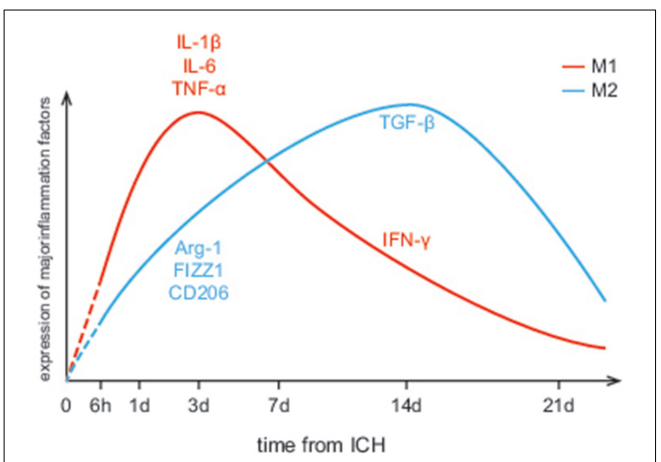

FIGURE 1 | Dynamic changes of M1/M2 microglial activation levels after intracerebral hemorrhage (ICH). It provides a visual expression that M1/M2 microglia take on different activation characteristics. The red curve represents M1 microglia while the blue curve represents M2 microglia. Yet, the referenced researches about microglial spatiotemporal features are all animal experiments, leaving the human brain as an unknown area.

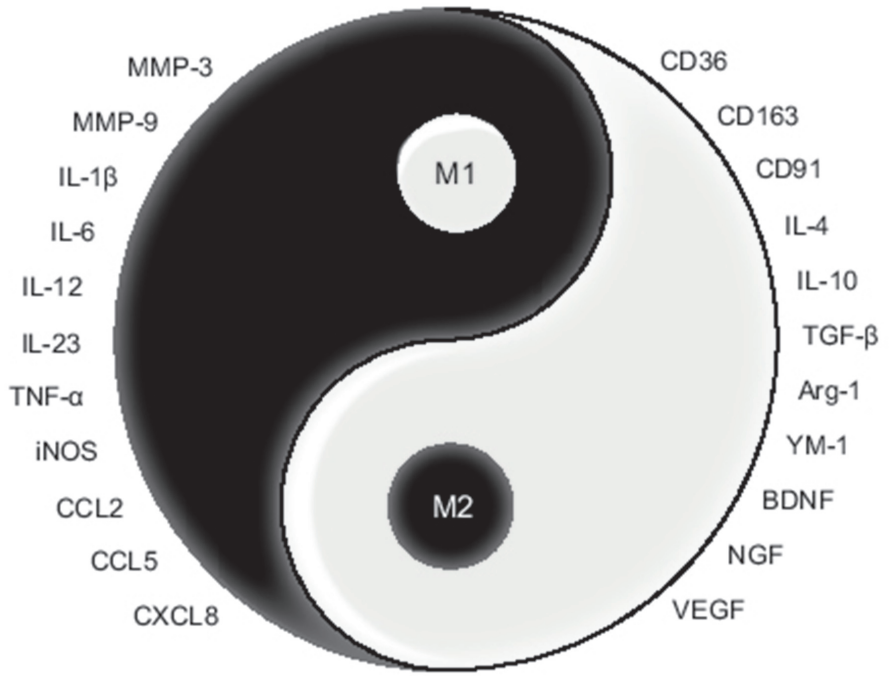

FIGURE 2 | Sketch map for the opposite function of M1/M2 microglia. In this Tai Chi-diagram, the half-filled-out symbols with left half black represent M1 microglia, with the expression of MMPs, pro-inflammatory cytokine, and chemokine. And the right half white represents M2 microglia, with the expression of phagocytic receptors, anti-inflammatory cytokine, and growth factors.

towards the M1 phenotype, cause the inflammatory region to expand, which forms a vicious circle. In patients with ICH, the levels of IL-1β, TNF-α, and IL-6 in plasma and brain tissues are significantly increased within 1–3 days, and the increasing degree is related to 90-days poor prognosis (Jiang et al., 2020). During pathological processes, oxidative stress and inflammation mutually reinforcing, which is no exception in ICH (Hu et al., 2016; Yao et al., 2021). M1 microglia express large amounts of peroxidases, iNOS, and reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase, which produce excessive free radicals and damage surrounding cells by attacking cellular membranes and DNA (Yang et al., 2013; Duan et al., 2016; Hu et al., 2016; Xiong et al., 2016). Moreover, M1 microglia contribute to the activation of matrix metalloproteinases (MMPs), including MMP2 and MMP9, which markedly destruct the blood-brain barrier (BBB) and cause severe vasogenic brain edema by degrading extracellular matrix constituents and attacking endothelial claudin-family tight junction proteins (Montaner et al., 2019). In ICH patients, increased MMP2/9 levels were independently associated with perihematomal edema volume (Li et al., 2013). In addition, M1 microglia also release chemokines including CXCL8, CCL2, and CCL5, which diffuse into peripheral blood through the ruptured blood vessel and attract peripheral leukocytes such as neutrophils, monocytes, and lymphocytes into brain parenchyma through disrupted BBB (Trettel et al., 2020). It was reported that chemokines concentrations in plasma were proportional to the infiltration degree of peripheral immunocytes in ICH patients (Guo et al., 2020). The infiltrated immunocytes not only express and secrete inflammatory factors and aggravate inflammatory response but also release toxic substances after their apoptosis (Lambertsen et al., 2019). In ICH patients, CCL2 concentrations in plasma within 24 h were associated with poor functional outcomes at day 7 after ICH (Hammond et al., 2014). Also, inhibiting CCL2 in animal models reduced brain edema and improved neural function (Yan et al., 2020).

Noticeably, there is an evident cooperativity effect on tissue damage induced by inflammatory cytokines, protease MMPs, and chemokines. Inflammatory cytokines not only attack vascular endothelial cells and tight junction proteins but also induce endothelial cells to secrete intercellular cell adhesion molecule-1 (ICAM-1), which promotes the adhesion and infiltration of peripheral leukocytes (Aslam et al., 2012). The direct damage on neurons induced by MMPs exacerbates inflammatory response, disrupts BBB to facilitate peripheral leukocytes infiltration (Kim et al., 2005). The infiltrated peripheral leukocytes secrete inflammatory factors and MMPs, which aggravates inflammatory response and BBB destruction in turn (Tschoe et al., 2020).

Although the treatments aiming at inflammatory cytokines are currently limited in animal experiments, TNF-α antibody has shown huge therapeutic potential by significantly reducing the number of perihematomal activated microglia and improving neurological outcomes in mouse stroke models (Mayne et al., 2001; Lei B. et al., 2013; Chen A.-Q. et al., 2019). Inhibition of TNF-α not only reduces the microglial activation/macrophage

recruitment *via* decreasing cleaved caspase-3 level (Mayne et al., 2001; Lei B. et al., 2013; Chen A.-Q. et al., 2019) but also reduces the activation of TNF receptor 1 (TNFR1) on endothelium therefore reducing endothelium necroptosis and ameliorating disruption of BBB (Mayne et al., 2001; Lei B. et al., 2013; Chen A.-Q. et al., 2019). Predictably, inhibition of specific inflammatory factors is becoming the central theme of ICH therapeutic researches.

M2 Microglia

M2 microglia primarily express anti-s and facilitate tissue regeneration (Lan et al., 2017b). Thereby, the injured brain acquires comprehensive and effective recovery. Due to the large amounts of anti-inflammatory cytokines and antioxidants, the inflammatory response and oxidative become diminished tardily (Zhu et al., 2019). More importantly, the anti-inflammatory factors promote surrounding microglia and other immune cells to transform into anti-inflammatory phenotype. It's found that patients with higher TGF-β levels in plasma had a better prognosis at 90 days after ICH (Jiang et al., 2020).

At the same time, M2 microglia engulf the hematoma and cells debris, remove harmful substances and provide space for tissue regeneration. With the increase of the number of M2 microglia, the volume of the hematoma is eliminated promptly in 7–21 days after ICH. B-scavenger receptor CD36, one of the M2 microglial makers, is the main executive of microglial phagocytosis activity, which is obviously induced to upregulate by IL-10 (Fang et al., 2014; Yang et al., 2015; Li et al., 2021). In the mouse ICH model, CD36 knockout significantly inhibits hematoma absorption, and leads to the aggravation of neurological disorders (Fang et al., 2014). Instead, adoptive transferring CD36-positive microglia to CD36 knockout mice showed a significant improvement of neurological function after ICH (Yang et al., 2015). In fact, M2 microglia express CD163 and CD91 to absorb hemoglobin and heme, respectively (Dang et al., 2017; Garton et al., 2017). It should be noted that CD163 levels expressed by microglia may not be the only limiting factor in hematoma clearance. As a protective mechanism against severe hemolysis, the Haptoglobin (Hp) secreted by oligodendrocytes can capture free hemoglobin (Hb) to form a stable Hp-Hb complex, which is then englobed through CD163, thus reducing the toxicity of Hb. Similarly, hemopexin (Hx), secreted by neurons, binds with heme and is devoured *via* CD91 (Ma et al., 2016).

Particularly, M2 microglia are the drivers of brain tissue regeneration and remodeling. M2 microglia express various growth factors and trophic factors, such as insulin-like growth factors-1 (IGF-1), Brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), neurotrophin 3 (NT-3), NT-4/5, which could promote neurogenesis and neural circuit reframing (Xi et al., 2014; Ma et al., 2017). IGF-1 promotes the proliferation, migration, and differentiation of the neuro precursor cells in the subventricular zone, and facilitates the regenerated neurons' functional integration into a new neural circuit (Thored et al., 2009). In a mouse ICH model, IGF-1 antibody promotes microglial M1 polarization, leading to more residual behavioral defects (Sun et al., 2020). BDNF and GDNF stimulate axon regeneration, which takes part in new neural connections (Madinier et al., 2009). The neurotrophic factors, including NT3 and NT4/5, are not only beneficial to the survival of residual neurons but also essential for the improvement and stability of the newborn neuron (Ma et al., 2017). During the remodeling of brain tissue, M2 microglia secrete clotting substance chitinase 3-like 3 (Ym-1) to prevent the degradation of extracellular matrix components (Girard et al., 2013). M2 marker Arg-1 not only converts arginine into polyamine which contributes to extracellular matrix subsidence but also competes with iNOS for reaction substrates to inhibit the excessive oxidative stress (Munder, 2009).

In general, M2 microglia resist inflammation and engulf hematomas to create a calm and stable microenvironment, which contributes to the neuro-angiogenesis and matrix deposition, and allows brain tissue to regain structure and function. Nevertheless, because of M1 microglia domination, only a third of new neurons survive inflammation in the acute phase. Therefore, promoting a beneficial microglial phenotypic transformation is a promising way in ICH treatment.

POLARIZATION MECHANISM OF MICROGLIA AFTER ICH

In order to regulate microglial polarization accurately and effectively, it is necessary to understand the mechanism of microglial polarization, including the source of extracellular stimuli and intracellular signaling pathways, which has been briefly summarized in **Table 2**.

Extracellular Agents

After ICH, blood carrying red blood cells (RBCs) and plasma proteins including thrombin and fibrinogen infiltrate into the brain parenchyma, and trigger the initiation of early cellular and molecular pathological processes. Hematoma not only contains the agents that directly activate microglia but also promote microglial M1-polarization indirectly through tissue damage. **Figure 3** provides an overview of M1-polarization.

Because of energy exhaustion and cytotoxicity, RBCs in the hematoma begin to lyse within 1 day and continue for weeks after ICH (Righy et al., 2016). The damaged RBCs release Hb, peroxiredoxins (Prxs), and Carbonic Anhydrase-1 (CA-1), which induce microglia differentiating into M1-phenotype (Guo et al., 2012; Liu et al., 2016; Bian et al., 2020). Hb and the decomposed product hemin can directly promote microglial M1-polarization through Toll-like receptors (TLRs; Lin et al., 2012; Wang et al., 2014). Therefore, clearing hematoma is of importance in reducing brain damage. Since no reliable clinical benefits are provided from surgical hematoma removal at present, promoting hematoma devouring by microglia is of great significance.

During the formation of hematoma, thrombin and complements are produced in the brain, which are also important factors for M1-polarization. Thrombin, a serine protease that promotes blood clotting, is detected in the brain within 1 h after ICH (Zhu et al., 2019). Thrombin directly activates M1 microglia by binding to the proteinase-activated receptor-1 (PAR-1; Wan et al., 2016). In mouse models, delayed administration of thrombin inhibitor hirudin

TABLE 2 | Signaling pathways of microglia polarization.

Microglia phenotype	Intracellular signal molecule	Extracellular agents	Effect molecules
M1	TLRs-NF-κB	Hb, hemin fibrinogen HMGB1, nucleic acids, heat shock protein Prxs	NLRP3; IL-1β, IL-6, TNF-α
	MAPK-NF-κB	IL-1β, IL-6, TNF-α thrombin glucocorticoid	
	STAT1	IL-1β, IL-6, TNF-α	
M2	PPAR/Nrf2	Peroxisome	Arg-1, IL-4, CD36, HO-1
	STAT4/6	IL-4, IL-10	Ym-1, Fizz1

in 7–28 days after ICH significantly reduced the number of pro-inflammatory microglia (Li et al., 2019). However, thrombin regulation is difficult to apply to clinical therapy because of its two-sided effects. Though the inhibition of thrombin shows a beneficial effect in inflammation reduction, a suitable thrombin concentration is necessary for helping stop hemorrhage and protect neurons. Complements, anaphylatoxins, are activated within 24 h through various proximal cascaded pathways (Ducruet et al., 2009; Yuan et al., 2017). Complement composition C3a activates microglia cells by binding to the specific receptor C3aR. Membrane attack complex (MAC), the end product of complement cascade, attacks cell membrane, and leads to erythrocyte lysis and neuronal death, which indirectly exacerbates microglial M1-polarization. In animal models, complement inhibitor N-acetyl heparin inhibits microglia activation and ameliorates neurological deficits (Wang M. et al., 2019).

Besides, brain tissue primary damage also contributes to microglial polarization (Zhang et al., 2017). Neurons and astroglia around the hematoma express inflammatory factors such as IL-15 and IL-17, playing a vital role in M1 polarization (Yu et al., 2016; Shi et al., 2018, 2020). Likewise, damaged neurons and glia release damage-associated molecular patterns (DAMPs), including high mobility group protein-1 (HMGB1), heat shock proteins, and extracellular matrix fragments (Mracsko and Veltkamp, 2014; Bobinger et al., 2018). HMGB1 is a non-chromosome-related protein widely expressed in the nucleus of all eukaryotic cells (Mu et al., 2018). Under physiological conditions, HMGB1 helps stabilize chromosomes and regulate the transcription of many survival-based genes, but once it is dissociated from the nucleus and released outside the cell, HMGB1 becomes a powerful inflammatory mediator that promotes microglial M1 polarization by binding to TLRs on microglia (Ohnishi et al., 2011; Wang D. et al., 2017). In rodent ICH models, glycyrrhizin attenuates intracerebral hemorrhage-induced injury in a concentration-dependent manner *via* inhibiting HMGB1 (Ohnishi et al., 2011; Mu et al., 2018). HMGB1 inhibitor Ethyl-pyruvate significantly reduced microglia activation and inflammatory factors levels *via* inhibiting nuclear factor kappa B (NF-κB) DNA binding activity (Su et al., 2013).

In the later phase of ICH, an anti-inflammatory pathway, enlisting native microglia, occurs alongside neuroinflammation (Shtaya et al., 2021). Anti-inflammatory factors such as IL-4, IL-33, IL-10, TGF-β increase distinctly around the hematoma, which are mostly released by macrophages, mature lymphocytes, and mast cells (Taylor et al., 2017; Zhou et al., 2017; Chen Z. et al., 2019). The immune microenvironment changes shift microglial polarization from M1 to M2. Intraventricular injection of IL-4 in mice increases the proportion of M2 microglia and accelerates the recovery of neurological function after ICH (Yang J. et al., 2016). Some other molecular targets have also been recently identified up-regulated on microglia during M2-polarization after ICH, including Dopamine D1 receptor (DRD1), Cannabinoid receptor-2 (CB2R), Melanocortin receptor 4 (MC4R), and especially sphingosine-1-phosphate receptor (S1PR; Xu et al., 2013; Li L. et al., 2015; Zhang et al., 2015).

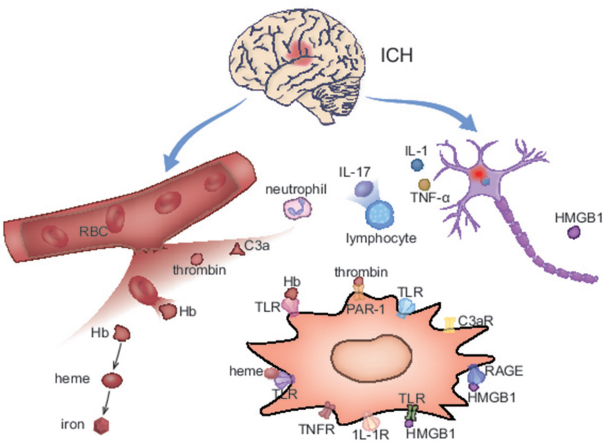

FIGURE 3 | The activation mechanism of M1 microglia after ICH. In ICH acute phase, M1 microglia are activated on account of the blood composition and neuron primary damage. Meanwhile, microglia up-express corresponding receptors for activation.

Intracellular Signal Transduction

To recognize extracellular agents and transduce extracellular signals, microglia express various membrane receptors, nuclear receptors, and executive proteins to play roles in morphological and functional changes such as secretion, phagocytosis, and movement. Understanding microglial signal transduction is beneficial to the exploration of clinical targets.

Here, we briefly introduce several important receptors and signaling molecules.

TLRs-NF-κB

TLR is a type I transmembrane protein that plays an important role in the innate immune and inflammatory response (Alvarado and Lathia, 2016). So far, 10 functional TLRs have been found in humans, and microglia mainly express TLR4, TLR2, and heterodimer TLR2/4 (Fang et al., 2013; Hayward and Lee, 2014; Wang et al., 2014). Hb, hemin, fibrinogen, HMGB1, heat shock protein, Prxs, and nucleic acids generated during ICH are all TLRs ligands (Lin et al., 2012; Fang et al., 2013; Zhou et al., 2014; Wan et al., 2016; Fu et al., 2021). After binding to these ligands, TLRs signaling is activated. TLR4 simultaneously activates two parallel downstream pathways of myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) while TLR2 recruits only MyD88. Both of them lead to the activation of transcription factor NF-κB (Sansing et al., 2011; Wang Y.-C. et al., 2013; Fei et al., 2019). NF-κB is a crucial signal for microglial M1-polarization and inflammatory factors expression. During the process, inhibitors of NF-κB kinase (IKK) are activated firstly, which cause the phosphorylation and degradation of NF-κB inhibitor (Iκb; Fei et al., 2019). After that, NF-κB dimer is released and enters the nucleus to regulate transcription for M1-polarization. Of note, NF- κB can be detected in the peripheral circulation, which is a biomarker to determine the severity of brain damage.

MAPK

Mitogen-activated protein kinase (MAPK) is a member of the serine/threonine kinase family, which includes P38, Extracellular Signal-Regulated Kinase1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) pathways (Sun and Nan, 2016). MAPK not only enters the nucleus to regulate the transcription processes but also increases the activity of NF-κB in the cytoplasm (Wei et al., 2019). After ICH, MAPK is activated by inflammatory factors, thrombin, and glucocorticoid, MAPK signaling plays a critical role in microglia survival and M1-polarization.

NLRP3

Inflammasome NLR Family, Pyrin domain containing protein 3 (NLRP3) is a kind of intracellular multi-molecular protein complex that is involved in inflammation (Walsh et al., 2014; Luo et al., 2019). NLRP3 activates lyase caspase-1, an enzyme that trims microglia secreted pre-IL-1β and pre-IL-18 into mature IL-1β and IL-18 (Ren et al., 2018), which makes NLRP3 a promising target of inflammatory regulation. In the mouse ICH model, intraventricular injection of NLRP3 siRNA immediately reduced inflammatory response and brain damage.

PPAR-γ and Nrf2

Peroxisome proliferator-activated receptor (PPAR-γ) and Nuclear erythroid 2 related factor 2 (Nrf2) are important signals of M2-polarization (Zhao X.-R. et al., 2015). Nrf2 is a basic leucine zipper (bZIP) protein that enters the nucleus to regulate transcription. PPAR-γ is a highly-expressed nuclear hormone receptor in microglia. PPAR-γ and Nrf2 actually work together with overlapping functions. They enhance the expression of Arg-1, IL-4, and CD36, which enables microglia in phagocytosis and tissue repair (Xia et al., 2015; Wang J. et al., 2018). Except for that, PPAR-γ and Nrf2 jointly regulate the expression of hundreds of antioxidant genes including heme oxygenase-1 (HO-1; Culman et al., 2007; Shang et al., 2013).

STATs

As a common transcription signal for cytokines, signal transducer and activator of transcriptions (STATs) family exert their effect on both M1 and M2 polarization (Tschoe et al., 2020). Microglia express a large number of cytokine receptors, such as IL-1R, TNFR, IL-4R, which activate the downstream Janus kinase (JAK)-STATs signal. Among STATs, STAT1 promotes M1 polarization and inflammatory factors expression (Bai et al., 2020). STAT4/6 promotes M2 polarization and the expression of Ym-1 and Fizz1 (Righy et al., 2016). Intriguingly, STAT3 was demonstrated to be involved in both M1/M2 polarization (Hu et al., 2015).

M1-M2 Phenotypic Transformation

It is observed that single microglia express both M1/M2 phenotypic markers (Ransohoff, 2016; Tschoe et al., 2020). Neither M1 nor M2 should be considered as a microglial final differentiation form. The ability of microglia to switch between M1/M2 phenotypes is always a fascinating topic. However, the mechanism for this phenotypic transformation is really elusive. M1 and M2 microglia not only perform distinct cellular functions but also have incompatible polarization processes. For example, *in vitro*, PPAR-γ significantly inhibits the activation of NF-κB and STAT1/3 (Fang et al., 2014). In like manner, inflammatory cytokines and TLRs inhibit microglia in CD36 expression (Zhou et al., 2014; Yuan et al., 2015).

Recently, the relationship between microglia phenotype and metabolic status has attracted much attention. Microglia in different phenotypes show different oxidative metabolism (Eun Jung et al., 2020). Compared to M1 microglia, M2 microglia have significantly lower oxygen consumption (Orihuela et al., 2016). Therefore, it has been speculated that intracellular stress environment and energy crisis promote M2 polarization by influencing mitochondrial metabolism. The reactive oxygen species (ROS) released by M1 microglia has been found to activate Nrf2, which contributes to microglial M2 polarization (Duan et al., 2016; Hu et al., 2016; Qu et al., 2016). In addition, Adenosine 5'-monophosphate activated protein kinase (AMPK), as a key molecule regulating bioenergy metabolism, is activated under cellular energy crisis and oxidative stress (Saikia and Joseph, 2021). Evidence indicates that AMPK contributes to Nrf2 activation as well (Zhao et al., 2018; Zheng et al., 2019). In other words, the initiative activation of M2 microglia may be a type of self-protection when M1 phenotype creates an immoderate oxidative stress (Barakat and Redzic, 2015). More in-depth research in the mechanism of microglial phenotypic transformation may provide insights into innovative therapeutic strategies for ICH.

Preclinical Researches Targeting Microglia

In view of the serious inflammatory brain injury, whole microglia population deletion by knocking out microglial survival signal

receptor colony-stimulating factor 1 receptor (CSF1R) achieved an early therapeutic effect in rodent experiments (Li et al., 2017; Shi et al., 2019). Of course, increasing the M2/M1 phenotypic proportion of microglia usually brings more satisfactory results (Wang J. et al., 2018; Bai et al., 2020), and it has become the most frequently studied therapeutic method. Relying on the aforementioned targets, experimental therapeutic studies on the precise regulation on microglia phenotype are developing rapidly, and relevant drugs are summarized in **Table 3** (Hu et al., 2011; Ohnishi et al., 2013, 2019; Yang et al., 2014a,b, 2018; Iniaghe et al., 2015; Zhao et al., 2015a,b, 2018; Flores et al., 2016; Shi et al., 2016; Sukumari-Ramesh and Alleyne, 2016; Zhang et al., 2016; Anan et al., 2017; Chen-Roetling and Regan, 2017; Lan et al., 2017a; Wang J. et al., 2017; Wei et al., 2017; Xu et al., 2017; Zeng et al., 2017; Chen C. et al., 2018; Chen S. et al., 2018; Fu et al., 2018; Han et al., 2018; Li X. et al., 2018; Qiao et al., 2018; Ren et al., 2018; Wang et al., 2018b; Liang et al., 2019; Song and Zhang, 2019; Xi et al., 2019; Zhou F. et al., 2019; Cheng et al., 2020; Ding et al., 2020).

CLINICAL RESEARCHES TARGETING MICROGLIA

Translational research in medication development has never been effortless. Although many preclinical researches have got positive results in ICH treatment, large clinical trials on microglia intervention are second to none. Conservatively, the therapeutic effect of minocycline, deferoxamine, fingolimod, thiazolidinediones (TZDs), and statins are relatively promising. Related clinical researches have been briefly summarized in **Table 4**.

Minocycline is an ordinary broad-spectrum antibiotic. It could pass through the blood-brain barrier freely and has a neuronal protection effect (Yang et al., 2019). With pleiotropic properties, minocycline scavenges free radical and promotes M1-M2 phenotypic transformation of microglia in piglet and rodent ICH models (Möller et al., 2016; Dai et al., 2019; Wang G. et al., 2019). When applied in ICH clinical trials, minocycline has not been demonstrated to produce favorable outcomes on 3-month functional independence and behavior score, but significantly depresses the levels of circulating inflammatory components (Fouda et al., 2017; Malhotra et al., 2018). It may be due to the fact that oral administration does not produce sufficient potency concentrations in brain parenchyma.

Deferoxamine is a classical iron-chelating agent. Except for reducing oxidative damage, it effectively reinforces the function of M2 microglia (Hu et al., 2019). RBC CD47 is a signal that stops itself from being swallowed by microglia (Song et al., 2021; Ye et al., 2021). Deferoxamine inhibited CD47 expression on RBCs and accelerated hematoma absorption conspicuously in pig models (Cao et al., 2016; Hu et al., 2019). In patients with spontaneous ICH, consecutive administration of deferoxamine mesylate for 5 days significantly reduces hematoma volume and brain edema progression (Yu et al., 2017).

Fingolimod is an S1PR agonist previously used for multiple sclerosis, which can directly activate M2 microglia. In ICH preclinical experiments, fingolimod has been demonstrated to inhibit brain edema and reduce the numbers of apoptotic cells (Rolland et al., 2013; Lu et al., 2014; Sun et al., 2016). When applied to clinical trials, 3 days consecutive oral administration of fingolimod shows beneficial effects on decreasing the numbers of lymphocytes and NK cells in circulation, controlling perihematomal brain edema (PHE), and ameliorating neurological deficits (Fu et al., 2014; Li Y.-J. et al., 2015).

TZDs, including pioglitazone and rosiglitazone, have a function in activating M2 microglia as PPAR-γ agonist (Song et al., 2018). In the rodent model, intraperitoneal injection of rosiglitazone increases the expression of CD36 on microglia, promotes hematoma clearance, and inhibits inflammatory factors expression (Chang C.-F. et al., 2017; Mu et al., 2017). TZDs have long been designed for clinical trials (Gonzales et al., 2013), but have not yet shown significant results.

Statins (HMG-CoA reductase inhibitors) are widely prescribed medications for the management of hypercholesterolemia. The potential of Statins for ICH treatment has been revealed recently (Chen Q. et al., 2019). Mechanistically, Statins regulate microglial phenotype by inhibiting inflammatory signals and enhancing PPAR-γ activity (Wang et al., 2018a; Bagheri et al., 2020). Although stains have been doubted for the safety of ICH treatment, they are ultimately deemed applicable in promoting neurological rehabilitation (Ribe et al., 2019). It has been demonstrated that statins improve the neurological function of ICH patients and reduce the mortality at 6 months (Tapia-Pérez et al., 2013; Witsch et al., 2019).

PERSPECTIVE

The Balance of Yin and Yang

Though how to regulate microglia to promote brain recovery remains worth pondering in some sense, there are latent misgivings that excessive inhibition of M1 microglia and promotion of M2 microglia may turn into adverse effects in ICH treatment, where we should keep watchful eyes.

On the one hand, the immunoreactive materials secreted from M1 microglia appear to have delayed beneficial effects on brain repair. Solid evidence indicates that MMPs are necessary for angiogenesis, myelin remodeling, and axonal regeneration in ICH later stage (Lei et al., 2015; Fields, 2019). As well, infiltrating neutrophils and monocytes have been found conducive to hematoma clearance and inflammation regression (Lambertsen et al., 2019). Besides, the over-suppressed inflammatory status may increase brain infection risk since the systemic immunity also decreases after ICH (Saand et al., 2019).

On the other hand, the early organizational disruption may build the basis of neogenesis. M1 microglia destroy dying and defunct neurons in pieces, which lends a convenience for M2 phagocytosis (Hu et al., 2015). In addition, the deconstruction of dense tissue matrix made by M1 microglia provides space for the migration of neural precursors and synaptic remodeling (Lei C. et al., 2013). Also, M1 microglia impair BBB integrity, which is in favor of the hematoma clearance by free diffusion, especially when microglial phagocytic

TABLE 3 | Preclinical researches on microglial regulation for ICH therapy.

Drugs	Targets	Species/Models	Results
Ginkgolide B	TLR4	rats/autologous blood	reduce inflammatory cytokine, lessen neuronal cell apoptosis.
Ligustilide	TLR4	mice/autologous blood	reduce inflammatory cytokine, induced neurological deficits.
Magnolol	TLR4	rats/collagenase	reduce the brain water content, attenuated neurological deficits.
Pinocembrin	NF-κB	mice/collagenase	reduce lesion volume and neurologic deficits.
Sparstolonin B	NF-κB	mice/autologous blood	reduce inflammatory cytokine and brain edema.
Curcumin	NF-κB	mice/autologous blood	inhibit inflammation and neurological impairment.
Protocatechuic acid	NF-κB	mice/collagenase	inhibit oxidative stress, inflammation and apoptosis.
Annexin A1	MAPK	mice/collagenase	attenuate brain edema, improved short-term neurological function.
Sesamin	MAPK	rats/collagenase	suppress microglial activation, prevent neuron loss.
Fisetin	NF-κB	mice/collagenase	reduce inflammatory cytokine, brain edema and cell apoptosis.
Theaflavin	NF-κB	rats/collagenase	alleviate the behavioral defects, inhibit the neuron loss and apoptosis.
fimasartan	NLRP3	rats/collagenase	attenuate brain edema and improve neurological functions.
dexmedetomidine	NLRP3	mice/autologous blood	reduce inflammatory cytokine, improve neurological function.
AC-YVAD-CMK	NLRP3	mice and rats/collagenase	reduce brain edema and improve neurological function.
MCC950	NLRP3	mice/autologous blood and collagenase	attenuate neuro-deficits and perihematomal brain edema.
Dimethyl fumarate	Nrf2	mice and rats/collagenase and autologous blood	improve neurological deficits.
Nicotinamide mononucleotide	Nrf2	mice/collagenase	suppress neuroinflammation and oxidative stress.
Shogaol	Nrf2	mice/collagenase	suppress oxidative stress and improve neurological function.
sulforaphane	Nrf2	mice and rats/ autologous blood	improve hematoma clearance.
Tert-butylhydroquinone	Nrf2	mice/collagenase	suppress oxidative stress and improve neurological function.
Isoliquiritigenin	Nrf2	rats/collagenase	alleviate neurological deficits.
Andrographolide		rats/autologous blood	alleviate neurobehavioral disorders and brain edema.
monascin	Nrf2	rats/collagenase	improve neurological deficits.
Sinomenine	Nrf2	mice/autologous blood	improve neurological deficits.

TABLE 4 | Clinical researches on microglial regulation for ICH therapy.

Drugs	Continent	No. of patients	Outcomes	Efficacy	References
minocycline	North America	10	NIHSS, mRS, mortality	NO	Chang J. J. et al. (2017)
	North America	8	mRS	NO	Fouda et al. (2017)
deferoxamine mesylate	Asian	47	hematoma volume, edema	YES	Yu et al. (2017)
fingolimod	Asian	23	hematoma volume, NIHSS	YES	Fu et al. (2014)
	Asian	11	edema	YES	Li Y.-J. et al. (2015)
statins	Europe	29	NIHSS, mortality	YES	Tapia-Pérez et al. (2013)
	North America	38	hematoma volume; edema	YES	Witsch et al. (2019)

NIHSS, National Institute of Health stroke scale; mRS, modified Rankin Scale.

receptors are of inefficiencies in the ICH early phase (Righy et al., 2016, 2018).

As for M2 microglia, superfluous and prolonged existing growth factors will predictably cause abnormal tissue repair. Overexpression of Arg1 has been found to cause tissue scarring and brain dysfunction (Hesse et al., 2001), and excessive polyamines extraordinarily promote inflammatory response (Dudvarski Stankovic et al., 2016). Resting microglia plays a special role in tissue repair and remodeling (Cherry et al., 2014), and M2-M0 may be a necessary functional transformation after ICH.

In summary, it is really improper to consider that M1 and M2 microglial phenotypes are thoroughly opposite. Instead, their interaction, cooperation, and even codependency are waiting to be explored in the future. A balance of M1 and M2 microglial, rather than extremely choosing M2 over M1, ought to be achieved for ICH individualized treatment, just like the balance of yin and yang.

Targeting Strategy

Although drugs with the pleiotropic ability of immune regulation may bring more benefits, not a few medical experiments failed just because of uncontrolled side effects. It is a neglected consensus that many microglial receptors and signaling molecules are meanwhile expressed or activated in other brain cells, such as astrocytes, oligodendrocytes, endothelial cells, and neurons. It is unwise to judge the holistic functions of concerned targets in the brain by taking only microglia into account. For example, CD163 helps microglia engulf and break down hemoglobin, whereas, inhibition of CD163 in the ICH acute phase unexpectedly reduces brain damage, possibly because inhibition of CD163 expressed on neurons decreases the Hb neurotoxicity induced neuronal death (Righy et al., 2018).

Hence, we need a kind of drug that has a high targeting specificity to microglia. Preferably, it's expected to have sufficient liposoluble ability to pass through BBB and concentrate on microglia. Furthermore, it's recommended to conjunctive use advanced medical technology such as intranasal administration, nanomaterials, and genetic technologies to achieve better intervention results for ICH treatment.

AUTHOR CONTRIBUTIONS

RB and ZF wrote and revised the manuscript. MY helped with the literature search and correction of the manuscript. BH and QH provided the conception and design of the review, and directed the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Sphingosine-1-Phosphate Signaling in Ischemic Stroke: From Bench to Bedside and Beyond

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Sphingosine-1-phosphate (S1P) signaling is being increasingly recognized as a strong modulator of immune cell migration and endothelial function. Fingolimod and other S1P modulators in ischemic stroke treatment have shown promise in emerging experimental models and small-scale clinical trials. In this article, we will review the current knowledge of the role of S1P signaling in brain ischemia from the aspects of inflammation and immune interventions, sustaining endothelial functions, regulation of blood-brain barrier integrity, and functional recovery. We will then discuss the current and future therapeutic perspectives of targeting S1P for the treatment of ischemic stroke. Mechanism studies would help to bridge the gap between preclinical studies and clinical practice. Future success of bench-to-bedside translation shall be based on in depth understanding of S1P signaling during stroke and on the ability to have a fine temporal and spatial regulation of the signal pathway.

Keywords: sphingosine-1-phosphate, ischemic stroke, fingolimod, neuroprotective agent, translational research

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INTRODUCTION

As the second largest cause of death and a leading cause of disability (Johnson et al., 2019), stroke produces immense health and economic burdens globally (Virani et al., 2020). With an aging population, the prevalence of stroke can be predicted to increase. More worrisome is the fact that the incidence of stroke in young adults has increased in recent years, which causes a profound socioeconomic impact due to high health-care expenditure and compromised labor productivity (Ekker et al., 2018). Most stroke survivors are unable to live independently and have greater risks of recurrence and other long-term disabling sequelae such as dementia (Levine et al., 2015).

Although the primary health goal is a decrease in stroke incidence as prevention is always better than cure. The increasing global stroke burden indicates that primary prevention strategies may not be sufficiently effective (Johnson et al., 2019), which calls for effective therapies in the acute phase and long-term follow-up rehabilitation for people who have developed stroke. About 87% of all strokes are ischemic (Virani et al., 2020). Despite a series of clinical trials with neuroprotective drugs, current treatment options remain limited to thrombolysis and mechanical recanalization for the acute phase of ischemic stroke (Powers, 2019). While effective, the treatments can only be applied to less than 10% of patients for a narrow treatment time window and strict exclusion criteria (Rinaldo et al., 2019). Moreover, even a successful recanalization may lead to enlarged infarction

due to ischemic-reperfusion (IR) injury (Mizuma et al., 2018). Novel safe and effective treatment strategies are therefore needed.

Sphingosine-1-phosphate (S1P) is a terminal breakdown product of sphingolipid metabolism discovered in the 1960s (Cartier and Hla, 2019). The degradation of plasma membrane sphingomyelin produces ceramide (Merrill, 2011). S1P is then produced through the metabolism of ceramide by ceramidase and two types of sphingosine kinases (SphK1 and −2). S1P can also be recycled back to ceramide or irreversibly degraded by S1P lyse (**Figure 1**; Hannun and Obeid, 2018). Many tissues keep a low intracellular S1P level by a rapid degradation of S1P. By contrast, S1P can be transported extracellularly allowing its extracellular action (Proia and Hla, 2015). Erythrocytes and endothelial cells are the major producers of S1P, while substantial amounts of S1P stored in platelets can only be released upon activation (Gazit et al., 2016). In circulation, S1P is bound mainly to high-density lipoproteins and albumin (Proia and Hla, 2015). These protein chaperones of S1P enable its solubility and specific biological activity. Outside the cell, S1P binds to S1P receptors (S1PR) to exert its biological actions. Vertebrates possess five S1PR (S1PR1-5) which are G-protein coupled receptors (Proia and Hla, 2015). These widely expressed S1PRs couple to key intracellular signaling pathways (Chun et al., 2010; Proia and Hla, 2015), thus coupling phospholipid metabolism with intercellular communication (Cartier and Hla, 2019). In the central nervous system, neuronal lineages express all S1PR (Soliven et al., 2011). Astrocytes and microglia express predominantly S1PR1 and S1PR3 (Benarroch, 2021). S1PR5 is mainly expressed in oligodendrocytes (di Nuzzo et al., 2014). As for peripheral immune cells, T cells are known to express S1PR1 (Baeyens et al., 2021), S1PR2 (Baeyens et al., 2015), and S1PR4 (Xiong et al., 2019). Human B cells express S1PR1, S1PR2, and S1PR4 at different levels by different B-cell subtypes but not S1PR3 (Sic et al., 2014). S1P signaling participates in many processes of growth and development and pathological conditions (Proia and Hla, 2015). It is essential for neural and vascular development (Mizugishi et al., 2005). S1P is in relatively high concentrations in circulation compared with tissue parenchyma. This concentration gradient, formed by the interplay of S1P synthetic and degradative enzymes as well as S1P exporters, is fundamental to S1P biology such as regulating lymphocyte migration (Cyster and Schwab, 2012) and supporting endothelial barrier function (Camerer et al., 2009).

The observation that *Rag1*$^{-/-}$ mice, that were mice devoid of lymphocytes, developed smaller infarct volume than that of wild-type mice (Yilmaz et al., 2006), pioneering a new field of immunomodulating therapies in ischemic stroke, especially those that have been used for the treatment of multiple sclerosis (MS) (Dreikorn et al., 2018). One of the best-studied drugs is fingolimod (FTY720), a S1P modulator that causes rapid induction of lymphopenia (Cyster and Schwab, 2012) and is approved for the treatment of relapsing-remitting multiple sclerosis (RRMS). Moreover, recent studies suggest that the function of S1P signaling in ischemic stroke includes but goes far beyond immunomodulating (Li et al., 2020; Chua et al., 2021; Nitzsche et al., 2021). Emerging experimental models and small-scale clinical trials have shown promise of S1P modulator for the treatment of stroke (Cyster and Schwab, 2012; Kraft et al., 2013; Fu et al., 2014; Qin et al., 2017). Herein, we highlight S1P signaling pathway in ischemic stroke and the translation from biomedical research basis into clinical stroke applications.

S1P IN CEREBRAL ISCHEMIA

We attempt to have a brief summary from the aspects of brain inflammation and immune interventions, sustaining endothelial functions, regulation of blood-brain barrier (BBB), and functional recovery (**Figure 1**).

S1P Signaling in Post-ischemic Brain Inflammation and Immune Interventions

Inflammatory reactions and immune responses have long been recognized as important elements during ischemic stroke (Fu et al., 2015). Neural cell death after ischemia releases damage-associated molecular patterns (DAMPs) and triggers immune responses including activation of microglia and astrocytes, recruitment of resident and peripheral immune cells (Shi et al., 2019). Inflammation can be both detrimental and beneficial at certain stages after stroke (Lambertsen et al., 2018).

Microglia are among the very first cell types to be activated and recruited to the site of ischemia (Ma et al., 2017). Upon activation, microglia produce numerous mediators such as cytokines and chemokines, growth and trophic factors (Ma et al., 2017). Studies showed that Sphk1 level was elevated in microglia and S1P production was enhanced mainly in activated microglia after ischemia (Kimura et al., 2008; Zheng et al., 2015). The production of S1P on the other hand enhanced the release of proinflammatory mediators from microglia (Nayak et al., 2010; Moon et al., 2015). In addition, S1PR1, S1PR2, and S1PR3 were shown to influence microglial activation and inflammation (Gaire et al., 2018b, 2019; Sapkota et al., 2019; Gaire and Choi, 2021). S1PR1 knock-down reduced microglial activation and microglial proliferation after ischemia (Gaire et al., 2018a). Therefore, the pathogenic role of S1P signaling may have a close relationship with microglia activation after ischemic stroke.

As the most numerous cells in the brain, astrocytes interact extensively with microglia (Liddelow et al., 2020) and help recruit immune cells (Li M. et al., 2017). S1P was also shown to activate astrocytes (Sorensen et al., 2003) while S1PR3 could promote astrogliosis after ischemic stroke (Gaire et al., 2018a,b). An antagonist of S1PR3, CAY10444 was found to attenuate astrocyte activation after transient middle cerebral artery occlusion (tMCAO) (Gaire et al., 2018b). Furthermore, S1PR3 deletion could attenuate S1P-induced inflammatory responses in astrocytes (Dusaban et al., 2017).

Invasion of peripheral lymphocytes drives the progression of inflammation (Shi et al., 2019). During IR injury, T cells interact with platelets and formulate a complex process called thrombo-inflammation which leads to infarct expansion (Stoll and Nieswandt, 2019). Experiments on a series of ischemic stroke mouse models indicated that the protective effect of S1P modulators such as fingolimod could be largely attributed to

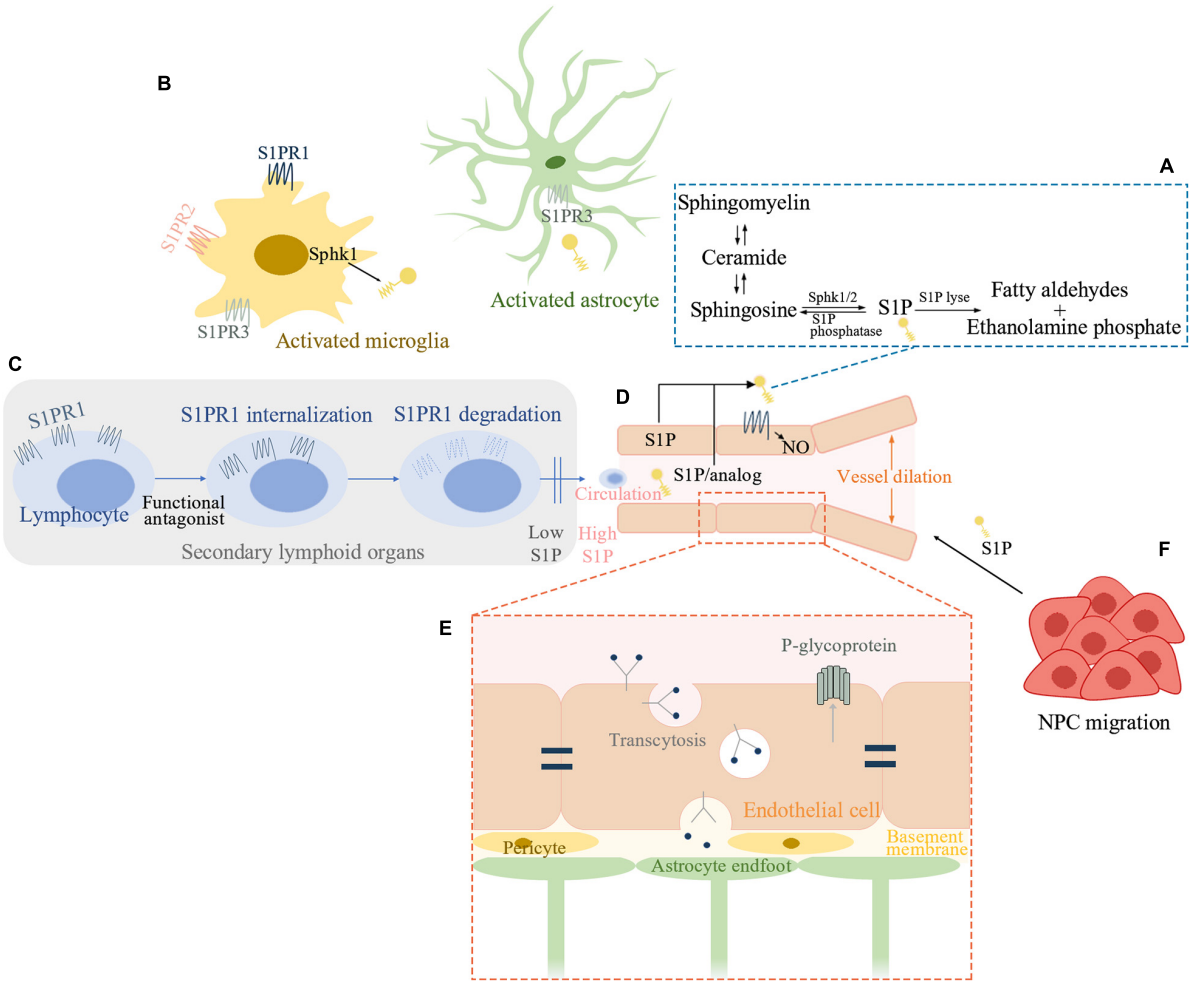

FIGURE 1 | Sphingosine1-phosphate (S1P) signaling pathway involved in ischemic stroke. **(A)** Schematic outline of S1P metabolism. **(B)** S1P signaling in post-ischemic brain inflammation. Sphk1 level is elevated in microglia and S1P production is enhanced in activated microglia. S1PR1, S1PR2, and S1PR3 influence microglial activation. Astrocytes interact extensively with microglia. S1P can activate astrocytes while S1PR3 is known to promote astrogliosis in ischemic brain. **(C)** S1P and recruitment of peripheral lymphocytes. Functional antagonist of S1PR1 can induce receptor internalization and degradation and thereby inhibits peripheral lymphocytes' recruitment. **(D)** S1P signaling in sustaining endothelial functions. S1P-S1PR1-nitric oxide regulates vessel dilation to flow. S1PR1 distributes to the abluminal surface of the endothelial cells. Either cell-autonomous S1P or system S1P/analog which penetrates the blood-brain barrier (BBB) can activate endothelial S1PR1 and sustains endothelial function. **(E)** S1P inhibits the function of P-glycoprotein at the BBB. Brain endothelial S1PR1 may help maintain BBB function by sustaining a proper distribution of tight junction proteins. Endothelial S1PR1 may participate in regulating vesicular transport in the early phase of BBB opening after ischemic stroke. **(F)** S1P is a chemoattractant for neural progenitor cells (NPC) toward the infarcted area thus facilitating neurogenesis. Sphk, sphingosine kinase; S1P, sphingosine-1-phosphate; S1PR, S1P receptor; NO, nitric oxide; NPC, neural progenitor cells.

impairment of lymphocyte trafficking and thereby, lymphocyte-driven thrombo-inflammation (Kraft et al., 2013). A marked decrease in infarct volume and improvement of functional outcome were found after fingolimod administration in wild type but not lymphocyte-deficient *Rag1*$^{-/-}$ mice after tMCAO, highlighting the key mechanism of lymphocytopenia in its protective effect (Kraft et al., 2013). Lymphocytopenia could then attenuate thrombo-inflammation in microvasculature and increase cerebral blood flow (CBF) after tMCAO (Kraft et al., 2013). However, in another permanent occlusion of the middle cerebral artery (pMCAO) mouse model, infarct volume and behavioral dysfunction were not reduced by fingolimod (Liesz et al., 2011). Similar results were found in pan-hematopoietic *S1pr1* knockout (KO) mice. Hematopoietic S1PR1 deficiency induced lymphopenia and exerted some neuroprotection after tMCAO, but not pMCAO (Nitzsche et al., 2021). These unexpected negative findings in pMCAO can be explained by the difference in the contribution of neuroinflammation in transient and permanent ischemia (Stoll and Nieswandt, 2019). In contrast to tMCAO where recanalization occurs, and the function of T cells is well established as contributing to IR injury in an antigen-independent fashion, in pMCAO, the contribution of T cells is less clear and more complex as secondary phenomena such as gut microbiome mediated systemic immunomodulation and stroke-related immunodepression syndrome may participate in the process (Stoll and Nieswandt, 2019).

S1P Signaling in Sustaining Endothelial Functions

Cerebral endothelial dysfunction contributes to stroke-induced brain injury. Despite successful recanalization, as many as 25–50% of patients still have undesirable long-term outcomes (Meinel et al., 2020). A main reason for this futile recanalization is poor reperfusion in the microvasculature downstream of an occlusion (Tian et al., 2018). In addition, to counteract the expansion of the infarct core, adequate and timely perfusion is needed to rescue the ischemic penumbra, the area surrounding the necrotic core, where CBF is still sufficient to keep neurons alive (Manning et al., 2014). Not only the number and size but also the dilatory capacity, integrity, and patency of collateral anastomose are important for successful reperfusion (Bonnin et al., 2019). Ischemia impairs endothelium-dependent vasodilation (Hu et al., 2017) and induces a proinflammatory endothelial phenotype (Ishikawa et al., 2003) which may promote thrombus formation and reduce blood flow. Sustaining endothelial functions in ischemic stroke may therefore constitute a therapeutic opportunity (Shuaib et al., 2011).

S1PR1, S1PR2, and S1PR3 are found in endothelial cells and S1P signaling help sustain endothelial functions (Proia and Hla, 2015). S1P is believed to act on endothelial S1PR1 to reduce vascular leakage (Camerer et al., 2009) and improve endothelial barrier (Huwiler and Zangemeister-Wittke, 2018). S1P-S1PR1-nitric oxide signaling was found to be a new regulatory pathway of vessel dilation to flow (Cantalupo et al., 2017). As for endothelial S1PR1 in the brain, a recent study demonstrated that after the formation of BBB, S1PR1 distributed to the abluminal surface of the endothelial cells which shielded them from ligands in circulation (Nitzsche et al., 2021). Therefore, cell-autonomous S1P is required to activate these endothelial S1PR1. While BBB penetration is needed for synthetic ligands to reach these receptors (Nitzsche et al., 2021). S1PR1 maintained endothelial function during cerebral ischemia as endothelial cell *S1pr1* knockout ($S1pr1^{ECKO}$) mice showed impaired microvascular perfusion after pMCAO (Nitzsche et al., 2021). Moreover, failure of collateral formation was found in $S1pr1^{ECKO}$ mice after pMCAO (Nitzsche et al., 2021). Based on these findings, the study showed that a S1PR1-selective agonist which can readily penetrate BBB targeting at endothelial S1PR1 receptor pool provided protection against ischemic injury (Nitzsche et al., 2021). And this beneficial effect was independent of reperfusion, which was in contrast to that of lymphocytopenia.

S1P Regulation of BBB Integrity

Lying between peripheral circulation and the brain parenchyma, BBB serves as both structural and metabolic barriers that restrict the access of many compounds while keeping the transport of nutrients and oxygen to the brain, thus maintaining the extracellular environment (Profaci et al., 2020). BBB permeability is controlled by cerebral endothelial cells along with pericytes and astrocytes through the presence of endothelial tight junctions (TJ), efflux and solute transporters, and low levels of transcytosis (Obermeier et al., 2013). During reperfusion, oxidative stress-induced BBB disruption causes vascular leakage which leads to vasogenic edema and aggravated brain damage. Moreover, extreme barrier disruption will result in intracerebral hemorrhage (Jickling et al., 2014). In experimental stroke studies, BBB opening is biphasic. A partial recovery was found between the initial and second increase in BBB permeability (Pillai et al., 2009). Two different mechanisms participated in this process, beginning with upregulation of endothelial transcytosis (6 h after tMCAO). Dynamic remodeling of TJ complexes forming gaps or protrusions is not obvious until in the late phase (24–48 h after tMCAO) (Knowland et al., 2014).

S1P has been reported to inhibit the function of P-glycoprotein at the BBB (Cannon et al., 2012). Since P-glycoprotein is an efflux pump for small-molecule drugs, S1P might therefore enhance drug transportation across the BBB (Cannon et al., 2012). $S1pr1^{ECKO}$ mice showed a size-selective BBB disruption and different subcellular distribution of tight junctional proteins in brain microvessels (Yanagida et al., 2017). Thus, brain endothelial S1PR1 may help maintain BBB function by sustaining a proper distribution of tight junction proteins. During ischemia, endothelial cell S1P signaling also plays important roles. Profound edema can be seen in $S1pr1^{ECKO}$ mice shortly after tMCAO by MRI (Nitzsche et al., 2021), which suggests that endothelial S1PR1 may participate in regulating vesicular transport in the early phase of BBB opening. In line with this, in brain arterioles, ApoM-S1P regulates vesicular transport through S1PR1 signaling (Janiurek et al., 2019). In contrast, S1PR2 was shown to induce cerebrovascular permeability in tMCAO mice by experiments using genetic approaches and a S1PR2 antagonist (Kim et al., 2015).

S1P Signaling in Functional Recovery Following an Ischemic Stroke

A series of events such as neurogenesis, synaptogenesis, angiogenesis, and white matter remodeling (Sommer and Schabitz, 2021) happen after ischemic stroke that contribute to neural repair and functional recovery (Overman and Carmichael, 2014). S1P signaling could also be involved in these events. The migration of neural progenitor cells (NPC) is an important step in neurogenesis (Koh and Park, 2017). S1P has been shown as a chemoattractant for NPCs released from infarction zone and S1PR2 antagonism enhanced the migration of NPCs toward the infarcted area thus facilitating neurogenesis (Kimura et al., 2008). Besides, fingolimod was found to enhance angiogenesis in a photothrombotic stroke model (Shang et al., 2020). In another photothrombotic stroke model, fingolimod significantly decreased astrogliosis and increased post-synaptic densities up to a month after the onset of ischemia (Brunkhorst et al., 2013). Other roles of S1P signaling in functional recovery following an ischemic stroke are poorly understood and shall constitute a promising direction for future studies.

The contributions of S1P signaling in ischemic stroke are primarily through diverse mechanisms as mentioned above. Additional mechanisms warrant further exploration. Some researches supported a possible direct action of S1P signaling on neuronal function after ischemic stroke (Hasegawa et al., 2010, 2013), while others proposed that such influence might be

limited. As fingolimod, an analog of sphingosine, penetrated the blood-brain barrier, but it was not primarily located in neurons (Miron et al., 2008). Besides, in neuronal cell cultures under hypoxic conditions, fingolimod did not reduce cell death (Kraft et al., 2013). With more studies being undertaken, joint targeting of diverse mechanisms is suggested.

INSIGHTS INTO CURRENT AND FUTURE THERAPEUTIC PERSPECTIVES

Fingolimod

Fingolimod (Gilenya^{TM}, Novartis), is an analog of sphingosine and was the first oral treatment approved by the United States Food and Drug Administration for RRMS (Brinkmann et al., 2010). Fingolimod is phosphorylated by Sphk2 to fingolimod-phosphate (fingolimod-P) (Zheng et al., 2015), which is an agonist for four S1PR (S1PR1, 3, 4, and 5) (Brinkmann et al., 2002). Fingolimod-P activates S1P receptors with very high potency and efficacy. The over-activation leads to rapid receptor internalization and degradation, especially for S1PR1 (Cyster and Schwab, 2012). Thus, fingolimod serves as a functional antagonist of S1PR1. While S1PR3, 4, 5 are also internalized but can come back to the cell surface without being degraded (Huwiler and Zangemeister-Wittke, 2018).

The mechanism of action of fingolimod in ischemic stroke is mainly mediated by the functional antagonism and agonism of S1P receptors (Wang et al., 2020). Fingolimod binds to S1PR1 on lymphocytes and leads to transient receptor degradation, thereby preventing lymphocytes from releasing to the bloodstream (Brinkmann, 2007). As discussed above, this decreases post-stroke lymphocyte infiltration and therefore thrombo-inflammation formation and inflammatory response. Besides its role in immunomodulation, fingolimod can also enhance the endothelial barrier function (Peng et al., 2004; Dudek et al., 2007). However, this effect is likely to be dose-dependent, as higher concentrations or prolonged fingolimod treatment increase endothelial permeability and vascular leakage instead (Shea et al., 2010; Muller et al., 2011). Fingolimod is highly lipophilic and can cross the BBB so it may exert a direct effect on CNS (Fu et al., 2015).

Based on previous studies that fingolimod reduced IR-induced tissue injury in the kidney (Troncoso et al., 2001) and liver (Anselmo et al., 2002), animal models of stroke were performed (**Table 1**). Most results indicated a beneficial role of fingolimod in tMCAO (Czech et al., 2009; Shichita et al., 2009; Wacker et al., 2009; Hasegawa et al., 2010; Pfeilschifter et al., 2011a,b; Wei et al., 2011; Kraft et al., 2013), thromboembolic stroke model (Campos et al., 2013) and photothrombotic stroke model (Brunkhorst et al., 2013; Li X. et al., 2017; Shang et al., 2020). In contrast, a few negative results were found in a pMCAO model (Liesz et al., 2011) and a large hemispheric stroke model when co-administration with rt-PA (Cai et al., 2013).

Encouraged by favorable preclinical data, clinical trials on fingolimod in patients with ischemic stroke have also been conducted. In an open-label pilot trial of patients presented beyond the 4.5 h time window for thrombolytic therapy with anterior circulation infarction, oral fingolimod was given 0.5 mg daily for 3 consecutive days (Fu et al., 2014). Results showed that from baseline to 7 days, enlargement of infarct volume was more restricted and microvascular permeability was reduced in patients who received fingolimod plus standard treatment than in patients receiving standard treatment alone (Fu et al., 2014). Fingolimod treatment was also associated with better neurological function recovery (Fu et al., 2014). In a multi-center trial of patients with anterior or middle cerebral artery occlusion, alteplase plus oral fingolimod 0.5 mg daily for 3 consecutive days were given (Zhu et al., 2015). Patients who received the combination therapy had smaller lesion volume at day 1 and better clinical outcomes at day 90 than patients who received solely alteplase (Zhu et al., 2015). In extended time windows from 4.5–6 h post stroke, patients receiving fingolimod along with alteplase also had a favorable shift of 90-day modified Rankin Scale score (Tian et al., 2018). No adverse effect was reported in the clinical trials mentioned above. More studies aiming to determine whether fingolimod enhances the action of endovascular treatment (NCT04629872) and a combination of fingolimod with alteplase in conjunction with thrombectomy (NCT04675762) are underway. Although the available clinical data are promising, it is noteworthy that current trials were not double-blinded and the number of participants was small and included mainly Asian patients. Therefore, it is still too early to confirm the role of fingolimod in the treatment of ischemic stroke. Additional large-scale, well-designed experiments are warranted.

Other S1PR Modulators

As potential substitutes to fingolimod, other S1PR modulators with better specificity, improved pharmacokinetic properties are under study. SEW2871 and LASW1238 are selective agonists of S1PR1 and were shown to reduce infarct volume after tMCAO (Hasegawa et al., 2010; Brait et al., 2016). CYM-5442 is another S1PR1-selective agonist reported to have preferential distribution to the brain after systemic administration (Gonzalez-Cabrera et al., 2012). Besides, CYM-5442 is shown to induce a shorter duration of lymphopenia than fingolimod (Gonzalez-Cabrera et al., 2012). In a recent experiment of a pMCAO model, CYM-5442 reduced 24-hour infarct size when administered 0-6 hours after occlusion and the beneficial effect was demonstrated to depend on endothelial S1PR1. In contrast, in another tMCAO model, siponimod, a S1PR modulator of S1PR1 and S1PR5, did not reduce stroke size in middle-aged mice despite significant lymphopenia. More basic and clinical researches are in progress to dig deeper into the therapeutic potential of other S1PR modulators for ischemic stroke.

FUTURE THERAPEUTIC PERSPECTIVES

The Underlying Mechanisms of S1P Signaling in Ischemic Stroke Need Further Study

S1P modulators have shown promise in experimental models and some small-scale clinical trials of stroke. Yet their mechanisms

TABLE 1 | Animal studies of S1P modulators in ischemic stroke.

Stroke models	Animal species	Drug name	Drug doses	Drug application	Results	References
TMCAO (90 min)	C57Bl/6 mice (male, 10 weeks old)	FTY720	1 mg/kg	At the onset of ischemia (i.p.)	↓ Infarct volume ↑ neurological function 24 h after reperfusion	Czech et al., 2009
TMCAO (60 min)	C57Bl/6 mice (male, 9–17 weeks old)	FTY720	1 mg/kg	5 min before reperfusion/before reperfusion and every 24 h for 3 days (i.v.)	↓ Infarct volume on day 4	Shichita et al., 2009
TMCAO (60 min)	Swiss-Webster ND4 mice (male, adult)	FTY720	0.24 or 1 mg/kg	48 h before ischemia (i.p.)	1 mg/kg FTY720 or 0.24 mg/kg FTY720 combined with hypoxic preconditioning ↓ Infarct volume ↑ neurological function 24 h after reperfusion	Wacker et al., 2009
TMCAO (120 min)	Sprague–Dawley rats (male)	FTY720, SEW2871, VPC23019	FTY720 (0.25 mg/kg, 1 mg/kg), SEW2871 (5 mg/kg), VPC23019 (0.5 mg/kg)	Immediately after reperfusion (i.p.)	FTY720 and SEW2871 ↓ Infarct volume ↑ neurological function at 24 and 72 h after tMCAO, VPC 20319 abrogated the protective effects of FTY720	Hasegawa et al., 2010
PMCAO	C57BL/6 mice (male, 8–10 weeks old)	FTY720	1 mg/kg	48 h before or 3 h after ischemia (p.o.); 48 h before ischemia (i.p.)	Infarct volume and behavioral dysfunction were not altered 7 days after pMCAO	Liesz et al., 2011
TMCAO (90 min for mice, 120 min for rat), pMCAO	C57BL/6 mice (male), Sprague–Dawley rats (male)	FTY720	0.5 mg/kg, 1 mg/kg, 3 mg/kg	Mice tMCAO: 0.5 mg/kg, 1 mg/kg at reperfusion and at 24 h (i.p.); 3 mg/kg 2 h, 24 h, 48 h after reperfusion (p.o.). Rat tMCAO: 1 mg/kg 30 min after reperfusion (i.p.). Mice pMCAO: 1 mg/kg (i.p.) 2 or 4 h after ischemia	Mice tMCAO: FTY720 (0.5 mg/kg, 1 mg/kg, i.p.) ↓ Infarct volume 48 h after tMCAO, FTY720 (1 mg/kg, i.p.) ↑ neurological function 48 h after tMCAO; FTY720 (3 mg/kg, p.o.) ↓ Infarct volume ↑ neurological function at 14 days. Rat tMCAO: ↓ Infarct volume at 22 h after reperfusion. Mice pMCAO: ↓ Infarct volume 20 h after pMCAO.	Wei et al., 2011
TMCAO (90 min or 3 h)	C57BL/6 mice (male, 10 weeks old)	FTY720	1 mg/kg	2 h after onset of ischemia (i.p.)	↓ Infarct volume (tMCAO 3 h, 90 min) and ↑ neurological function (tMCAO 3 h) at 24 h after the induction of ischemia	Pfeilschifter et al., 2011a
TMCAO (120 min)	C57BL/6J mice, *SphK1*$^{-/-}$ and *SphK2*$^{-/-}$mice (10–12 weeks old)	FTY720	1 mg/kg	At the onset of ischemia (i.p.)	FTY720 ↓ Infarct volume ↑ neurological function after 24 h. The protective effect was not shown in *SphK2* $^{-/-}$mice	Pfeilschifter et al., 2011b
Photothrombosis (PT) (15 min)	C57BL/6 mice (male, 6–12 weeks old)	FTY720	1 mg/kg	Beginning 3 days after PT, for 5 days b.i.d. (i.p.)	↑ Neurological function over 31 days, ↓ reactive astrogliosis ↑ synapse size at day 7	Brunkhorst et al., 2013
TMCAO (3 h)	C57Bl/6 mice (10–12 weeks old)	FTY720, rt-PA	FTY720 (1 mg/kg), rt-PA (10 mg/kg)	Rt-PA (i.v.), FTY720 (i.p) at the end of the tMCAO period.	FTY720 with rt-PA did not alter mortality rate or neurological function at 24 h after the onset of ischemia	Cai et al., 2013
Thromboembolic occlusion	C57BL/6 mice (male)	FTY720, tPA	FTY720 (0.5 mg/kg), tPA (10 mg/kg)	FTY720 (i.p.) 45 min,24 h, 48 h after occlusion; FTY720 + early tPA: tPA 30 min (i.v.) after thrombin injection + FTY720 (i.p.) 30 min, 24 h, 48 h after occlusion; FTY720 + delayed tPA: tPA 3 h (i.v.) after thrombin injection + FTY720 (i.p.) 3 h, 24 h, 48 h after occlusion;	FTY720 or early tPA: ↓ Infarct volume ↑ neurological function 3 days after occlusion; FTY720 + early tPA: further ↑ neurological function; FTY720 + late tPA: ↓ Infarct volume ↑ neurological function 3 days after occlusion; FTY720 ↓ hemorrhagic transformation associated with late tPA.	Campos et al., 2013
TMCAO (2 h)	Sprague–Dawley rats (male)	FTY720	0.25 mg/kg	Immediately after reperfusion (i.p.)	↓ Infarct volume ↑ neurological function 24 h after tMCAO	Hasegawa et al., 2013

(Continued)

TABLE 1 | (Continued)

Stroke models	Animal species	Drug name	Drug doses	Drug application	Results	References
TMCAO (60 min or 90 min)	C57Bl/6 mice, *Rag1*$^{-/-}$ mice (male, 6–8 weeks old)	FTY720	1 mg/kg	Immediately before reperfusion (i.p.)	↓ Infarct volume on day 1 and day 3, ↑ neurological function on day 1 after 60-min tMCAO in wild-type mice but not in lymphocyte-deficient *Rag1*$^{-/-}$ mice after 90-min tMCAO	Kraft et al., 2013
TMCAO (60 or 90 min)	ICR mice (male, 7 weeks old)	S1P, FTY720	S1P (1 nmol/0.5 μL) FTY720 (3 mg/kg)	FTY720 (i.p.) immediately after reperfusion; S1P + FTY720: microinjection of S1P into the corpus callosum 24 h before tMCAO, FTY720 (i.p.) 30 min before S1P microinjection or tMCAO	FTY720 ↓ Infarct volume ↑ neurological function 22 h after reperfusion of 90-min tMCAO. S1P ↑ Infarct volume ↓ neurological function 22 h after reperfusion of 60-min tMCAO, FTY720 attenuate the augmented damage caused by S1P.	Moon et al., 2015
TMCAO (45 min)	C57BL/6J mice (male, adult)	LASW1238, FTY720	LASW1238 (3 mg/kg, 10 mg/kg), FTY720 (1 mg/kg)	Immediately after reperfusion (i.p.)	LASW1238 (10 mg/kg) ↓ Infarct volume at 24 h after reperfusion	Brait et al., 2016
TMCAO (1 h)	Sprague–Dawley rats (male)	FTY720	0.5 mg/kg	24 h before surgery, and continued every other day (i.p.)	↓ Infarct volume and ↓ memory deficit on day 7 after the surgery	Nazari et al., 2016
PT (20 min)	C57/B6 mice (male, adult)	FTY720	0.5, 1, or 2 mg/kg	2 h after ischemia induction and continued every day (i.p.)	↓ Infarct volume at day 1 and 3 after PT, ↑neurological function at day 1,3,5,7 after PT	Li X. et al., 2017
TMCAO (45 min)	C57BL/6 and lymphocyte-deficient *Rag2*$^{-/-}$ mice (male, 12–16 weeks old)	FTY720	1 mg/kg	Immediately after reperfusion (i.p.)	↓ The degree of hemorrhagic transformation in *Rag2*$^{-/-}$ mice at 48 h of reperfusion	Salas-Perdomo et al., 2019
TMCAO (90 min)	Sprague–Dawley rats (male)	FTY720	0.5, 1, and 2 mg/kg	Immediately at 1 h after reperfusion (i.p.)	FTY720 (2 mg/kg) ↓ infarct volume ↑ neurological function 24 h after reperfusion	Ji et al., 2019
PT (15 min)	C57BL/6 mice (male)	FTY720	0.3 mg/kg	First dose given 24 h post-stroke, then for 1, 7, 14 consecutive days (i.p.)	↑ Neurological function at day 7 after PT, ↑ angiogenesis in the ischemic boundary at day 14	Shang et al., 2020
TMCAO (60 min)	Wistar rats and CD-1 mice	Probucol (inhibitor of S1P transporter)	30, 60 mg/kg	Immediately after tMCAO (i.p.)	↓ Infarct area after 24 h of reperfusion	Nakagawa and Aruga, 2020
TMCAO (60 min), pMCAO	C57BL/6J mice (adult)	CYM-5442	3 mg/kg	0–6 h after occlusion (pMCAO); immediately before reperfusion (tMCAO)	↓ Infarct size 24 h after pMCAO, ↓ Infarct size 24 h after tMACO.	Nitzsche et al., 2021

TMCAO, transient middle cerebral artery occlusion; pMCAO, permanent occlusion of the middle cerebral artery; PT, photothrombosis; i.p., intraperitoneally; i.v., intravenously; p.o., by oral gavage; h, hours; min, minutes.

of action are not fully understood. Optimal targeting strategies remain to be defined. A recent study revealed the pivotal role of endothelial S1P signaling in supporting BBB and maintaining perfusion in the penumbra area of ischemic stroke (Nitzsche et al., 2021). This argues against the application of functional S1PR1 antagonists, which may also block endothelial S1P signaling. Since most S1PR1 agonists also induce lymphopenia (Brait et al., 2016; Nitzsche et al., 2021), joint targeting of lymphocyte and endothelial cell receptors with S1PR1 agonists is suggested. Future application of S1P modulation in ischemic stroke treatment depends on an in-depth understanding of the mechanism of action, and on the ability to have a fine temporal and spatial regulation of the signal pathway. With more studies being undertaken, strategies for developing new-generation drugs with superior attributes will be provided.

To Develop More Specific S1PR Modulators and Seek Other Targets in S1P Signaling Pathway in Ischemic Stroke Treatment

Fingolimod, the S1PR modulator used in most preclinical and clinical studies so far, is non-specific and desensitizes brain endothelial S1PR1 at high doses as discussed above. Moreover, it induces long-lasting lymphopenia (Gonzalez-Cabrera et al., 2012). As the contribution of different S1PR on ischemic stroke

is not fully elucidated (Brait et al., 2016), drugs with more specificity could be not only safer but also more efficacious. Besides S1PR, S1P signaling is providing more therapeutic targets such as S1P transporters and metabolic enzymes. New biased S1PR agonists have been developed that do not cause receptor desensitization and do not induce lymphopenia (Poirier et al., 2020). ApoM-Fc is a soluble carrier for S1P, which can specifically activate endothelial S1P receptors but do not influence circulating lymphocyte numbers (Swendeman et al., 2017). With genetic approaches being applied, the roles of SphK and other enzymes in S1P signaling shall be further elucidated (Gaire and Choi, 2021). New pharmacological advances can be expected.

Timing, Drug Formulation Shall Be Considered and Catering to the Therapeutic Strategy of Ischemic Stroke

The appropriate timing for drug administration is vital for ischemic stroke treatment, especially when considering the narrow time window for thrombolytic therapy and dramatically different series of events occurring at different time points after stroke. Medications for stroke treatment therefore shall be fast-acting. As for fingolimod, lymphopenia is induced within 6 h of the first dose and the lymphocyte count readily returns to baseline within 72 h of the last dose (Fu et al., 2015). Treatment duration is another problem in need of attention. A study showed that S1P1 agonist LASW1238 reduced infarct size in a tMCAO mouse model only when the lymphopenia state was induced for 24 hours. Defining the best dose and duration of drugs to ensure sufficient but not excessive lymphopenia is thus critical (Brait et al., 2016). Besides, the optimal route of drug delivery should also be considered. In cases of severe stroke, intravenous administration is preferable to oral administration for the impaired ability of swallowing and absorption in these patients (Fu et al., 2015).

To Reveal the Role of S1P Signaling in Functional Recovery and Post-stroke Sequelae

As mentioned above, the role of S1P signaling in neurogenesis, synaptogenesis, angiogenesis, white matter remodeling, and other processes of functional recovery after stroke is poorly studied. Besides, due to a reduced mortality of ischemic stroke, stroke prevalence is on the rise. Other than sensorimotor deficits, neuropsychiatric sequelae such as depression dramatically reduce the quality of life. In light of this, assessing the role of S1P signaling in post-stroke sequelae is promising.

Methodological Quality of Preclinical Trials Shall Be Improved and Close Cooperation Between Preclinical and Clinical Studies Are Called For

Although there are dozens of preclinical trials on S1P modulators in ischemic stroke treatment as described above, it should be noted that these experiments are heterogeneous in design. The ischemic stroke models, occlusion times, doses of drug, timing, and duration of treatment, route of administration are different (**Table 1**). Methodological problems might limit the effective translation from bench to bedside. In light of this, preclinical studies should be better designed to incorporate sex, age, and comorbidity factors. On the other hand, problems encountered in clinical trials can be brought back to mechanism research. The close cooperation between preclinical and clinical studies would be valuable to bring new insight into S1P signaling modulation in ischemic stroke treatment.

CONCLUSION

Numerous preclinical and clinical studies suggest that S1P signaling pathway is actively engaged in ischemic stroke pathology. Several small-scale clinical trials investigating the effect of fingolimod in ischemic stroke patients have shown promising results. Other S1PR modulators with better specificity and pharmacokinetic property are under development. Synergistic interaction between preclinical and clinical studies would help to achieve new pharmacological advances in ischemia stroke treatment.

AUTHOR CONTRIBUTIONS

S-QZ drafted the initial version of the manuscript. JX, MC, L-QZ, and KS collected information and edited the manuscript. CQ and D-ST directed the work, reviewed and edited the manuscript. All authors contributed to the article and approved it for publication.

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Sepsis-Exacerbated Brain Dysfunction After Intracerebral Hemorrhage

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Sepsis susceptibility is significantly increased in patients with intracerebral hemorrhage (ICH), owing to immunosuppression and intestinal microbiota dysbiosis. To date, ICH with sepsis occurrence is still difficult for clinicians to deal with, and the mortality, as well as long-term cognitive disability, is still increasing. Actually, intracerebral hemorrhage and sepsis are mutually exacerbated *via* similar pathophysiological mechanisms, mainly consisting of systemic inflammation and circulatory dysfunction. The main consequence of these two processes is neural dysfunction and multiple organ damages, notably, *via* oxidative stress and neurotoxic mediation under the mediation of central nervous system activation and blood-brain barrier disruption. Besides, the comorbidity-induced multiple organ damages will produce numerous damage-associated molecular patterns and consequently exacerbate the severity of the disease. At present, the prospective views are about operating artificial restriction for the peripheral immune system and achieving cross-tolerance among organs *via* altering immune cell composition to reduce inflammatory damage.

Keywords: intracerebral hemorrhage, sepsis, inflammatory, neuronal death, brain dysfunction

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INTRODUCTION

Intracerebral hemorrhage (ICH) is frequently accompanied by infection ranging from 11 to 31% and long-term functional impairment (Ali et al., 2009; Lord et al., 2014). The majority of infectious patients will rapidly deteriorate and finally develop sepsis, due to systemic metabolic disorders and stress caused by excessive release of inflammatory factors and immunosuppression after ICH (Berger et al., 2014; Cheng et al., 2018). Clinically, sepsis complicated by the ICH is common but tricky in the neurosurgical intensive care unit and kills as many as a half (Goncalves et al., 2019). Our retrospective cohort study has shown that approximately 28% of patients with ICH would accompany sepsis, and sepsis is the leading cause of poor outcomes. Furthermore, approximately 80% of survivors will face severe sequelae of various organ damages, especially in the brain (Adam et al., 2013). Actually, there are many synergies in the pathophysiological mechanism of both ICH

and sepsis. For example, systemic inflammation after either ICH or sepsis emerges as a crucial trigger and mediator in the progression of secondary insult to the brain (Adam et al., 2013; Fu et al., 2015). In addition, subsequent circulatory dysfunction can be observed in both situations, leading to worse damage progress (Taccone et al., 2010; Kopeikina et al., 2020).

To date, ICH with sepsis is still difficult for clinicians to deal with, and the mortality and long-term cognitive disability are still increasing. Thus, understanding the relevant pathophysiology seems to be imminent and will be beneficial for the exploration of specific therapies. In this review, we focus on the crosstalk between ICH and sepsis and attempt to identify the mechanism of cerebral dysfunction, aiming to provide a unique and systematic insight into the interaction of the two diseases and guide indications for clinical treatment.

PATHOPHYSIOLOGY

Intracerebral hemorrhage and sepsis are mutually exacerbated *via* several pathophysiological mechanisms mainly consisting of systemic inflammation and circulatory dysfunction (see **Figure 1**).

Systemic Inflammation

On the onset of ICH, primary damage caused by disruption of normal anatomy occurred pathologically in a limited area and time window (Sun et al., 2016). Subsequently, the release of blood components [red blood cells (RBCs), thrombin (Babu et al., 2012), hemoglobin, and hemin (Robinson et al., 2009; Babu et al., 2012)], coagulation factors, complement components, and immunoglobulins activate multiple cerebral cells such as endothelial cells, microglia, and astrocyte, which is followed by proinflammatory cytokines release (Wagner et al., 2002; Nakamura et al., 2005; Aronowski and Zhao, 2011). As a result, the expression of Toll-like receptors (TLRs) and adhesion-related molecules (ARMs) is upregulated (Kodali et al., 2021). Furthermore, TLRs, as a group of class I transmembrane proteins, are critical to identifying the pathogen-associated molecular patterns (PAMPs) from bacteria (Zhu and Mohan, 2010; Fitzgerald and Kagan, 2020) and damage-associated molecular patterns (DAMPs) from systemic inflammatory injury (Kong and Le, 2011). Owing to the above contributors, the cerebral cells will transform into a "hyper-alert state" and become highly sensitive to exogenous substances and active signals. Thus, it is sepsis insult in patients with ICH, which is similar to adding fuel to the fire. The peripheral immune cells are selected and activated by invasive bacteria or toxins in circulation, secreting a series of inflammatory cytokines to induce the systemic inflammatory responses, which further amplify cerebral cell signal cascades (Singer et al., 2018) and bring catastrophic damage to the central nervous system (CNS).

Circulatory Dysfunction

In parallel, circulatory dysfunction can be observed after ICH and sepsis owing to pathological hypoperfusion and coagulation system disorders (Zheng and Wong, 2017; Font et al., 2020). During this process, inflammatory mediators first trigger the endothelial cells to express typical ARMs such as vascular cell adhesion molecule-1 (VCAM-1), intracellular cell adhesion molecule-1 (ICAM-1), endothelin-1, and platelet/endothelial cell adhesion molecule (Machado-Pereira et al., 2017). Especially, endothelin-1 is associated with continuous cerebral vasospasm resulting in local brain ischemia and hypoxia (Zheng and Wong, 2017). Activation of coagulative factors and formation of white/red blood cell plugs are also participating in the ischemic process. Excessive thrombin activation and platelet consumption are implicated with disseminated intravascular coagulation in the late stage (Goyette et al., 2004). Virtually, in the setting of systemic inflammation, the cerebral blood vessels are initially affected by CNS and that mediates further cytokine-dependent signals (Wong et al., 1996; Laflamme and Rivest, 1999). It has been confirmed that macro- and microcirculatory failure occurred rapidly and that is attributed to neurovascular coupling disorder (Rosengarten et al., 2009). Subsequently, extensive cells, especially brain cells, are damaged by compromised supplements of oxygen, nutrients, and metabolites (Sharshar et al., 2004). In turn, these damage signals can feedback to the central and peripheral cells and further augment systemic inflammation, which predisposes to a vicious circle of CNS dysfunction.

SEPSIS SUSCEPTIBILITY INCREASED BY INTRACEREBRAL HEMORRHAGE

Immunosuppression

The immune system will undergo a profound attenuation process in the setting of severe CNS injury (including traumatic brain injury, stroke, and spinal cord injury) (Fu et al., 2015). A meta-analysis consisting of 137,817 patients has identified the correlation between the high rates of systemic infections and stroke, and reported that approximately 30% of patients with stroke were along with infection including pneumonia or urinary tract infection (Westendorp et al., 2011). Temporary lymphopenia and splenic shrunk can be observed in both humans and animals at the early stage of stroke, *via* activation of the sympathetic, parasympathetic (cholinergic anti-inflammatory), and hypothalamus-pituitary-adrenal (HPA) axis pathways (Ajmo et al., 2009; Sahota et al., 2013). Thereby, the levels of noradrenaline, acetylcholine, and glucocorticoids in circulation are abruptly elevated by the promotion of these active neuroendocrine pathways, which are responsible for apoptosis and atrophy of lymphoid organs (Wong et al., 2011; Mracsko et al., 2014). This downregulation of immune cell generation and function that originates from the injured brain is aiming to avoid autoimmunity against brain antigens from the death or impaired cells (Fu et al., 2015), whereas it causes systemic immunosuppression and makes the body vulnerable to infections simultaneously (Hug et al., 2009).

Intestinal Microbiota Dysbiosis

The gut vascular barrier (GVB) mainly comprises three defense lines including the biological barrier set up by gut microbiota

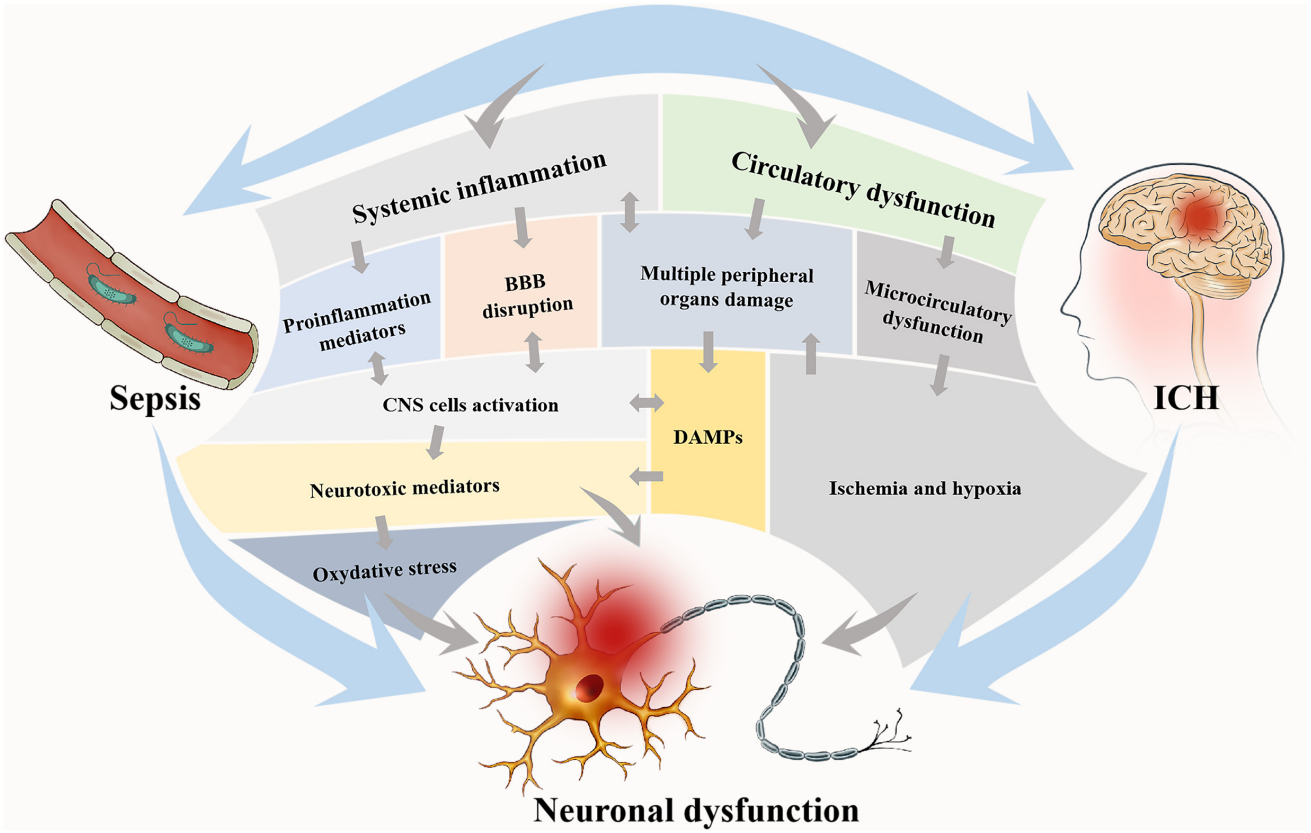

FIGURE 1 | Two main pathophysiological processes are involved in brain dysfunction in ICH with sepsis. These two processes are interdependent, on one hand, and mutually independent, on the other hand. Circulatory dysfunction will be followed by microcirculatory dysfunction and finally result in ischemia and hypoxia of tissues and multiple peripheral organ damages. In systemic inflammation, proinflammatory mediators will be released, and the DAMPs from the periphery will be allowed into the brain due to BBB disruption, which consequently activates CNS cells. The main consequence of these two processes is neural dysfunction, notably *via* oxidative stress and neurotoxic mediation. Neural dysfunction widely exists in the brain and accounts for the brain dysfunction *via* the alteration of neurotransmission. DAMPs, damage-associated molecular patterns; BBB, blood-brain barrier; CNS, central nervous system.

(Assimakopoulos et al., 2007) and keeps intestinal homeostasis. Intestinal flora displays important metabolic, immunologic, and gut protective functions modulated by the so-called "gut-brain axis" (Haak et al., 2018). Numerous models have shown that microbiota have the potentials to augment the proinflammatory effect of immune cells and even conduct the influx of immune effector cells into distant organs, probably mediated by microbe-associated molecular patterns including lipopolysaccharide (LPS), peptidoglycan, flagellin, and microbiota-derived metabolites (Magnotti et al., 1998; De-Souza and Greene, 2005). Moreover, the microbiota can induce the secretion of antibacterial factors from the gut-epithelial cells and, consequently, augment humoral responses against invading pathogens (Kim et al., 2017). ICH occurrence disturbs GVB integrity and intestinal hemostasis and, ultimately, alters the microbiota composition (Patterson et al., 2019; Rice et al., 2019). A recent study also has confirmed the prominent reduced species diversity and microbiota overgrowth in the dysbiosis induced by ICH, which may reduce intestinal motility and increase gut permeability. While recolonizing, normal health microbiota therapy ameliorated neural deficits and inflammation after ICH (Yu et al., 2021). Thus, it provides an opportunity for the potential translocation of aerobic opportunistic pathogens whereby impaired GVB and, finally, results in the onset of gut-origin sepsis (Donskey, 2004; Haak et al., 2017; Huber-Lang et al., 2018).

SEPSIS-ASSOCIATED INFLAMMATORY SIGNALS TO CENTRAL NERVOUS SYSTEM ENHANCED ON INTRACEREBRAL HEMORRHAGE

Signal Pathways in Central Nervous System Response to Sepsis Threat: Neural, Humoral, and Blood-Brain Barrier Alteration

There are three pathways to capture sepsis signals by CNS (Ali et al., 2009). Nervous pathways, mainly initiated by PAMPs and inflammatory cytokines *via* the primary afferent (vagal

and trigeminal) and sensorial (olfactory) nerves (Sonneville et al., 2013). At this time, visceral inflammation due to sepsis can be detected by the vagal nerve depending upon its terminal cytokine receptors (Tracey, 2009). In addition, the vagal nucleus transmits signals to the central autonomic system, the neuroendocrine centers, and the amygdala, leading to the alteration of behaviors and emotions (Adam et al., 2013; Lord et al., 2014). Humoral pathways, conducted by circulating inflammatory mediators through choroid plexus (CP) and circumventricular organs (CVOs) (Sonneville et al., 2013). CVOs are defined as the fenestrated regions lacking the intact blood-brain barrier (BBB) around the brain, and hence, molecules and peripheral cells can directly access the cerebral parenchyma through these regions (D'Mello and Swain, 2014). Similar to CVOs structure lacking BBB, CP is constituted of cuboidal epithelium cells and is responsible for secreting cerebrospinal fluid (Ghersi-Egea et al., 2018). Both of these structures express receptors of innate and adaptive immune systems, allowing them to detect central and peripheral inflammatory signals (Adam et al., 2013; Ghersi-Egea et al., 2018). Once relevant signals were captured, they will be amplified and transmitted to deeper areas implicated with controlling behavioral, neuroendocrine, and neurovegetative responses *via* above two structures (Adam et al., 2013; Berger et al., 2014). BBB alteration, enabling monocytes infiltration, and inflammatory molecules invading in the systemic inflammation (Meneses et al., 2019). Activated endotheliocytes express ARMs and release several inflammatory mediators such as cytokines, prostaglandins, and nitric oxide (Johnston and Webster, 2009). It is involved in the regulation of neurotransmission and neurosecretion (Johnston and Webster, 2009). Studies have reported that both pro-inflammatory cytokines [i.e., tumor necrosis factor (TNF)-α and interleukin (IL)-6 and IL-1β] and anti-inflammatory cytokines (i.e., IL-1Ra and IL-10) collectively participated in these systemic responses and formed inflammation homeostasis (Wong et al., 1997; Ilyin et al., 1998; Pintado et al., 2011). However, the abrupt presence of ICH or sepsis breaks the balance and causes an inflammatory signal cascade.

Central Nervous System Innate Cells-Associated Responses: Endothelial Cell Activation, Immunocyte Activation, and Blood-Brain Barrier Alteration

Endothelial cell activation is a crucial step in the CNS responses to sepsis, which affects microcirculatory function and BBB integrity. Evidence confirmed that LPS could induce various ARM expressions on the endothelial cells such as CD40, E-selectin, VCAM-1, and ICAM-1 (Hess et al., 1996; Wong et al., 1997; Omari and Dorovini-Zis, 2003; Hofer et al., 2008). In addition, several receptors for IL-1, TNF-α, and TLR4 were also upregulated (Wong et al., 1997; Zhou et al., 2009). And these receptors contribute to the secretion of TNF-α, IL-6, and IL-1β, followed by the generation of endothelial/inducible nitric oxide synthase (Freyer et al., 1999; Handa et al., 2008) and type-2 cyclooxygenase (Matsumura et al., 1998). In virtue of these inflammatory mediators, microglia are mobilized to secrete several cytotoxic molecules (Meneses et al., 2019), and astrocytes are also triggered to produce chemokines (the C-C chemokine ligand 2, IL-6, chemokine C-X-C ligand10) *via* NF-κB pathways (Mayo et al., 2014). Furthermore, the expression of ARMs and TLR4 as well as the conduction of chemokines collectively choose infiltration of peripheral monocytes and their participation in neuroinflammation (Adam et al., 2013). Microcirculatory dysfunction has been widely observed in multiple sepsis models owing to the aggregation of circulating white cells and monocytes in the CNS capillaries, compromising supplements of oxygen, nutrients, and metabolites (Bohatschek et al., 2001; Hofer et al., 2008; Zhou et al., 2009; Taccone et al., 2010). The BBB alteration has been clearly confirmed whereby versatile methods, including blue Evans, fluorescent-labeled dextran clearance, labeled granulocytes, electron microscopy, and magnetic resonance imaging in sepsis models and also in patients (Papadopoulos et al., 1999; Esen et al., 2005, 2012; Sharshar et al., 2007; Handa et al., 2008; Bozza et al., 2010). Furthermore, a recent study has confirmed that BBB displayed a short-term closure at the early stage of inflammation and gradually opened up as the disease progressed (Carloni et al., 2021). Nonetheless, it eventually allows for the entry of neurotoxic molecules, particularly inflammatory mediators, consequently giving rise to brain cell death in systemic inflammation. Likewise, extensive studies have found that the neuroinflammation in the ICH was complicated with gliacyte activation and BBB alteration (Anrather and Iadecola, 2016; Wofford et al., 2019). Therefore, all above mentioned mechanisms indicate that the occurrence of ICH greatly increases the risk of sepsis.

NEURAL DEATH ON INTRACEREBRAL HEMORRHAGE WITH SEPSIS

Intracerebral hemorrhage and sepsis can cause similar patterns of neural death and are discussed in the following text (**Table 1**; Castagna et al., 2016; Oberst, 2016; Lewerenz et al., 2018; Li et al., 2018; Takashima et al., 2019; Nagase et al., 2020). Reactive oxygen species (ROS), a kind of physiological defense molecule, can be maintained at a steady level *via* mitochondrial oxidative phosphorylation and antioxidant mechanisms (Zhou et al., 2020). However, the onset of ICH or sepsis overgenerates ROS and then causes mitochondrial damage, leading to the deterioration of iron metabolism (Zhou et al., 2020; Liu et al., 2021). Subsequently, hemin released from RBC lysis owing to the elevation of cytokines or bacterial toxins also accounts for excessive free iron in the extracellular matrix (Soares and Weiss, 2015; Ganz, 2016). Subsequently, extracellular iron bounding to the transferrin receptor is internalized by the cells under the drive of inflammatory cytokines (Ludwiczek et al., 2003). Excess iron in the cytoplasm significantly dampens enzyme activity and typically causes potent oxidization, resulting in various cell ferroptosis including neurocytes (Liu et al., 2021). Numerous reports have indicated that ferroptosis is invariably followed

TABLE 1 | Neuronal death patterns of both ICH and sepsis.

Types	Activators	Characterization	References
Ferroptosis	Iron and extracellular glutamine	Plasma membrane integrity loss, organelles disruption and swelling, mitochondria shrunk, without DNA fragmentation	Li et al., 2018
Necroptosis	Inflammatory factors	Plasma membrane integrity loss, organelles disruption and swelling, without mitochondria shrunk, without DNA fragmentation	Oberst, 2016; Li et al., 2018
Apoptosis	Inflammatory factors	Chromatin condensation, nuclear shrinkage, and DNA fragmentation	Castagna et al., 2016; Li et al., 2018
Oxytosis	Glutamate; ROS	Mitochondrial fragmentation, without DNA fragmentation	Lewerenz et al., 2018; Takashima et al., 2019; Nagase et al., 2020
Pyroptosis	Inflammatory factors	Nuclear condensation, cell swelling, lipid membrane vacuole formation, lipid membrane ruptures, without DNA fragmentation	Li et al., 2018

by necroptosis (Zhou et al., 2020), and NADPH might be the connectional mediator between the two patterns of cell death (Hou et al., 2019). In systemic inflammation, necroptosis can be initiated by several cytokines including, but not limited to, TNF (Oberst, 2016). Once ferroptosis or necroptosis happened, adjacent cells are likely predisposing to another kind of death pattern especially oxytosis (Zhou et al., 2020). Highly similar to the mechanism of ferroptosis, oxytosis occurrence is also related with the extensive ROS failed to be metabolized because of glutathione depletion (Landshamer et al., 2008; Grohm et al., 2010). Many studies even regarded oxytosis as a component of ferroptosis (Soares and Weiss, 2015; Zhou et al., 2020), and this needs further research to clarify. Apoptosis has been well studied by numerous researchers and mainly conducted by two pathways, namely extrinsic and intrinsic pathways. Among them, the extrinsic pathway is activated by cell surface receptors including TNF receptors (Hasegawa et al., 2011; Fricker et al., 2018; Zhao et al., 2018). Under the condition of systemic inflammation in ICH or sepsis, cerebral proinflammatory factors (e.g., TNF) are released in large quantities, and consequently, the Fas-associated death domain protein can be chosen to activate caspase-8 causing neural apoptosis (Micheau and Tschopp, 2003). Pyroptosis is one of the characteristic manners of cell death upon inflammation. In experimental models, it has been demonstrated to be induced by proinflammatory cytokines (i.e., IL-1β and IL-18) *via* the combination on the cell membrane between the lipid-selective N-terminal domain and phosphatidylinositol of the lipid plasma membrane (Shi et al., 2015; Ding et al., 2016; Feng et al., 2018). To sum up, inflammatory cytokines and metabolites are prioritized to induce neural death in the ICH with sepsis. Therefore, inhibition of neuroinflammation might be important for curbing brain function deterioration and warrant to be further explored.

MULTIPLE ORGAN DAMAGE-ASSOCIATED MOLECULAR PATTERNS RELEASE EXACERBATES CENTRAL NERVOUS SYSTEM DYSFUNCTION

Inflammatory Imbalance

Acute brain injury including ICH occurrence will initiate neuroinflammation and then spread inflammatory signals to the periphery, and monocyte infiltration might be a crucial mediator in this process. In an LPS-induced neuroinflammatory mouse model, the infiltrated neutrophils exhibited reverse trans-endothelial migration back to the bloodstream after interacting with microglia (Kim et al., 2020). Subsequently, these reverse-moving neutrophil-transported signals to several organs have been reported in numerous studies. For example, the upregulation of inflammatory cytokines (e.g., IL-8 and IL-10) were observed in the kidney used for organ donation, resulting in the reduction of allograft survival *via* increasing the number of trafficking inflammatory cells (i.e., $FoxP3^+$ regulatory T cells) (Morariu et al., 2008; Kim et al., 2020). In addition, in the brain injury models, infiltrated monocytes and macrophages were demonstrated to produce several chemoattractants (i.e., leukotriene-B4) and other cytokines (e.g., IL-1β, IL-6, and TNF-α), causing the amplification of pulmonary inflammation (Kalsotra et al., 2007; Mrozek et al., 2015). In addition, other organ damages such as the spleen (Li et al., 2011), gastrointestinal tract (Fung et al., 2017), and liver (D'Mello and Swain, 2014) owing to neuroinflammation have been reported in previous studies. Meanwhile, in sepsis, the reactions of the host to invasive bacteria or toxins typically induce phagocytosis in macrophages and secrete a series of proinflammatory cytokines (Takeuchi and Akira, 2010). Besides, this so-called "cytokines storm" subsequently activates the innate immune system (D'Elia et al., 2013). Apparently, the activation of the innate immune system, which is modulated by pattern-recognition receptors, upregulates the expression of associated inflammatory genes *via* detecting PAMPs or DAMPs (Raymond et al., 2017). Thus, the simultaneous occurrence of ICH and sepsis can elicit the superposition of inflammatory effects and cause extensive organ damages. Obviously, the inflammatory imbalance represents the most important basis for brain dysfunction pathogenesis in the ICH with sepsis and occurs throughout the whole process of the ICH with sepsis.

Ischemia Process

Ischemia processes, consisting of microcirculatory dysfunction and macrocirculatory dysfunction, can be observed in the ICH and sepsis development. Furthermore, microcirculatory dysfunction arising from endothelial cell activation has been well-demonstrated in various sepsis models (Taccone et al., 2010). Meanwhile, endothelial cell dysfunction also activates the coagulation system to participate in the ischemia process (Adam et al., 2013). Similar processes have been reported in acute cerebral injury patients with vasospasm. It is presented

as persistent narrowing of cerebral arteries and is believed to be contributed by spasmogenic or neuroinflammatory factors (Kassell et al., 1985; Taccone et al., 2010), which indicates the role of inflammation in the ischemia process. In addition, severe inflammatory responses caused by sepsis will disturb neurovascular coupling, followed by a disorder of heart rate and blood pressure and deterioration of macrocirculation (Godin and Buchman, 1996; Jafari and Damani, 2020). It has been reported the autonomic controlling system of the heart and vessel is compromised in polymicrobial sepsis because of the degraded autonomic nervous system (Pancoto et al., 2008). Besides, the parasympathetic nervous system (e.g., vagal nerve) is regarded as one of the critical pathways connecting the center and periphery, suggesting that the autonomic nervous system functions and inflammation may be interdependent (Huston and Tracey, 2011). The damage to the susceptible regions of the CNS, whether chemical or mechanical nature, can augment the sympathetic nerve or HPA axis, further causing the dysregulation of catecholamine and dopamine secretion (Lattanzi et al., 2018). The amount of catecholamine released into the bloodstream will activate α-receptors on the cell surface to produce vasoconstriction for abdominal viscera, leading to consequent hypoperfusion and ischemic injury (Lattanzi et al., 2018). In conclusion, the ischemia process in the ICH accompanied with sepsis is under the co-modulation of inflammation and neuroendocrine changes.

Damage-Associated Molecular Patterns Generation and Insult

Damage-associated molecular patterns are non-microbial molecules in the host nucleus or cytoplasm and consist of high mobility group box 1 (HMGB1), histones, and adenosine triphosphate (Sunden-Cullberg et al., 2005; Ekaney et al., 2014; Zhou et al., 2015). Once released to the extracellular matrix from injury cells, they will act as the effective activators of the immune system and perpetuate non-infectious inflammatory responses to cause systemic inflammation and cellular injury, even death (Matzinger, 1994; Seong and Matzinger, 2004; Rubartelli and Lotze, 2007). In addition, the exact mechanisms may be implicated with the proinflammatory cytokines and chemokines secreted from active macrophages/microglia, which facilitates excessive neutrophil activation and infiltration into the tissues (Denning et al., 2019). Activated neutrophils can generate several kinds of toxic mediators including ROS and inducible nitric oxide synthase (iNOS) to cause oxidative stress and cellular injury (Gentile and Moldawer, 2013; Schaefer, 2014; Brinkmann, 2018). TLRs are considered as the pivotal signal receptors for DAMPs. Rodriguez-Yanez et al. (2012) have reported in a recent clinical study that the upregulation of TLR2 and TLR4 in the peripheral monocytes is closely related to the undesired prognosis in patients with ICH. In addition, the improved neurological function after ICH onset was demonstrated in the TLR4-knockout rodent (Sansing et al., 2011; Lin et al., 2012). HMGB1, as one of the DAMPs extensively discussed, can enable microglia to increase the NF-κB activity and the transcription of cyclooxygenase-2, TNF-α, and IL-1β (Yang et al., 2011). In turn, the TNF-α can feedback on microglia to facilitate the release of HMGB1 (Wang et al., 2015). Evidence indicated that HMGB1 may be contributive to the poor outcomes after CNS injury and the serum levels, which was associated with the disease severity (Nakahara et al., 2009; Zhou et al., 2010).

In summary, under the effect of inflammatory imbalance and ischemia, injury and/or death cells in multiple organs release DAMPs into circulation and amplify systemic inflammation, accompanying oxidative stress and cytotoxic mediator production, further causing more damage and brain dysfunction worsening.

PERSPECTIVE AND CONCLUSION

Increasing evidence has indicated that there is an inextricable inflammatory association between the center and periphery. CNS injury will spread the danger signals to the periphery, and *vice versa*. Under the mediation of systemic inflammation and circulatory dysfunction, the pathological changes can be observed in multiple organs of the patients with ICH or sepsis. Recent advances in the multi-omics analysis could provide vivid evidence regarding the cascade of biofluids from the injured brain, namely, cerebrospinal fluid and circulation blood, and urine and saliva. In contrast, clinical therapies for patients with ICH with sepsis frequently focus on a single organ or system lacking holistic ideas, causing dissatisfied outcomes. For the ICH process, sepsis presence displays an aggravation to peripheral inflammation imbalance that should not be ignored. Although there are many methods attempting to modulate the peripheral immune system, such as antibiotic prophylaxis and probiotic therapy, and have shown limited achievement (Kim and Cho, 2021), the prospective views considered that we should operate artificial restriction for the peripheral immune system and achieve the cross-tolerance among organs *via* altering immune cell composition. Based on that, stem cell therapy was extensively used in clinical trials for diverse diseases including hemorrhagic stroke, and exhibited many advantages. Therefore, further study for crosstalk between center and periphery might be beneficial for us to explore potential methods for improving brain dysfunction and prognosis in patients with ICH or sepsis or combination.

AUTHOR CONTRIBUTIONS

JL, BT, and YL drafted the manuscript, and prepared the figure and table. HF and YC proofread and revised the manuscript, then gave the final approval for this submission. All authors contributed to the article and approved the submitted version.

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REVIEW
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Clearance Systems in the Brain, From Structure to Function

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As the most metabolically active organ in the body, there is a recognized need for pathways that remove waste proteins and neurotoxins from the brain. Previous research has indicated potential associations between the clearance system in the brain and the pathological conditions of the central nervous system (CNS), due to its importance, which has attracted considerable attention recently. In the last decade, studies of the clearance system have been restricted to the glymphatic system. However, removal of toxic and catabolic waste by-products cannot be completed independently by the glymphatic system, while no known research or article has focused on a comprehensive overview of the structure and function of the clearance system. This thesis addresses a neglected aspect of linkage between the structural composition and main components as well as the role of neural cells throughout the clearance system, which found evidence that the components of CNS including the glymphatic system and the meningeal lymphatic system interact with a neural cell, such as astrocytes and microglia, to carry out vital clearance functions. As a result of this evidence that can contribute to a better understanding of the clearance system, suggestions were identified for further clinical intervention development of severe conditions caused by the accumulation of metabolic waste products and neurotoxins in the brain, such as Alzheimer's disease (AD) and Parkinson's disease (PD).

Keywords: brain waste clearance, glymphatic system, meningeal lymphatic vessels, blood-brain barrier, neuroglia

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INTRODUCTION

It has previously been observed that despite a high metabolic rate, the brain lacks an actual lymphatic system that aids in removing metabolic waste and toxic agents from the brain (Kumar et al., 2019). Therefore, the clearance system in the brain has long been a subject of great interest in a wide range of neuroscience fields since it was discovered in the 1960s (Bedussi et al., 2015). In recent years, there has been growing recognition of the vital links between the clearance system and disease of the central nervous system (CNS). Therefore, there is an urgent need to have a comprehensive understanding of the clearance system (Zhang et al., 2019). So far, however, the whole clearance system, including the structural characterization and a possible connection between them, has received scant attention in the research literature. Some of the previous studies have generally been focused on analyzing the glymphatic system (Jessen et al., 2015). To some extent, the relationship between other important structures in the brain and the glymphatic system needs further investigation to provide robust evidence for promising clinical intervention based

on the clearance system in brain disorders due to the accumulation of waste products (Sun et al., 2018). This article seeks to remedy these problems and propose a theoretical framework based on the clearance system in the brain by systematically reviewing the literature of main structural components, including nerve cells and extracellular vesicles (EVs) at present (**Figure 1**). The viewpoint presented in this review is to explore the overall structure and function of the clearance system, which will shed new light on future research that will lead to the further potential development of preventive and therapeutic interventions of brain disorders mentioned earlier.

STRUCTURAL BASIS OF CLEARANCE SYSTEM IN THE BRAIN

The proper functioning of the clearance system in the brain requires a stable structure. Also, it has been reported that the glymphatic and meningeal lymphatic have brain lymphoid function. Thus, the "Glymphatic system" section will establish the framework of the structure, function, and interactions of two systems, which contributes to a better understanding of physiological and pathological conditions of the clearance system in the brain.

GLYMPHATIC SYSTEM

The first pioneering study in 2012 identified a brain-wide pathway in mice with small fluorescent tracers and termed it as a "glymphatic" system (Iliff et al., 2012). With the development of the diffusion MR technique and diffusion tensor imaging (DTI), subsequent researches indicated the presence of the glymphatic system in the human brain (Taoka et al., 2017; Yokota et al., 2019). The glymphatic system supports interstitial solute and cerebrospinal fluid (CSF) clearance from the brain and shares functions with the lymphatic vessels.

The glymphatic system is a brain-wide route that comprehends the entrance, interstitial movement and exchange, and exit of CSF. There are several critical components in this system, including CSF, interstitial fluid (ISF), perivascular space (PVS), cerebral vascular, glial cells, and the astrocyte aquaporin 4 (AQP4)-controlled water channels. Produced by the choroid plexus, CSF flows through the lateral ventricles, the third ventricles, and the fourth ventricles into the subarachnoid compartment connected to the PVS (Iliff et al., 2012; Nedergaard and Goldman, 2020). Cerebrospinal fluid is then driven into the PVS under the pulsatility of the penetrating arteries (Benveniste et al., 2019). PVS is an open, low-resistance space formed by the vascular endfeet of the astrocyte, which serves as a wall, strengthening the entire cerebral vascular bed (Nedergaard and Goldman, 2020). Therefore, CSF can be considered as river-carrying sediment, which is metabolic waste, and PVS is the conduit where CSF flows (Benveniste et al., 2019). AQP4 is one of the subtypes of AQP that are highly expressed in the endfeet of the astrocyte. Working similar to sluice gates, AQP4 allows CSF to enter the brain parenchyma to exchange metabolites (Rasmussen et al., 2018). Interstitial fluid takes up 12%–20% of the fluid compartments in the brain, and it mixes with the CSF influx within the tissue toward the perivenous spaces (Benveniste et al., 2019). Eventually, the CSF-ISF fluid and small-size and hydrophilic waste drain to meningeal lymphatic vessels (MLVs) and the blood circulation (**Figure 2**).

Having discussed how to construct the glymphatic system, it is essential to probe into the significant roles in neurophysiology (Plog and Nedergaard, 2018). The function of the glymphatic system in waste clearance includes three aspects: (1) Since the PVS serves as a conduit sink for CSF/ISF movement, the glymphatic system constructs a trans-arteriovenous network in transmitting hydrophilic waste, such as lactate (Lundgaard et al., 2017). (2) The perivascular influx of CSF is involved in the clearance of macromolecules that are then absorbed by the downstream lymphatic network, forming a "front end" of the glymphatic-lymphatic connection (Da Mesquita et al., 2018b; Benveniste et al., 2019). (3) It serves complementary roles with the blood-brain barrier (BBB) in providing a well-conditioned neuronal environment in the prevention of mechanical disruption and waste accumulation (Verheggen et al., 2018).

With multiple critical roles in CNS physiology, it is not difficult to comprehend that the lesions of the major structures of the glymphatic system can weaken its capabilities to remove waste and have a devastating impact on brain health. For example, the depolarization, mislocalization, and deletion of AQP4 in the aging brain are associated with the impairment of perivascular CSF recirculation, which may together contribute to the accumulation of amyloid β (Aβ) protein and Tau proteins in the brain (Kress et al., 2014; Zeppenfeld et al., 2017) (**Figure 2**). A hypothesis might be further explained as follows: due to a loss of AQP4 localization, the extracellular fluid containing Aβ, which should have proceeded on to the perivenous spaces, is now attenuated in the brain interstitial space and flows disorderly (Jessen et al., 2015; Reeves et al., 2020). Also, it results in the elevated extracellular concentration of the protein (Nedergaard and Goldman, 2020). Given the combination of other factors, such as shear stress, ionic strength, and local pH, waste accumulation comes into existence (Burke et al., 2013). A recent study that used pharmacological blockade to perturb AQP4 polarization in rTg4510 mice has observed an ∼85% reduction in MRI-quantified CSF-ISF exchange and a similar decrease in tau clearance from the brain (Harrison et al., 2020), which is consistent with what was mentioned earlier. It suggests that AQP4 may serve as a significant predictor for the status of certain diseases, including chronic sleep disruption (Zhang et al., 2020), Alzheimer's disease (AD) (Zeppenfeld et al., 2017), and traumatic brain injury (TBI) (Piantino et al., 2019), whose pathogeneses are all related to the inefficient waste removal process. To further discuss the imaging progress of AQP4 detection, it is known that the diffusion methods can evaluate the molecular dynamics of water in brain tissue by the signal changes of motion-probing gradients (Taoka and Naganawa, 2020). Diffusion tensor imaging analysis along the PVS (DTI-ALPS) has the advantage of being non-invasive. It is applied to measure the motion of water molecules along the PVS direction

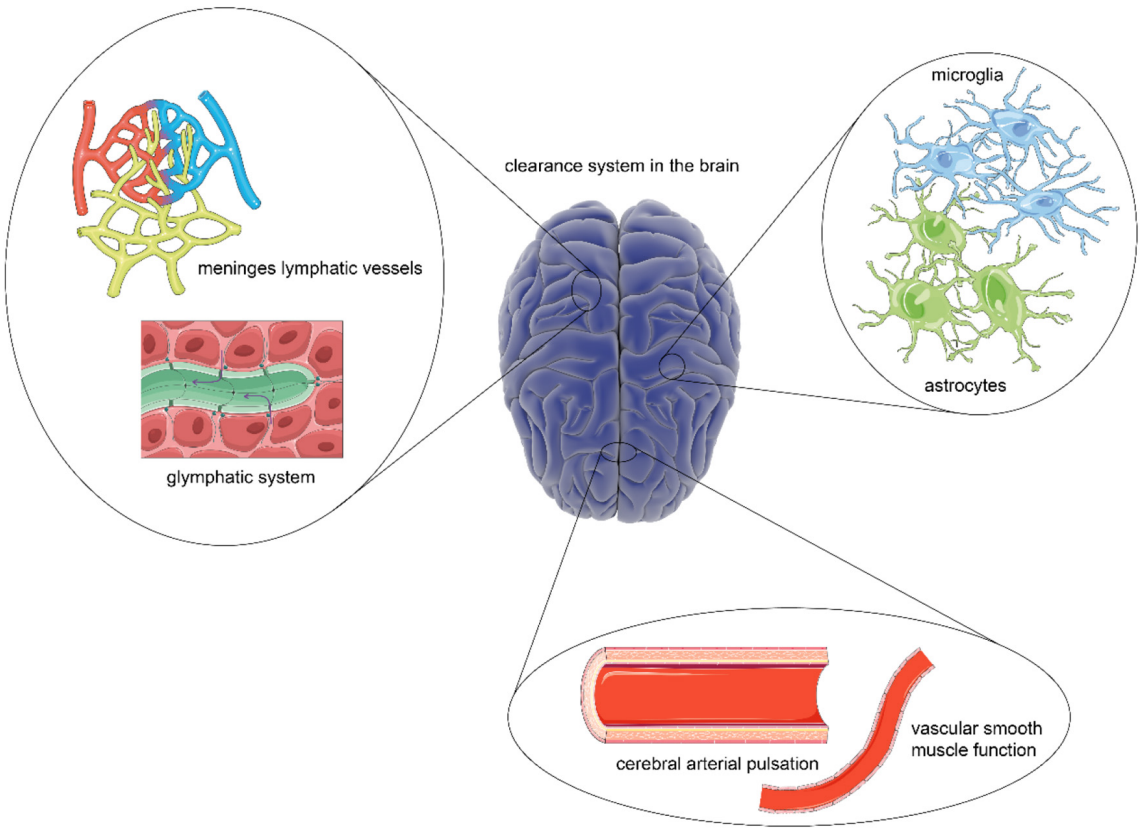

FIGURE 1 | A comprehensive overview of the clearance system in the brain. As the scarcity of lymphoid tissue, the brain has a unique clearance system to eliminate metabolic waste. The construction of a framework based on the glymphatic system and meningeal lymphatic vessels provides a platform for cerebrospinal fluid (CSF) flow, which is essential for the exchange and transport of metabolic waste. Besides, the driving force for CSF flow is provided by cerebral arterial pulsation and smooth muscle function. While the steady states of the process are guaranteed by astrocytes and microglia.

in recent studies, indicating that diffusion MRI can evaluate the CSF-ISF exchange (Harrison et al., 2018; Taoka, 2021). The expression of AQP4 plays an essential role in CSF-ISF exchange, and the apparent diffusion coefficient (ADC) can be regarded as a biomarker of AQP gene expression (Ohene et al., 2019), suggesting that diffusion MRI can monitor the changes of AQP4 molecules while evaluating the glymphatic system. What is more, a study of Sindex and shifted water diffusion coefficient (sADC), i.e., the two biomarkers that are more sensitive to tissue microstructure than ADC, further makes it possible to monitor the expression of AQP4 *in vivo* (Debacker et al., 2020).

The PVS is the space filled with CSF-like fluid, which follows penetrating vessels (Iliff et al., 2012; Mestre et al., 2017). The pulsatility of the penetrating arteries drives CSF into the neuropil along with the periarterial spaces (Aldea et al., 2019; Sloots et al., 2020). Typically, vascular cell debris, particulate, and protein deposition can cause the enlargement of PVS (Brown et al., 2018), but the driving forces within the glymphatic system do not change synchronously (**Figure 2**). Therefore, it is reasonable to work on the principle that enlarged perivascular space (ePVS) might be the pathological changes that occur early in minor vessel diseases (Brown et al., 2018; Wardlaw et al., 2020), in which secondary neuroinflammation is often observed. In addition to the associations between ePVS burden and minor vessel diseases, mounting evidence also suggests that ePVS may be modulated by sleep and TBI (Opel et al., 2019), revealing the presence of ePVS as a potential state marker for impaired clearance. While researchers have recently started to assess the role of astroglial biology in ePVS (Boespflug et al., 2018), more studies are needed to confirm the relationship between the pathological states of AQP4 and ePVS. Previous studies have been carried out on AQP4 dysfunction and its role in waste accumulation and disease, which may neglect its interrelation and interaction with other components and cells in the glymphatic system. Further study is necessary to uncover their close connection to the waste clearance process. Thus, new insights can be given to discover drugs and therapeutics for neurodegenerative diseases that involve multiple pathological changes.

MENINGEAL LYMPHATIC VESSELS

The first serious discussions and analyses of MLVs emerged during the 1800s with Paolo Mascagni (Lecco, 1953). So far, evidence of MLVs was obtained at autopsy (Visanji et al., 2018) that at the level of the superior sagittal sinus, there were lymphatic

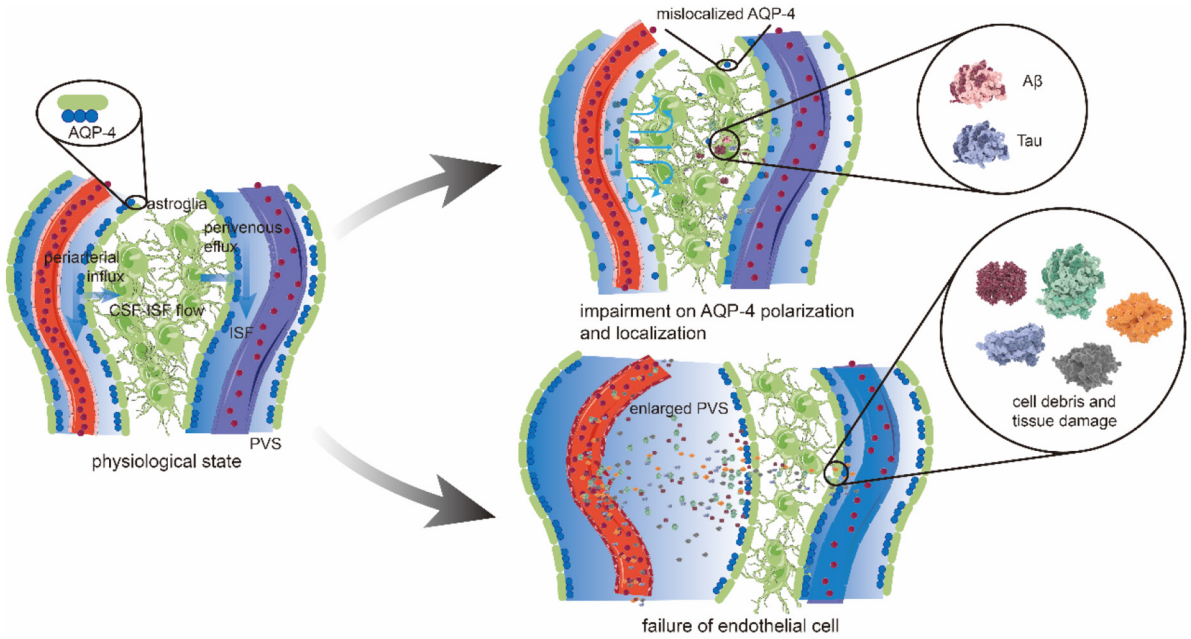

FIGURE 2 | The glymphatic pathway and pathological changes. CSF in periarterial spaces flows across the glial basement membrane and astroglial endfeet to enter the brain parenchyma. The aquaporin 4 (AQP4) is highly expressed on the endfeet of astrocytes, forming the outer wall of the perivascular space (PVS) and facilitating the CSF influx into the interstitium. After mixing with interstitial fluid (ISF) and waste metabolites, CSF is transported toward to perivenous space in a polarized net fluid movement. Ultimately, CSF exits along the lymphatic vessels and arachnoid granulations for the removement of solutes from the brain parenchyma accumulated during neural activity. Under pathological conditions, the brain may have an impairment on AQP4 polarization and localization. In patients with Alzheimer's disease (AD), AQP4 depolarization leads to the reduction in CSF influx from periarterial space. At the same time, the direction of CSF-ISF flow is disrupted or even reversed, which reduces the ability and efficiency to transport amyloid β (Aβ) protein. The concentration of Aβ increases gradually, and deposition occurs in the brain parenchyma and periarterial space. Vascular pathology causes endothelial cell (EC) failure, producing the cell debris and protein deposition that gives rise to the obstruction and inflammation of PVS. Increased PVS fluid and the abovementioned factors causes enlarged PVS, which then leads to a further accumulation of toxic metabolic by-products, such as Aβ and tau.

vessels in the human dura mater. In 2017, Absinta et al. (2017) visualized the lymphatic vessels in the dura mater by brain MRI (Absinta et al., 2017) which innovatively and concretely demonstrates the existence of MLVs.

The MLVs were initially considered mainly residing in the base of the skull (Bakker et al., 2016). The development of MRI has enabled a more delicate and precise structure of MLVs. Through the 3D-rendering of subtraction MRI images, dural lymphatics are discerned running parallel to the dural venous sinuses and along with the branches of the middle meningeal artery (Absinta et al., 2017). Besides, arteries, veins, and cranial nerves draining the contents into the deep cervical lymph nodes (dCLNs) were also detected by MRI (Bakker et al., 2016; Ha et al., 2018), which includes immune cells, CSF, and ISF from the subarachnoid space. Accordingly, MLVs are the downstream drainage of soluble and cellular components in CSF. In T2-fluid attenuated inversion recovery (FLAIR) MRI, lymphatic of the CSF was observed outflow through the jugular foramen into the cisterna magna. Also, most of it flew to the basal MLVs, rather than dorsal MLVs, and reached dCLNs from base MLVs (Ahn et al., 2019).

Similar to the initial lymphatic vessels in anatomy and molecular characteristics, the MLVs express all the traditional markers of endothelial cells (ECs) of the lymphatic vessels, without smooth muscle cells and valves (Louveau et al., 2015). While, a potential lymphatic valve has been detected at the bottom of the skull (Ahn et al., 2019), suggesting that these lymphatic vessels transition from the initial vessel to the collection vessel. Moreover, the diameter of MLVs is smaller than peripheral lymphatic vessels (Louveau et al., 2015).

A way to remove cellular debris and toxic molecules, such as Aβ peptides in the brain, is that CSF influx and ISF efflux through the paravascular pathway (Iliff et al., 2012; Peng et al., 2016), as previously demonstrated. As far as pathological changes are concerned, MLV disorders can impair the efflux of substantial/ISF macromolecules and their drainage to dCLNs, which functionally links the meningeal lymphatics with the CSF influx/ISF efflux mechanism (Da Mesquita et al., 2018b).

As the functions of CSF and ISF in the glymphatic system mentioned earlier, it is speculated that the glymphatic system and the meningeal lymphatic system are inextricably linked in exerting immune and clean functions. CSF, ISF, and meningeal lymphatic flows can be regarded as a holistic model when exploring the mechanism of brain immune surveillance and waste removal. MLVs were proved to be located downstream of the glymphatic system using MRI (Zhou et al., 2020), and rather than dorsal MLVs, basal MLVs are the main pathways to uptake and clear macromolecule and could be hot spots for the drainage

of CSF and ISF (Ahn et al., 2019). Researches have shown that injected brain-derived antigen efflux by CSF through MLVs can trigger T cell immune response after passing through dCLNs (Noé and Marchi, 2019). In comparison, the impairment of CSF-ISF exchange leads to reduced clearance of tracers in the brain and damage of drainage to dCLN (Iliff et al., 2012).

The regular operation of the CNS of the brain has strict requirements for the brain environment. Therefore, when the MLVs are damaged or CNS changes, it will affect the function. Recent evidence from animal studies demonstrates that impaired meningeal lymphatic drainage function caused spatial learning and memory impairment (Wang et al., 2019). Additionally, mice with the long-term ablation of MLVs show abnormal gene expression in the hippocampus related to neurodegenerative diseases (Lundgaard et al., 2017), which all suggest that neurodegenerative diseases might be related to meningeal lymphatic disorders. As mentioned earlier, the MLV is an immune tissue structure closely related to CSF. The impaired function of the MLV may affect the removal of metabolic waste in CSF, providing new possibilities for the preventive treatment of certain elder brain diseases manifested as cognitive impairment.

Alzheimer's disease has been taken as an example, which is one of the common neurodegenerative diseases. We inferred earlier that the obstruction of Aβ precipitation removal may lead to AD. As previously mentioned, the drainage of MLVs is one of the pathways for Aβ clearance, so it is inferred that the damage of meningeal lymphatics is related to AD. Recent observations suggest that the decline in meningeal lymphatic function during aging may aggravate the pathological state of the brain and meningeal amyloid (Ma et al., 2017). It is hypothesized that impaired meningeal lymphatic drainage of CSF will affect Aβ clearance, thus increasing the amyloid load of the brain (Da Mesquita et al., 2018a). Since vascular endothelial growth factor C (VEGF-C) can regulate the development of MLVs, promoting the clearance of amyloid (Wang et al., 2019) makes hope for a novel therapy for elderly AD.

Previous studies have confirmed CSF efflux movement from the brain parenchyma to downstream lymphatic circulation (Iliff et al., 2012). With new studies on the function of the glymphatic system and MLVs emerging (Louveau et al., 2017), it is significant to understand their interaction in detail.

Based on former research, the connection between the glymphatic system and MLVs was demonstrated with fluorescent tracer and dye in 2015 (Louveau et al., 2015). It is found that the MLVs could absorb the fluid from the glymphatic system and transport it into dCLN (Aspelund et al., 2015). Anatomically, Aspelund et al. indicated that the waste products might gain access to the lymphatic vessels through the adjacent subarachnoid space and drainage veins merging into the dura mater (Aspelund et al., 2015). The meningeal lymphangiogenesis was found coupled with the enhanced glymphatic influx under chronically implanted electroencephalography electrodes, which confirmed a close association between glymphatic activity and the meningeal lymphatic vasculature (Hauglund et al., 2020). When devastating cerebrovascular events, such as subarachnoid hemorrhage occurred, a decrease of meningeal lymphatic drainage and the depolarization of APQ4 were observed at the same time (Pu et al., 2019), suggesting a pathological link under the glymphatic-lymphatic connection. In conclusion, the function and dysfunction of MLVs and their relationship to the glymphatic system shed light on a hypothesis that the two systems work as a whole clearance system in the brain. Still, the hypothesis needs to be validated and strengthened by future studies.

COMPONENTS AND BARRIER PROTECTION OF CLEARANCE SYSTEM IN THE BRAIN

Based on the complete structure, maintaining the normal biological function of the clearance system in the brain still requires that waste capture and directional movement and barrier protective effects of the process ensure metabolic homeostasis in the brain. A more detailed account of components and barrier protection, including drivers and impact factors, is given in the following section.

CEREBROSPINAL FLUID

Produced by the choroid plexus, CSF fills the brain ventricles and the subarachnoid space and surrounds the spinal cord in the adult human brain (Benveniste et al., 2017; Marques et al., 2017). Cerebrospinal fluid flows are a prerequisite for a clearance system to maintain normal biological function. Therefore, exploring the effect of the driving force is required to construct a framework for a complete system (Benveniste et al., 2017). The flow direction of CSF along the intracranial artery was confirmed according to the studies mentioned earlier, which implies the importance of the pulsation of cerebral arteries, such as leptomeningeal arteries, to the driving force (Ringstad et al., 2017). Further observations relating to the flow velocity in CSF confirmed the pulsatile flow of CSF that matches the cerebral arterial pulse (Mestre et al., 2018). Subsequently, more and more evidence has indicated that the pulsatile flow in the arterial wall is a significant driver of the CSF (Taoka and Naganawa, 2018). However, as research in the fields of the clearance system surges forward, the cerebral arterial pulsation driven by the cardiac pump is strong enough to support efficient CSF flows, which is questionable (Aldea et al., 2019). Arguments have been put forward that vascular smooth muscle function is also considered important for CSF pressure and dynamics based on increased vascular smooth muscle reactivity modulating the clearance of intracranial metabolic waste (Cheng et al., 2019).

In addition, the circulation of CSF is also one of the factors affecting brain clearance system function. According to the "glymphatic system" hypothesis, CSF enters the brain *via* periarterial spaces, passes into the interstitium *via* perivascular astrocytic AQP4, and then drives the perivenous drainage of ISF and its solute (Plog and Nedergaard, 2018). Previous researches pointed out that this cerebral CSF circulation is only active during sleep or general anesthesia (Winsky-Sommerer et al., 2019). More and more evidence suggests that regular sleep time ensures the efficiency of the brain clearance system (Aguirre, 2016). Given

the latest discoveries, the brain clearance system is closely related to neurodegenerative diseases, such as AD, improving sleep is an essential means of preventing neurodegenerative diseases (Albrecht and Ripperger, 2018). However, the effect of anesthesia on CSF circulation is still controversial (Taoka et al., 2018). So far, the most influential account of anesthesia on CSF circulation is found in the study by Gakuba et al. (2018), which used MRI and near-IR fluorescence imaging to investigate the impact of general anesthesia on the intracranial CSF circulation in mice. Contrary to what was initially expected, they found that CSF circulation was more active when awake while significantly impaired during general anesthesia, suggesting that the effects of anesthesia on the brain clearance system are related to the dose (Gakuba et al., 2018). Clinical studies on whether anesthesia can be used to regulate the function of the clearance system are still lacking.

BLOOD-BRAIN BARRIER

The BBB cooperates with the glymphatic system in waste clearance and plays a dominant role in separating blood cells, exogenous pathogens, and circulatory wastes from the brain to maintain brain homeostasis (McConnell et al., 2017; Sweeney et al., 2019; Ueno et al., 2019) (**Figure 3**). With carrier-mediated transport on epithelium performing waste efflux and the clearance of its cellular components, BBB prevents CNS from accumulating metabolites and xenobiotics (Ueno et al., 2019). It is acknowledged that BBB can be divided into a basic functional unit called neurovascular unit (NVU), which consists of neurons, vascular cells, such as EC, pericytes, and gliocytes, including astrocytes and microglia. Mounting evidence suggests that NVU regulates BBB permeability, cerebrovascular net function, and neurogenesis (Obermeier et al., 2016; Saint-Pol et al., 2020). Under pathological conditions, the dysfunction and breakdown of constituent cells and relevant structures, such as tight junction (TJ), will lead to neurological disorders and deficits. The "Blood-brain barrier" section will attempt to describe how the cellular components with their junctions realize barrier function in BBB and elimination of wastes in the clearance system.

TIGHT JUNCTION

The TJ is the most substantial supporting structure in BBB formed on continuous capillary endothelium (Van Itallie and Anderson, 2014; Tietz and Engelhardt, 2015). TJ has plenty of specifically marked transmembrane proteins, including the occludin and claudin family, which communicate with other intracellular scaffolding proteins, such as the zonula occludens family (Tietz and Engelhardt, 2015; McConnell et al., 2017; Abdullahi et al., 2018) (**Figure 3**). However, the TJ can also be passive and changeable for nutrients and metabolites. It has been demonstrated that TJ synergizing with adherens junctions acts as an adjuster to the diffusion of solutes in plasma (Tietz and Engelhardt, 2015). Researchers have identified that Aβ itself could downregulate claudin-5 and occludin levels at the posttranslational levels to regulate TJ function, thereby promoting the efficiency of brain turnout and clearance of Aβ *via* the paracellular pathway through transendothelial cell receptors (Keaney et al., 2015). Hence, an appropriate opening of the TJ may promote the values of clearance of CSF and ISF through perivascular gaps. For example, Zn + highly accumulated in synapses can help open the TJ reversibly *via* the Glycogen synthase kinase-3 signaling (GSK3β)/snail-mediated signal transduction, i.e., the opening facilitates expelling macromolecular metabolites and toxins out of the brain cleaned (Xiao et al., 2018).

Under pathological conditions, the structure of TJ can be damaged, causing BBB to degrade even break down. Reactive oxygen species (ROS) may affect the integrity of TJ through the degradation of occludin, as evidenced by the elevated levels of occludin phosphorylation caused by ROS hydrogen peroxide (H_2O_2) at low concentrations, and occludin cleavage caused by H_2O_2 at moderate-to-high concentrations (Lischper et al., 2010). Neurons may thus be subjected to exogenous toxicants (Blanchette and Daneman, 2015). Besides, the TJ leakage can also result in the solute heaped up in vessels and tissues, hindering the drainage of ISF and CSF and causing ischemic lesions, such as ischemic stroke (Abdullahi et al., 2018). From the above mentioned text, opening the TJ can be helpful to deliver drugs targeted at brain parenchyma. Drugs targeting TJ-associated proteins, such as borneol, bradykinin, and hyperosmotic mannitol, and approaches, such as focused ultrasound, have been proven effective in opening TJ currently (Duan et al., 2016; Gonzalez-Mariscal et al., 2016).

CELLULAR COMPONENTS OF CLEARANCE SYSTEM IN THE BRAIN

The glial cell is the executor of the function of the clearance system in the brain. As more adequately correlate with functional status, the "Vascular EC" section involves physiological fluctuations and pathological shifts, which can provide perspective guidance and meaningful solutions of diseases caused by the accumulation of metabolic waste products and neurotoxins in the brain in the future.

VASCULAR ENDOTHELIAL CELL

Forming blood vessels by chimerism and connection with each other, vascular ECs build a core barrier between brain parenchyma and external environment, with the assistance of their junctions, such as TJ. Besides the physical function in BBB mentioned earlier, vascular ECs also provide a selective passage for the import of macromolecular nutrients and circulating proteins and the efflux of toxins and metabolites (McConnell et al., 2017). Carrier-mediated transporters, such as nucleotide, hormone, and amino acid transporters, and receptor-mediated transporters, such as insulin and lipoprotein receptors, ATP-binding cassette (ABC), and ion transport channels, are all endothelium-dependent, regulating the homeostasis of the material delivery (Sweeney et al., 2019;

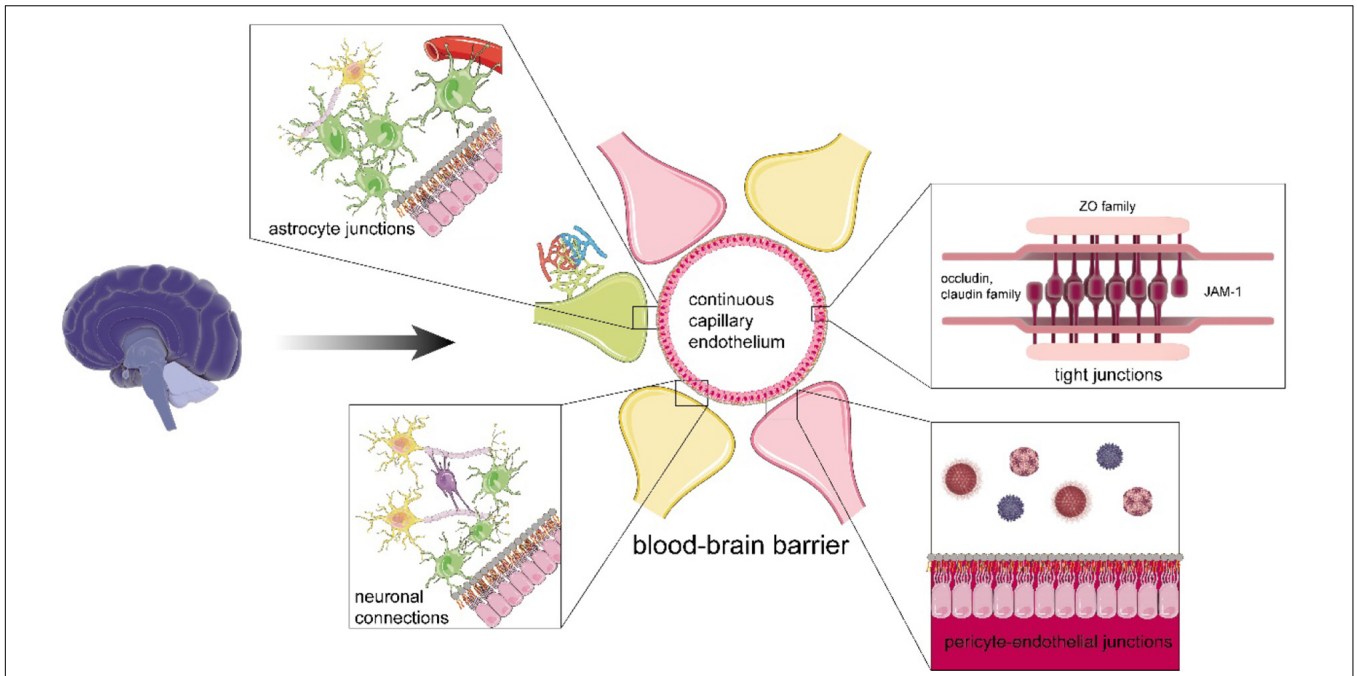

FIGURE 3 | The functional structure of the blood-brain barrier (BBB). The BBB includes continuous capillary endothelium with tight junction (TJ), adherens junctions and gap junctions, pericyte-endothelial junctions, astrocyte junctions, neural connections, intact basement membrane, and glial membrane. The TJ is abundant in TJ proteins. For instance, claudin and scaffolding protein, such as zonula occludens family, form the backbone of TJ, occludin helps maintain the integrity and stability of TJ, and junctional adhesion molecule A mediates cell adhesion to restrict epithelial permeability. In terms of astrocytes, they can strengthen the BBB by junctions with ECs, communicate with myelinated nerve fiber and synapses, and support them. For neurons, their connections are enhanced by glial cells, such as oligodendrocytes, which can assist efficient jump transmission of bioelectrical signals and maintain the normal function of neurons.

Shubbar and Penny, 2020). For example, ABC family, such as ABCB1 and P-glycoprotein, on the endothelium has been proved effective in the removal of Aβ out of the brain (Pan et al., 2020; Shubbar and Penny, 2020). Factors, such as gene mutation, that can alter the phenotype of vascular ECs may result in the diminishment or disappearance of the waste clearance function (Oikari et al., 2020). In further research, the specific relationship between EC function-related gene mutation and disease still needs to be clarified, benefiting the symptomatic treatment of specific neurological disorders (Oikari et al., 2020). What is more, the upregulated expression of translocators on ECs and induced release of endothelial relaxation factor, such as NO may also offer a possibility to accelerate the flow of CSF and ISF in PVS, enhancing the function of the clearance system in the brain consequently (Cheng and Haorah, 2019).

ASTROCYTE

Astrocytes, the coadjutant to build BBB with their endfeet attached to the vascular interface, also function as a doorman to control and clear CSF flow from paravascular spaces (Albrecht and Ripperger, 2018). In the last few years, more researchers shift their sights to the clearance of metabolic wastes and exogenous toxins that astrocytes perform, during which AQP4 takes a critical part. The AQP4 helps to remold to promote synaptic and vascular plasticity and assist astrocytes in eliminating dendritic spines (Valenza et al., 2019). In the clearance system, the AQP4 functions as a circulator participating in regulating the diameter of brain capillaries to guarantee interstitial flow and accelerate ISF circulation. It has been demonstrated that the polarized localization of AQP4 to perivascular endfeet can accelerate the CSF from subarachnoid to flow to brain interstitium in a convective bulk way, helping the glymphatic system clear the ISF and CSF simultaneously (Yang et al., 2017; Valenza et al., 2019; Zhang et al., 2020). Suppose the AQP4 localization has been altered or lost. In that case, astrocytes tend to release VEGFs and matrix metalloproteinases (MMPs) to increase BBB permeability, which will result in the accelerating process of BBB disruption and the deposition of insoluble Aβ along with subsequent reactive gliosis (Michinaga and Koyama, 2019; Valenza et al., 2019).

Interestingly, researchers also find a positive correlation between age and the expression of AQP4, especially in neurodegenerative disorders (such as AD) (Denver et al., 2019). Therefore, the downregulation and the fine control of the AQP4 show potential in the healing and prognosis of these diseases. Studies have shown that drugs, such as zonisamide and topiramate, can inhibit the function of AQP4. At the same time, the synthetic antibody to AQP4 also benefits a great deal in diseases caused by the autoantibody to AQP4, such as neuromyelitis optica (Verkman et al., 2017).

From the perspective of regulating BBB structure, astrocytes are also the hinge cable between BBB and the glymphatic system. As far as can be determined, consciousness and body

posture affects the clearance rate of brain waste, in which sleep is a stimulating factor (Natale et al., 2021). While asleep, astrocytes may increase the interstitial volume by shrinking themselves, promoting ISF flow and the transformation between CSF and ISF. Also through this process, the glymphatic clearance is straightly facilitated, and the scavenging efficiency of BBB is also indirectly improved by increasing the flow of interstitial solute (Mendelsohn and Larrick, 2013; Verheggen et al., 2018; Zhang et al., 2021). Accordingly, BBB will also upregulate the activity of efflux transporters, such as ABCB1, under the control of the circadian clock (Zhang et al., 2021). Consequently, the BBB and the glymphatic system can cooperate and influence each other.

MICROGLIA

Microglia are resident cells in the innate immune system performing as CNS parenchymal macrophages, taking charge of digesting and degrading endogenous wastes in the brain, such as cellular debris, to accomplish neuronal and synaptic surveillance, developmental neuronal apoptosis clearance, and dendritic spine pruning (Casano et al., 2016; Dudvarski Stankovic et al., 2016; McConnell et al., 2017). Once microglia are exposed to declining and apoptotic cells in the very first stage of apoptosis, the expression of clearance-related genes, such as PRC2, is promoted, launching a series of clearance reactions in the very first stage of apoptosis (Ayata et al., 2018). Besides, microglia tend to share mutual support and promote phagocytosis, especially the one young microglia give to the older (Daria et al., 2017). Furthermore, microglia can be divided into pro-inflammatory (M1-like) phenotypes to immunosuppressive (M2-like) phenotypes according to the degree of activation at present (Loane and Kumar, 2016). Suppose microglia are aberrantly activated, which is common in acute CNS injury and neurodegenerative diseases. In that case, they can shift to M1-like phenotype, which results in the M1/M2-like polarization, and can release relative cytokines to speed up the disruption of BBB, consequently exacerbating the destruction of vessels and neurons (Loane and Kumar, 2016; Hammond et al., 2018; Hickman et al., 2018).

Moreover, their increased intrinsic function of phagocytosis and elimination can also damage neuron morphology and interconnection with others. Hence, it has been suggested that the detection of regions where the M1-like phenotype of microglia proliferate becomes increasingly significant in diagnosing neurodevelopmental disorders, such as autistic spectrum disorders, most of which are characterized by microglioma (Hammond et al., 2018). However, microglial activation also has several serious drawbacks, such as transferring microtubule-associated protein tau *via* exosomes in the brain and failing to clear the toxic metabolite (Asai et al., 2015; Li and Barres, 2018). Altogether, the mechanism of microglia causing aberrance and dysfunction of neurons in over-clearance circumstances remains to be elucidated, and the moderate regulation of microglia activation also needs to be explored (Dudvarski Stankovic et al., 2016). In the next step of research, discovering biomarkers of the M1-like phenotype microglia can be a target, which is beneficial in prompt diagnoses and treatment of neurodevelopmental disorders by more advanced pathological examinations, such as neuroimaging method.

PERICYTE

Pericytes are a group of cells embedded in the basement membrane of capillary ECs in the vasculature, act as ancillary cells to strengthen BBB, and assist functional members in the clearance system. As a member of NVU collaborates with astrocytes, microglia, and other neurons, pericytes participate in vascular development, permeability, and remodeling. Currently, pericytes have been proved to have the ability to differentiate into the neural lineage, contributing to the neurogenesis and inflammatory factor clearance after the brain injury or the chronic neuroinflammation, such as glioblastomas (Birbrair et al., 2017; Andreotti et al., 2018; Santos et al., 2019). In addition, pericytes reach to regulate the blood flow and interstitial flow of capillaries, modulating the clearance values of ISF and CSF (Burdyga and Borysova, 2018). Under pathological conditions, degenerated pericytes can directly decrease the pericyte-glia interface at BBB and then disrupt BBB (Giannoni et al., 2018; Klement et al., 2019). Consequently, it allows blood-derived toxins, such as autoantibodies murine immunoglobulin G (mIgG), to invade and then deposit in the brain parenchyma, leading to the diminishing clearance of gliocytes (Villasenor et al., 2017; Santos et al., 2019). Although the specific functions and behaviors of various subtypes of pericytes displayed in different stages of life in humans are not clear currently, the possibility and potential pericytes that have shown in TJ opening and blood flow control are undoubted, which provides us with a novel idea in the therapeutic measures of relative neurodegenerative diseases, such as AD (Sweeney et al., 2016).

The BBB and the glymphatic system have in common that they all aim to remove waste from CSF and ISF in the brain and reach mutual promotion and cooperation (Verheggen et al., 2018). Nevertheless, they also differ in the specific way of performing the function on account of structure. For BBB, barriers formed by cells and tight connections and selection of channels for substances, such as proteins, are the primary way to execute clearance. For the glymphatic system, however, it mainly separates waste from circulating CSF by promoting the convective movement of CSF between PVS and interstitial space to achieve the purpose of clearance (Abbott et al., 2018). On the one hand, when the clearance of more metabolites or toxic substances of BBB transporters reaches operational saturation, it seems that the glymphatic system can undertake the scavenging effect. On the other hand, the activity of convection in the glymphatic system can also stimulate the enhanced responsiveness of the AQP4 water channel and promote the function of BBB, especially astrocytes (Kress et al., 2014). In this context, BBB is more susceptible to aggressive barrier destruction factors, such as trauma, the collapse of cell junctions, such as pericyte loss, and the internal lesion-like upregulation of lipoprotein receptors (Keaney et al., 2015; Kaur and Sharma, 2018; Wei et al., 2019). At the same time, the glymphatic system is more susceptible to interference with CSF convection factors,

such as altered pulsations and changed vasomotor tone (Brown et al., 2018; Rasmussen et al., 2018; Shackleton et al., 2019). In this process, inflammation and sleep disturbances may be the common factors that destroy both and cause the malignant cycle.

The NVU, composed of ECs, glial cells, pericytes, and neuronal cells, functions as a whole beyond the narrow scope of BBB operation, as well as performs and enhances the function of the removal of brain waste through intercellular communication and interaction (McConnell et al., 2017). As the cerebral vasculature extends, the vascular surface cover transforms from smooth muscle cells and pia mater to astrocytic endfeet and pericytes, during which the components of the NVU act synergistically to regulate vascular permeability, neuroimmune responses, and cerebral blood flow (CBF) for the transport of substances and waste products. Astrocytes play a critical role in this process by acting as a communication relay for neurons and blood vessels (Lv et al., 2021). Astrocytes transmit neuronal responses to changes in the surrounding environment (e.g., metabolite accumulation and hypoxia) in the form of cellular electrical and chemical signals, which ultimately regulate cerebral vascular tone and CBF. The impairment of NVU structures, such as the absence of normal vascular covering components in the intratumoral vasculature, can lead to changes in vascular permeability and corresponding alterations in the selective permeability of nutrients, metabolites, and exogenous toxicants (Lv et al., 2021). Simultaneously, the excessive activation of astrocytes may allow overproduction of Aβ in patients with AD; the impairment of the EC-mediated transport system could lead to decreased efficiency of Aβ clearance and its deposition in axonal compartments (Olabarria et al., 2010; Muoio et al., 2014; De Bock et al., 2016).

4 POTENTIAL THERAPEUTIC STRATEGIES TARGETING DEFECTS OF CLEARANCE SYSTEM IN THE BRAIN

Exploring the regulatory mechanism of deficiencies in the clearance system may help identify the precise therapeutic target for intracerebral hemorrhage (ICH). Notably, the multi-omics technologies have made significant achievements in the research of neurodegenerative diseases (Nativio et al., 2020; Petermann-Rocha et al., 2020). A comparative analysis of driving gene expression in the physiological and pathological states of the clearance system will establish preliminary evidence for a link between them (**Supplementary Table 1**). The application of systematic multiomics approaches to precision medicine and systems biology has great potential to improve the care of patients with dysfunction of clearance systems. Besides, the target gene identified by multiomics studies can potentially be used for drug repositioning in ICH, which is approved to be cheaper, quicker, and effective (Armando et al., 2020) (**Supplementary Table 2**). For example, Caffeine targeting the *PDE1C* that is related to brain metabolites has been approved by the FDA for the short-term treatment of apnea of prematurity. Given the same putative drivers of the clearance system, those may maintain clearance system stability *via* relaxing the vascular smooth muscle and alleviate some pathological conditions caused by the disturbed clearance system. At present, direct evidence linking disease-driven analyses and molecular network dynamics in pathological contexts is lacking. Further research in neurological disorders is the next essential step in confirming diseases-driven networks associated with clearance system, thus developing novel therapeutic strategies that are more pertinent and specific.

CONCLUSION

This review set out to provide a systematic account of the clearance system in the brain. The findings indicate that the flow of CSF through the glymphatic system and meningeal lymphatic vessels is driven by smooth muscle and cerebral arterial pulsation, thus clearing toxic and metabolic waste, including small molecules and biomacromolecules. Notably, BBB, microglia, and astrocytes seem to play a significant role in maintaining the process. These findings have substantial implications for understanding the clearance of metabolic waste from the brain. When the balance of the process is disturbed, how it contributed to pathological changes in the brain lays the groundwork for future research into physiological homeostasis and pathophysiological responses within the brain.

AUTHOR CONTRIBUTIONS

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

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SUPPLEMENTARY MATERIAL

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The Updated Role of Transcranial Ultrasound Neuromodulation in Ischemic Stroke: From Clinical and Basic Research

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Ischemic stroke is a common cause of death and disability worldwide, which leads to serious neurological and physical dysfunction and results in heavy economic and social burdens. For now, timely and effective dissolution of thrombus, and ultimately improvement in the recovery of neurological functions, is the treatment strategy focus. Recently, many studies have reported that transcranial ultrasound stimulation (TUS), as a non-invasive method, can dissolve thrombus, improve cerebral blood circulation, and exert a neuroprotective effect post-stroke. TUS can promote functional recovery and improve rehabilitation efficacy among patients with ischemic stroke. This mini-review summarizes the potential mechanism and limitation of TUS in stroke aims to provide a new strategy for the future treatment of patients with ischemic stroke.

Keywords: transcranial ultrasound stimulation, ischemic stroke, mechanism, review, limitation

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INTRODUCTION

Ischemic stroke is the most common type of cerebrovascular disease. According to the data of the global burden of disease (GBD) study, stroke is the most common cause of death among Chinese residents (GBD 2019 Stroke Collaborators, 2021). From 2010 to 2019, the incidence of ischemic stroke has increased from 129/1,00,000 in 2010 to 145/1,00,000 in 2019, and the prevalence of ischemic stroke has increased from 1,100/1,00,000 in 2010 to 1,256/1,00,000 in 2019. According to China's aging population trend and the seventh census data, in 2021, there will be approximately 17.8 million patients with stroke, with 3.4 million new stroke patients and 2.3 million stroke-related deaths among the population over 40 years of age in China each year. China is the largest developing country, accounting for one-fifth of the world's total population, with the highest number of patients with stroke worldwide (Wang et al., 2017). Current treatments for ischemic stroke include thrombolysis, mechanical thrombectomy, and neuroprotective therapies (Liaw and Liebeskind, 2020). However, intravenous thrombolytic therapy has a strict treatment time window, with the risk of rebleeding, making its clinical application limited.

Statistical data have shown that only 16% of patients with acute ischemic stroke in China are admitted to the hospital within 3 h of symptom onset, and only 1.3% of these patients receive thrombolytic therapy (Wu et al., 2019; Shen et al., 2020; Tu et al., 2021a,b). Transcranial ultrasound stimulation (TUS) is a non-invasive technique for patients with stroke, which can stimulate specific brain areas, and improve neural activity and connectivity. The advantages of transcranial

ultrasound for brain stimulation are that it does not necessitate surgery or genetic alteration but confers spatial resolutions superior to other non-invasive methods such as transcranial magnetic stimulation (TMS; Tufail et al., 2011; Kim et al., 2012). TMS uses magnetic fields to pass through the skull to stimulate the brain tissue and has been widely used for disorders caused by the brain lesions, such as those caused in the type of depression that does not respond to medication, and cognitive impairment after stroke. The disadvantage of TMS is the lack of good spatial resolution, which results in limitations in the application of neural rehabilitation (Dionísio et al., 2018; Krogh et al., 2021; Liu et al., 2021a). The characteristics of high penetration and high spatial resolution of TUS have shown therapeutic potential in stroke treatment (Guo et al., 2015; Li et al., 2017; Liu et al., 2019; Wu et al., 2020; Malinova et al., 2021). Transcranial ultrasound is roughly divided into two types based on frequency: one is diagnostic transcranial ultrasound, with a frequency of 1.0–15 MHz, whereas the other is transcranial aggregation ultrasound, with a frequency lower than 1.0 MHz (Yang et al., 2008; Deng et al., 2021). At present, most studies focus on the thrombolytic effect and the mechanism of neuroregulation in low-frequency TUS (Li et al., 2017; Fomenko et al., 2018; Liu et al., 2019). TUS is able to transmit a certain frequency of an ultrasonic wave on the human skull with a specific ultrasonic probe. Through the skull, the ultrasonic energy is transmitted to the brain tissues, stimulating the brain to produce a series of biological effects. TUS has been explored in arterial thrombolytic therapy and post-stroke rehabilitation therapy as an emerging and non-invasive brain stimulation method (Rubiera et al., 2008; Tsivgoulis et al., 2008; Barlinn et al., 2013).

NEUROPROTECTIVE EFFECT OF TRANSCRANIAL ULTRASOUND STIMULATION IN STROKE

The neuroprotective effect of TUS has become a hot topic in recent years, especially in the field of stroke. TUS mainly exerts a neuroprotective effect through the following mechanisms.

Rapid Restoration of Cerebral Blood Supply and Improvement of Cerebral Blood Flow

The earlier the restoration of cerebral blood supplies after acute cerebral infarction, the better the recovery of neurological function (Fisher and Bastan, 2008; Molina and Alvarez-Sabín, 2009). Therefore, improving cerebral circulation before irreversible changes occur in the brain tissue and alleviating neuronal damage are the key factors for the treatment of ischemic stroke and also have a positive significance in improving the rehabilitation efficacy post-stroke. After ischemic stroke, the neurons in the stroke center rapidly undergo apoptosis and necrosis because of ischemia and hypoxia, whereas the surrounding cells still have transient survival ability because of the existence of collateral circulation, thus forming an ischemic penumbra. Within a certain period of time, cells in this area can either undergo apoptosis or return to the normal brain tissue (Bonnin et al., 2021; Davis and Donnan, 2021; Yang and Liu, 2021). If the ischemic penumbra is rescued in time, it can effectively prevent stroke progression; further, the successful rescue of ischemic penumbra is also conducive to the recovery of the nervous system and physical function in the future. TUS has been proved to be beneficial for the improvement of cerebral blood circulation after acute ischemic stroke, and within a certain range, cerebral blood flow also shows a gradually increasing trend with the increase of stimulation intensity and duration (Yuan et al., 2020, 2021; Liu et al., 2021b). However, with an improvement in cerebral blood flow, the risk of rebleeding also increases. A previous clinical trial (Daffertshofer et al., 2005) has confirmed that low-frequency (300 kHz) TUS not only resulted in improvement of the thrombolytic efficiency of tissue plasminogen activator (tPA) but also caused an increased rate of a cerebral hemorrhage in patients concomitantly treated with intravenous tPA. The limitation of the study was that only 26 patients were included. Combined lysis of thrombus with ultrasound and systemic tPA for emergent revascularization in acute ischemic stroke (CLOTBUST-ER), an international four-center phase II trial, demonstrated that in patients with acute ischemic stroke, transcranial ultrasound augments tPA-induced arterial recanalization with a non-significant trend toward an increased rate of clinical recovery from stroke, compared with the control group. The rates of symptomatic intracerebral hemorrhage were similar between the active and control groups (Schellinger et al., 2015; Katsanos et al., 2020). Further studies are still awaited on this important issue. A previous study has shown that the earlier the transcranial ultrasound intervention, the better the neuroprotective effect (Liu et al., 2019). Therefore, early use of TUS after stroke may effectively improve the brain–blood supply, restore local blood circulation, rescue the ischemic penumbra, and ultimately reduce brain tissue damage.

Transcranial ultrasound stimulation has also been shown to improve the vascular recanalization rate, which is an important index to evaluate the treatment effect in acute ischemic stroke. Evgenii et al. (Kim et al., 2021) reported a wireless, wearable system to achieve ultrasound brain stimulation in freely behaving animals. The brain activity induced by the system was monitored as cerebral hemodynamic changes *via* near-infrared spectroscopy. The system was also applied to stroke rehabilitation after temporal middle cerebral artery occlusion (MCAO) in rats. The stimulation was found to induce hemodynamic changes in the sonicated area, whereas open-field tests showed that ultrasound applied to the ipsilateral hemisphere for 5 consecutive days after stroke facilitated recovery. Another study conducted by Wu et al. (2020) aimed to determine the neuroprotective effect of low-intensity TUS at different time points using endothelin-1-induced MCAO in rats. The results showed that the rats that received low-intensity TUS exhibited reduced damage of the affected brain tissue after cerebral ischemia. The greatest protective effect was found with ultrasound stimulation of 30 min after cerebral ischemia. Hameroff et al. (2013) found that using 8 MHz ultrasound to stimulate the upper frontotemporal cortex of patients for 15 s could increase arterial oxygen saturation, suggesting that ultrasound stimulation at a higher frequency may

alleviate ischemic hypoxic changes after stroke. Furthermore, one study (Alexandrov et al., 2019) found that within 3 h after ischemic stroke onset, low-frequency transcranial ultrasound therapy within 30 min after thrombolysis can significantly enhance atenolol enzyme-induced arterial recanalization ability compared with the control group, but the results also indicated that transcranial ultrasound therapy did not significantly improve patient outcomes in 90 days after stroke.

Reduced Inflammatory Response and Apoptosis

Inflammatory mediators can cause further damage to neurons in the ischemic penumbra. A recent study applied low-intensity TUS to the ischemic cortex after distal MCAO and found that ultrasound may activate coagulation factors through certain signal transduction pathways and reduce neutrophils in the ischemic region, thus reducing the inflammatory response and facilitating neuronal recovery in the ischemic penumbra (Guo et al., 2015). Similarly, another research on Parkinson's disease rat model found that the low-intensity pulsed ultrasound treatment significantly inhibited 6-OHDA-induced glial activation and the phosphorylation of nuclear factor-κB p65 in the substantia nigra pars compacta. Further evaluation revealed that low-intensity pulsed ultrasound effectively preserved the levels of neurotrophic factors, dopamine transporter, and tight junction proteins in the blood–brain barrier (Song et al., 2021). Furthermore, (Zhou et al., 2021) revealed that TUS reduced the chronic inflammatory response in microglia and astrocyte activation, whereas Pang et al. (2021) found that TUS could attenuate the level of TNF-α .

As ischemic stroke induces cellular apoptosis, especially neuronal apoptosis, which leads to neurological dysfunction, determining a means to alleviate neuronal apoptosis has become an important issue for researchers while evaluating the outcome of patients with ischemic stroke. A study conducted in 2021 (Zhou et al., 2021) found that TUS could reduce the level of apoptosis-related protein Bax, and improved the movement and learning in aging rats. Su et al. (2017) further observed that low-intensity pulsed ultrasound could inhibit the progression of apoptosis following traumatic brain injury. Thus, the neuroprotective effects of TUS may be associated with the TrkB/Akt-CREB signaling pathway. Another study from the same team (Chen S. F. et al., 2018) found that the low-intensity pulsed ultrasound significantly attenuated the brain edema and neuronal death, reduced neutrophil infiltration and microglial activation, increased the Bcl-2/Bax ratio, and enhanced the phosphorylation of Bad and FOXO-1, ultimately improving the functional outcomes. These results indicated that the neuroprotective effects of TUS are associated with a reduction of early inflammatory events and inhibition of apoptotic progression.

Promotion of the Release of Neurotrophic Factors

Neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), are considered to be involved in the regulation of key nerve functions and neuroplasticity in stroke (Wang et al., 2006; Luo et al., 2019; Liu et al., 2020). A previous study has found that TUS effectively prevents cerebral ischemia/reperfusion injury through apoptosis reduction and BDNF induction (Chen C. M. et al., 2018). Su et al. (2017) observed that low-intensity pulsed ultrasound could increase the BDNF protein levels following a traumatic brain injury. The neuroprotective effects of TUS may be associated with the enhancement of protein levels of neurotrophic factors. Similarly, another study conducting MCAO using a C57BL/6J mouse model found that the low-intensity pulsed ultrasound accelerated the expression of BDNF in the brain of stroke mice and significantly moderated neuronal function after injury including neurological score, motor activity, and brain pathological score. In addition, Yang et al. (2015) demonstrate that TUS could enhance the protein levels of neurotrophic factors (i.e., BDNF, GDNF, VEGF, and GLUT1), which could have neuroprotective effects against neurodegenerative diseases. In a study using an ischemic stroke mouse model, low-intensity pulsed TUS could induce BDNF expression, decreasing the percentage of damaged neurons and the loss of neurological function after stroke. Tsai et al. (Zhang et al., 2019) interestingly found that TUS can also exert antidepressant-like effects. TUS could change the expression of BDNF in the hippocampus of rats. Given that BDNF plays an important role in the pathogenesis of depression, promoting BDNF could have a therapeutic effect (Hashimoto, 2010).

Thrombolytic Effect of Transcranial Ultrasound Stimulation

Ultrasound has direct and indirect thrombolytic effects. Indirect thrombolytic effects of TUS mainly enhance the effectiveness of thrombolytic drugs. In addition, ultrasound can directly dissolve the thrombus through its special physical and chemical properties (Cao et al., 2021; Doelare et al., 2021; Mei and Zhang, 2021). The main thrombolytic effects of TUS are summarized as follows.

Cavitation Effect

Cavitation is the generation of a large number of small bubbles when ultrasound is applied to a liquid (Lahiri et al., 2021). These bubbles rapidly vibrate, expand, and burst. A large amount of energy is released at the moment of bubble explosion, causing the thrombus to tear and decompose, exposing the surface of the thrombus in large quantities, and further accelerating the dissolution of the thrombus (Ma et al., 2020; Jo et al., 2021; Singh et al., 2021). The cavitation-induced thermal effect has been confirmed as a potential mechanism underlying this phenomenon. When a certain amount of heat is generated at the moment of bubble expansion, the heat effect may be the main role of ultrasound therapy, because a certain amount of heat effect can increase the activity of fibrinolytic enzymes, which is conducive to its binding with thrombosis (Wu et al., 2020). Intensity-focused ultrasound can generate high heat for the ablation of tumors and other biological tissues (ter Haar, 2007; Bessonova et al., 2010; Simon et al., 2012). The mechanism underlying this is complex. Some studies have proposed that high-intensity focused ultrasound can affect the action potential

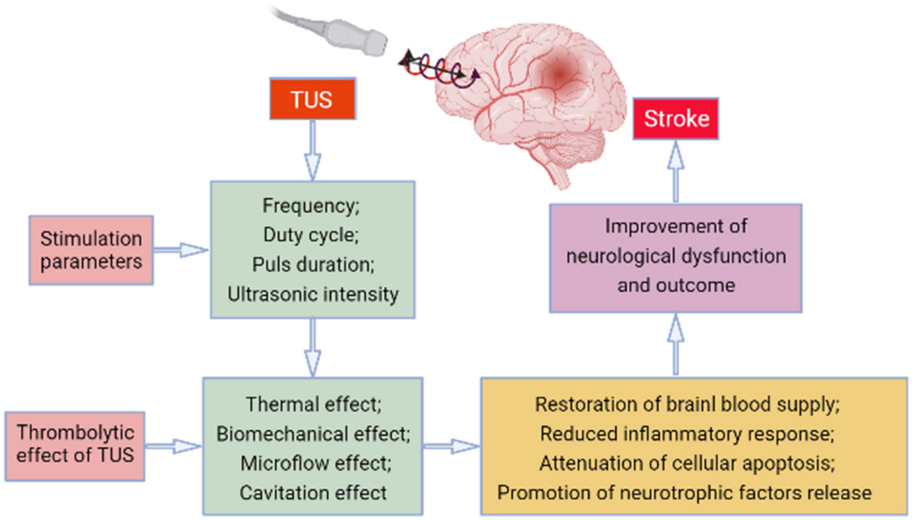

FIGURE 1 | The potential mechanism involved in the transcranial ultrasound stimulation (TUS)-induced effect on stroke.

of axons (Wahab et al., 2012); the electrical activity of neurons can be suppressed by disrupting the ultrastructure of synapses by blocking the connections between them (Borrelli et al., 1981; Tsui et al., 2005). Furthermore, the thermal effect of high-frequency and high-intensity focused ultrasound can damage nerve tissue and thus block transmission between synapses (Foley et al., 2008; Colucci et al., 2009). In contrast, one research found that although low-intensity TUS was eventually converted into heat energy when it passed through non-ideal media, the ultrasonic signal had almost no thermal effect on the brain tissue in the process of ultrasonic stimulation. The heat is extremely weak, much lower than the predicted heat required to produce obvious biological effects of temperature threshold (Liu et al., 2019). The differences in the abovementioned studies may be related to the differences between animal models and clinical patients. However, it is still controversial whether low-frequency TUS has a meaningful thermal effect during treatment. Whether the strength of the thermal effect is related to the frequency, intensity, and duration of TUS, and whether the thermal effect will cause damage to the brain tissue, needs further research.

Biomechanical Effect

The mechanical action is the main mechanism of ultrasound thrombolysis. The mechanical vibration of ultrasound can destroy blood clots, decompose thrombi, increase the contact between enzyme and fibrin, and promote the dissolution of thrombi. Ultrasound can also enhance the vitality of the brain cells and promote the repair of nerve cells after cerebral ischemia (Masomi-Bornwasser et al., 2021; Shin Low et al., 2021).

Microflow Effect

The microflow effect is caused by the cavitation effect. Here, the pressure generated by the burst microbubble causes the liquid to form microflow, which can accelerate drugs to the ischemic region (Matsievskii, 2003; Park et al., 2019; Li et al., 2021).

LIMITATIONS OF THE CURRENT TRANSCRANIAL ULTRASOUND STIMULATION STUDY

Treatment with TUS lacks clinical trials, as there are great differences between animal models and clinical patients, many problems remain to be solved in the clinical application of TUS. Although some clinical studies have proved that TUS can improve the clinical efficacy in ischemic strokes, these evaluation methods cannot reflect the improvement in a patient's ability during the rehabilitation process. More detailed and specific assessment scales should be used for evaluating the effect of TUS, such as assessment of cognitive, speech, sensory, motor, and other aspects. Moreover, most of the current clinical studies are about a combination of TUS and thrombolytic drugs to improve the thrombolytic effect. Clinical research in the future may explore TUS and other rehabilitation treatment methods, such as physical therapy, speech therapy, and low-frequency neuromuscular electrical stimulation, to improve the effectiveness of rehabilitation therapy in patients with ischemic stroke. For example, anodal transcranial direct current stimulation combined with constraint-induced movement therapy resulted in the improvement of functional ability of the paretic upper limb compared with constraint-induced movement therapy alone, indicating that TUS may enhance the effect of rehabilitation in patients with chronic stroke (Figlewski et al., 2017). In addition, there is no clear standard answer with regard to the specific parameters of TUS. Previous studies found that transcranial ultrasound with different frequencies can produce different excitatory or inhibitory effects on the brain (Krishna et al., 2018; Zhang et al., 2021). The ultrasonic stimulation parameters mainly include frequency, pulse repetition frequency, duty cycle, pulse duration, and ultrasonic intensity (Uddin et al., 2021). Frequency refers to the number of oscillation cycles per unit time, importantly, as

the frequency is inversely proportional to the wavelength, the higher the frequency, the smaller the focal spot volume, and the more significant the acoustic attenuation and scattering effect. Therefore, it is necessary to explore the most suitable frequency and intensity of TUS according to the location depth of the stimulation target and the thickness of penetration through the skull, and the safety of different parameter combinations in clinical application.

CONCLUSION

Ultrasound thrombolysis has already been used widely; low-frequency TUS can exert its effect based on the mechanical vibration, cavitation, or microflow, consequently dissolving the thrombus, reducing the infarct size in patients, promoting cerebral circulation, rescuing the ischemic penumbra, and improving the prognosis of patients with stroke. Low-frequency TUS is mostly used clinically at present. Numerous studies have explored the mechanism of low-frequency TUS and confirmed that low-frequency TUS has certain neuroregulatory effects, which have both excitatory and inhibitory effects on the human cerebral cortex (**Figure 1**).

In summary, although there are still many issues that need to be explored and solved in terms of clinical application, numerous studies have shown that TUS can promote thrombolysis, increase cerebral blood circulation, and improve neurological recovery. Thus, after ischemic stroke onset, timely and proper TUS intervention may improve the neurological function and quality of life of post-stroke patients.

AUTHOR CONTRIBUTIONS

QL participated in the design of the review. SZ, BM, JJ, XW, NLu, and NLi drafted the manuscript. HS and LW critically revised the text and figure. All authors read and approved the final manuscript.

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REVIEW
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Erythrophagocytosis by Microglia/Macrophage in Intracerebral Hemorrhage: From Mechanisms to Translation

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Intracerebral hemorrhage (ICH) is a devastating condition characterized by hematoma related mass effect. Microglia/macrophage (M φ) are rapidly recruited in order to remove the red blood cells through erythrophagocytosis. Efficient erythrophagocytosis can detoxify hemolytic products and facilitate neurological recovery after ICH. The underlying mechanisms include modulation of inflammatory response and oxidative stress, among others. It is a dynamic process mediated by a cascade of signal transduction, including "find-me" signals, "eat-me" signals and a set of phagocytotic receptors-ligand pairs that may be exploited as therapeutic targets. This review summarizes mechanistic signaling pathways of erythrophagocytosis and highlights the potential of harnessing M φ-mediated phagocytosis for ICH treatment.

Keywords: erythrophagocytosis, efferocytosis, macrophage, microglia, intracerebral hemorrhage, hematoma, phagocytosis

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INTRODUCTION

In intracerebral hemorrhage (ICH), the rupture of brain vessels results in the accumulation of millions of red blood cells (RBCs) within brain parenchyma. Surgical excavation of hematoma is not recommended for most ICH cases due to questionable clinical benefits and adverse effects of surgery (Hemphill et al., 2015). Hemolysis within the hematoma may cause significant secondary injuries and irreversible neurological deficits due to the toxic properties of hemolytic products (Ziai, 2013). Microglia and monocyte-derived macrophage (M φ) are rapidly recruited at the bleeding site and may aid hematoma resolution by phagocytosing RBCs through erythrophagocytosis (Zhao et al., 2009; Chang C. F. et al., 2018; Jing et al., 2019). This detoxication process helps alleviate the brain injuries results from secondary detrimental process such as neuroinflammation and oxidative stress. Increasing number of studies have investigated the mechanistic signaling and beneficial effects of erythrophagocytosis in ICH (Chang C. F. et al., 2018; Chang et al., 2020; Xu et al., 2020). However, this endogenous erythrophagocytosis tends to be incomplete, and hemolytic products-triggered brain damage remains common and detrimental to recovery after ICH. Thus, enhancing endogenous erythrophagocytosis is an important strategy for ICH treatment.

In this review, we highlight the therapeutic values of targeting erythrophagocytosis in ICH. Firstly, we introduce the neurotoxicity of hematoma and the role of M φ-mediated

erythrophagocytosis in hematoma clearance and ICH recovery. Then, we describe the dynamic process of phagocytosis, focusing on the essential membrane receptors in M φ. We further depict how erythrophagocytosis may be modulated by microenvironmental factors, including inflammatory cytokines and oxidative stress. Finally, we summarize the critical nuclear factors regulating erythrophagocytosis that could serve as druggable targets. The aim is to inform future pre-clinical and clinical studies on accelerating hematoma resolution as a means of improving patient outcomes in ICH.

NEUROTOXICITY OF HEMATOMA

The extravasated blood plays a critical role in ICH pathology. In acute ICH, the blood cells and other blood components from the ruptured vessels rapidly accumulate in the brain parenchymal, forming a hematoma with mass effect that can destroy the cerebral architecture (mass effect) (Xi et al., 2006). Subsequently, hemolysis occurs, releasing neurotoxic degradation products (hemoglobin, heme, and iron). These substances could cause detrimental effects on brain tissue (**Figure 1**; Wagner et al., 1996; Nakamura et al., 2005; Xue and Yong, 2020):

- **Inflammation** is represented by the rapid recruitment of immune cells and the inflammatory cytokines in perihematomal regions (Loftspring et al., 2009; Zia et al., 2012). The hemolysis products, especially hemoglobin and heme, may function as the ligands of Toll-like receptor 4 (TLR-4), which could activate proinflammatory M φ and elevate the levels of proinflammatory cytokines (Figueiredo et al., 2007; Kwon et al., 2015; Lan et al., 2017).
- **Oxidative stress** is featured by the accumulation of reactive oxygen species (ROS), which oxidize DNA, protein, and lipid, causing tissue damage (Marnett et al., 2003). Iron, generated from the heme degradation, can catalyze the well-studied Haber-Weiss reaction, yielding overwhelming ROS and resulting in oxidative stress (Wu et al., 2003; Nakamura et al., 2005).
- **Edema** develops as early as hours and peaks at a range of 10-20 days in patients after ICH (Xi et al., 2006). At the early phase (< 3 days), thrombin and serum proteins extruded from the hematoma are the leading cause of edema. At the later stage (> 3 days), the hemolysis products precipitate delayed edema (Wagner et al., 1996; Urday et al., 2015). Of note, the TLR-dependent inflammation by hemoglobin and heme, as well as the Matrix metallopeptidase 9 (MMP-9) activation and the oxidative stress by iron, could compromise blood-brain barrier integrity and aggravate edema (Katsu et al., 2010; Urday et al., 2015).

Post-ICH hematoma expansion and rebleeding occur commonly in patients, suggesting a continuous enlargement of blood burden within the brain (Brott et al., 1997; Morgenstern et al., 2001). To counteract it, erythrophagocytosis by M φ occurs at both the edge and the center of hematoma, resulting in hematoma reduction (Cao et al., 2016). Timely clearance of hematoma can maintain homeostasis in cerebrovascular units and allows neurological recovery after ICH (Zhao et al., 2009). As such, shrinking hematoma via boosting the endogenous removal of RBCs, the source of hemolysis, has attracted increasing scientific interest in the past decade.

MACROPHAGE/MICROGLIA: THE MAJOR CELL PERFORMING ERYTHROPHAGOCYTOSIS

At onset, ICH rapidly recruits microglia, neutrophil, monocytes, and T lymphocytes, successively (Xue and Yong, 2020). For hematoma clearance, microglia and monocyte-derived macrophage are 'professional' phagocytes uptaking the damaged cells, including RBCs, in ICH (Xu et al., 2020; Li Q. et al., 2021). Though some 'non-professional' cells, such as endothelial cells, are also involved in erythrophagocytosis in cerebral microbleeds, their roles in ICH remain unknown (Chang R. et al., 2018). Microglia are the brain resident macrophages, mediating diverse functions critical to brain development and injury, such as synaptic pruning and phagocytosis (Paolicelli et al., 2011). For macrophage, their precursor cells – monocyte – are thought to enter the brain as a component of blood at ICH onset while later migrate into the brain via cell adhesion pathways (Engelhardt, 2008; Mracsko et al., 2014). These peripheral monocytes might undergo *in situ* differentiation into mature macrophages in the ischemic brain (Gliem et al., 2012; Miro-Mur et al., 2016). Interestingly, a recent study found that skull and vertebral bone marrow also supplied monocytes, which infiltrated the brain border (e.g., the dura matter) and developed into macrophages in inflamed brains (Cugurra et al., 2021). Given the limited evidence on ICH, a review on the dynamic infiltration of monocyte-derived macrophage in the ischemic brain might shed light on future studies (Han et al., 2020). Moreover, perihematomal M φ might follow the route of an available scaffold - white matter fibers – and migrate into the hematoma core, aiding the hematoma clearance (Chen et al., 2021). In addition to the white matter, molecules from the scar tissue are also considered able to support M φ activity, including phagocytosis, in brain injuries (Rolls et al., 2009).

Notwithstanding the diverse functions of macrophage and microglia observed in other types of strokes, most ICH studies could not distinguish macrophage from microglia due to the obstacles in differentiating between the two cell types *in vivo* (Zarruk et al., 2018). Fortunately, with the help of more specific cell markers (e.g., Tmem119 for microglia) and multi-channel flow cytometry (Bennett et al., 2016; Li et al., 2018), some researchers had begun to study the two cell types separately in ICH (Chang C. F. et al., 2018; Li Q. et al., 2021). Due to the inconsistent gating strategy applied by these two studies, it remains inconclusive as to how the role of macrophage differs from that of microglia. Therefore, in this review, we use M φ to denote the two cell types except when discussing studies that clearly distinguish between the two. **Figure 2** summarizes the key findings on erythrophagocytosis after ICH.

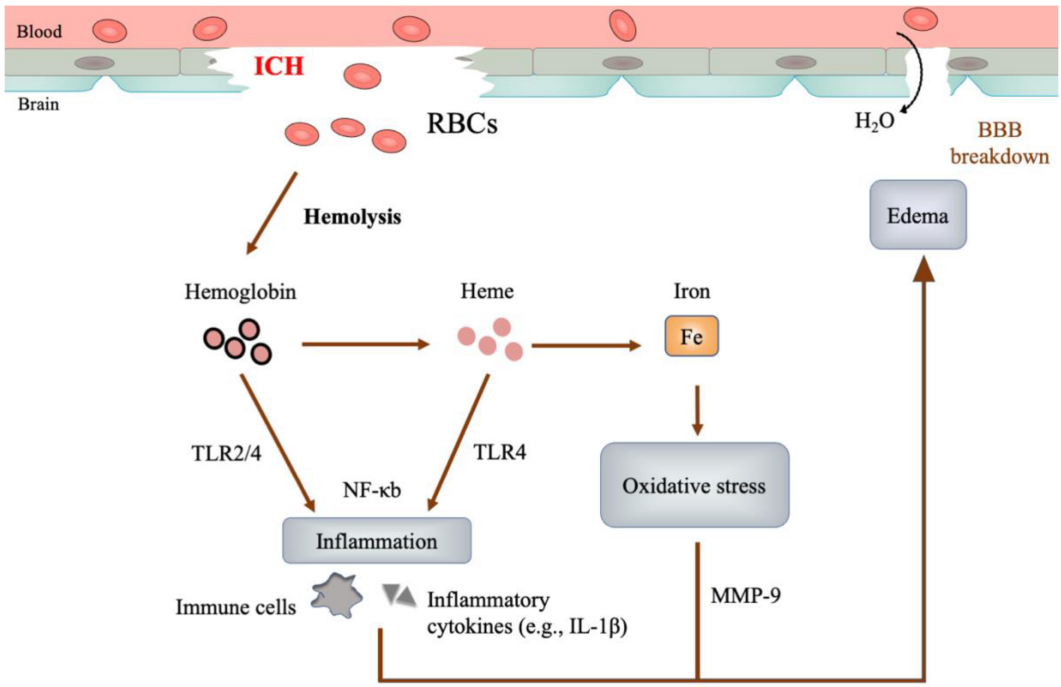

FIGURE 1 | Neurotoxicity of hematoma. After ICH, red blood cells (RBCs) would undergo hemolysis and degrade into hemoglobin, heme and iron consequently. These hemolysis products mediate cascades of inflammation, oxidative stress, and perihematomal edema, causing detrimental injury to the brain.

MEMBRANE RECEPTORS INITIATE ERYTHROPHAGOCYTOSIS

Exogenous stimulus, such as inflammation, could impair erythrocytes integrity and predispose them to become apoptosis-like cells (Lang et al., 2014). The apoptotic erythrocytes are then recognized and phagocytosed by M φ, a process termed efferocytosis (Trahtemberg and Mevorach, 2017). Erythrophagocytosis in ICH is a type of efferocytosis. Blocking the M φ receptors for recognizing the apoptotic markers can impair the erythrophagocytosis and impede hematoma resolution. Efficient efferocytosis is important for tissue homeostasis by reducing exposure to toxic components of hemolysis and self-antigens which can substantially induce tissue damage and autoimmune response (Sisirak et al., 2016; Herzog et al., 2019). In ICH, efficient efferocytosis leads to controlled clearance of damaged erythrocyte before injuries are inflicted by uncontrolled hemolysis (Chang C. F. et al., 2018).

As a type of efferocytosis, erythrophagocytosis is a highly orchestrated process which can be separated into four consecutive steps (Hochreiter-Hufford and Ravichandran, 2013; Doran et al., 2020):

(1) sensing: dying cells release "find-me" signals to attract phagocytes;
(2) recognition: aged or abnormal erythrocytes (apoptotic erythrocytes) externalize the "eat-me" signals, such as phosphatidylserine (PtdSer) (de Back et al., 2014; Klei et al., 2017); phagocytes upregulate specific surface receptors (e.g., CD36 and TAM family) to recognize the "eat-me" signal on the dying cells; interestingly, apoptotic erythrocytes also express the "don't eat me" signal, CD47, repelling M φ from efferocytosis (Ni et al., 2016); **Table 1** summarizes the phagocytosis-related receptors by RBCs.
(3) ingestion: phagocytes initiate cytoskeleton rearrangement to facilitate internalization of the dying cells;
(4) digestion and response phase: phagocytes process the corpses and produce anti-inflammatory mediators to suppress inflammatory response.

Of note, the current literature on erythrophagocytosis mainly focuses on the Step 3; the other steps remained largely unexplored in the context of ICH. Current studies have shown the therapeutic value of targeting the "don's eat me signal" from erythrocytes (CD47) and the surface phagocytosis receptors from M φ (CD36 and TAM family).

CD47

CD47 is an integrin-associated transmembrane protein ubiquitously expressed in many cell types including erythrocytes (Olsson et al., 2006). It regulates immune cell infiltration, phagocytosis, and the production of proinflammatory mediators and trophic factors by interacting with integrins and extracellular ligands (Brown and Frazier, 2001; Zhang et al., 2015). For phagocytosis, CD47 on erythrocytes acts as a "don't eat me" signal to block phagocytosis by binding to signal regulatory protein α (SIRPα) on macrophage (de Back et al., 2014). Preclinical studies have demonstrated the role and therapeutic

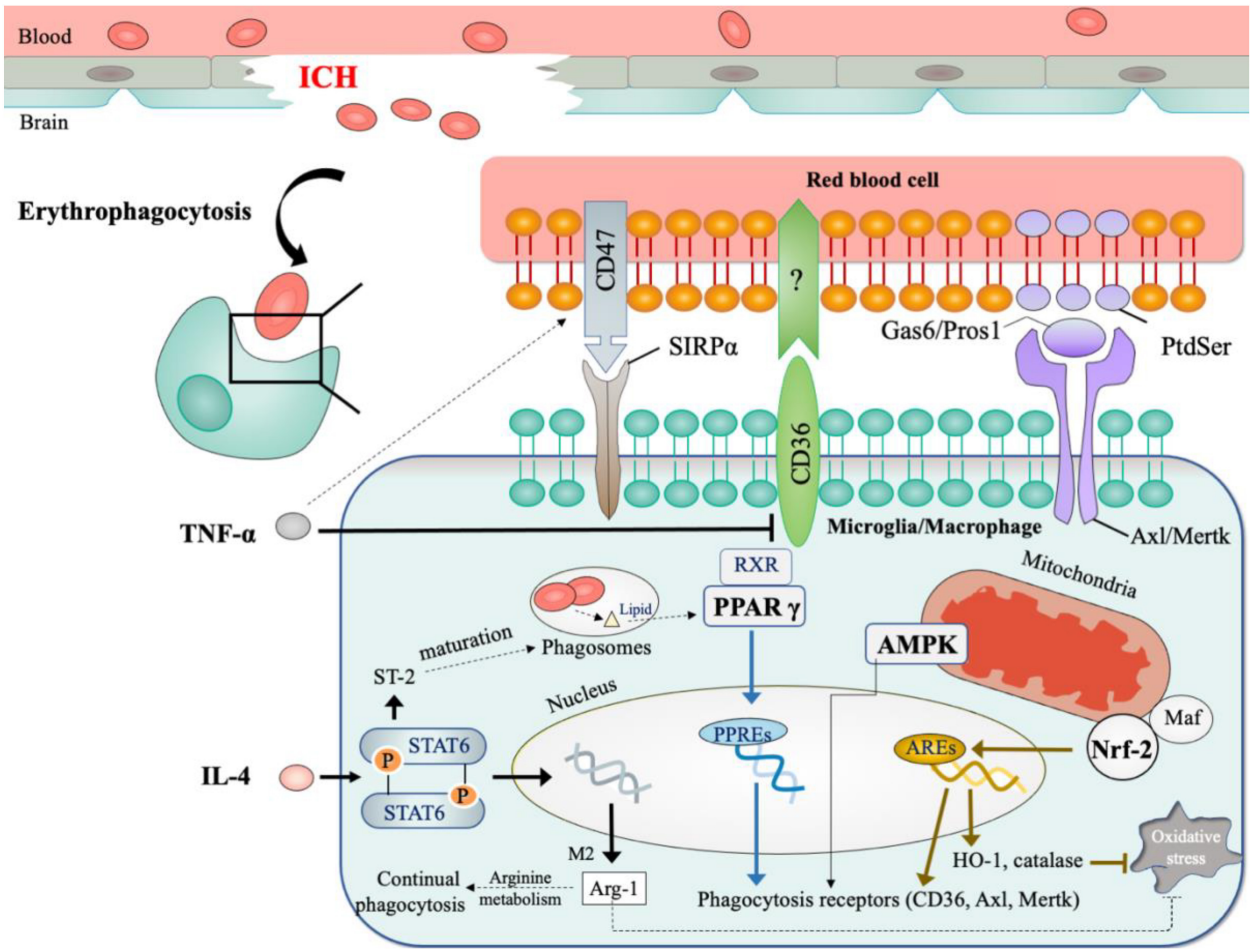

FIGURE 2 | Mechanisms of erythrophagocytosis in ICH. **(1)** Signals for recognition include: CD47 on erythrocytes serves as the inhibitory signal for erythrophagocytosis; CD36, Axl, and Mertk on phagocytes act as scavenger receptors for clearing erythrocytes. **(2)** Microenvironment factors (inflammatory cytokines and oxidative stress) shape the phagocytosis by M φ. **(3)** The critical modulators for phagocytosis include PPAR γ, Nrf2, AMPK. *The dotted lines showed results from other diseases models.

value of CD47 in ICH. The perihematomal level of CD47 increases within hours but decreases subsequently, accompanied by M φ infiltration and erythrophagocytosis (Zhou X. et al., 2014; Cao et al., 2016). This explains the reverse correlation between CD47 and M φ-mediated immune response. Intracranial injection of CD47 knock-out blood resulted in quicker hematoma resolution and milder brain edema (Ni et al., 2016). This effect was reduced by intracranial injection of clodronate liposomes, a specific phagocytes depletion drug. In all, CD47 may serve as an inhibitory signal in M φ-mediated hematoma resolution. Inspiringly, CD47 blocking antibody significantly enhanced hematoma removal after ICH, rendering CD47 a promising and draggable target in ICH treatment (Jing et al., 2019; Tao et al., 2020).

TABLE 1 | Receptors on the rede blood cells for erythrophagocytosis.

Function	Receptors	References
"Don't eat-me" signal	CD47	ICH: Ni et al., 2016; Jing et al., 2019; Tao et al., 2020 Other diseases: Oldenborg, 2004
"Eat-me" signal	Phosphatidylserine (PtdSer)	ICH: Chang C. F. et al., 2018 Other diseases: Boas et al., 1998; Sun et al., 2021
	Band 3 clustering	Other diseases: Klei et al., 2017
	Calreticulin	Other diseases: Gardai et al., 2005; Nilsson et al., 2012

CD36

Upon activation by "eat-me" signals, macrophage upregulates several membrane scavenger receptors to direct the ingestion process, including CD36 (Silverstein and Febbraio, 2009). The upregulated precursor intracellular CD36 undergoes glycosylation in the endoplasmic reticulum, followed by transportation to the cell membrane (Alessio et al., 1996; Roszer, 2017). Membrane CD36 cooperates with the αvβ3 receptor to engage with the eat-me signal thrombospondin (TSP) on

apoptotic cells, inducing the internalization and digestion of target cells (Savill et al., 1990, 1992).

In ICH, CD36-mediated apoptotic cells clearance is essential for hematoma resolution. Its transcription was upregulated in the erythrocytes-treated microglia culture as well as the perihematomal region after ICH (Zhao et al., 2007b). Both genetic deletion and antibody blocking of CD36 impeded the phagocytosis of erythrocytes by microglia (Zhao et al., 2007b; Fang et al., 2014). As M φ-mediated removal of erythrocytes is required for clot clearance, the roles and therapeutic values of CD36 in hematoma development has attracted considerable attention. For example, CD36 knock-out mice was found to have slower hematoma resolution and aggravated deficits when compared to wild-type mice after ICH (Fang et al., 2014). What's more, the same study also found that patients with CD36 deficiency showed impaired hematoma resolution and poorer clinical outcome. Consistently, the upregulation of CD36 generates faster speed of erythrophagocytosis and hematoma resolution (Zhao et al., 2007b; Flores et al., 2016; Wang Y. et al., 2018). Enhancing the upstream regulatory mechanism of CD36, including peroxisome proliferator-activated receptor γ (PPAR γ) and the nuclear factor erythroid 2–related factor 2 (Nrf2), is a potential approach to promoting CD36-mediated hematoma clearance.

Axl/Mertk

TAM – namely, Tyro3, Axl and Mertk – is a group of receptor tyrosine kinases functioning as macrophage scavenger receptor (Lemke and Burstyn-Cohen, 2010). Tyro3 is more highly expressed on neuron rather than on M φ in the brain, whereas Axl and Mertk are more abundant on M φ which makes them more relevant to erythrophagocytosis in ICH (Fourgeaud et al., 2016; Chang C. F. et al., 2018). Axl and Mertk participate in cell survival, migration, and phagocytosis by engaging with the ligands, growth arrest specific 6 (Gas6) and protein s (Pros1) (Shafit-Zagardo et al., 2018). Gas6/Pros1 acts by bridging Axl/Mertk with the "eat-me" signal (PtdSer) on apoptotic cells, initiating the phagocytosis process (Tondo et al., 2019).

In ICH, Axl, and Mertk might be required for erythrophagocytosis. In a murine model, the transcriptional level of Axl, Mertk and Gas6 ligand are increased within 24 h after ICH (Tong et al., 2017); deficiency of Axl/Mertk resulted in defective erythrophagocytosis by macrophage in ICH (Chang C. F. et al., 2018). However, results from double knockout Axl/Mertk were inconclusive as to whether both or either of them was responsible for these findings, it is likely that Mertk and Axl may have diversified functions, with Mertk playing important roles in homeostasis and Axl being more involved in inflammatory conditions (Zagorska et al., 2014). Further studies are necessary to define whether Axl or Mertk alone is indispensable for the removal of erythrocytes in ICH. Interestingly, Axl/Mertk also modulates M φ phenotypes and alleviates neuroinflammation in addition to its effects in phagocytosis. In ICH, Toll-like receptors (TLRs) polarizes M φ toward proinflammatory phenotype (M1), as opposed to anti-inflammatory (M2) phenotype (Lan et al., 2017). Axl/Mertk can activate the TLRs suppressors, SOC1 and SOC3, thereby inhibiting M1-like M φ activation and suppressing inflammatory response in a range of disease including in ICH (Rothlin et al., 2007; Tong et al., 2017; Chang C. F. et al., 2018; Wu et al., 2021).

From a therapeutic standpoint, exogenous ligands (e.g., recombinant Gas6) can be used to target Axl/Mertk-mediated beneficial effects. In inflammatory conditions, Axl/Mertk is prone to be cleaved from the cell membrane, generating the soluble but unfunctional Axl/Mertk (sAxl/sMertk). sAxl/sMertk competitively occupies the endogenous ligands (Gas6 and Pros1), resulting in the lack of ligands for regulating homeostasis in inflammatory conditions (Cai et al., 2016; Chang C. F. et al., 2018). Therefore, exogenous ligands, such as recombined Gas6 can compensate this insufficiency, serving as the druggable target in augmenting the effects of TAM (Di Stasi et al., 2020). For example, recombined Gas6 promoted inflammation resolution via Axl-dependent manner in experimental ICH (Tong et al., 2017). However, whether the exogenous ligands could facilitate the Axl/Mertk-mediated hematoma clearance warrants further investigation.

MICROENVIRONMENTAL FACTORS ORCHESTRATE THE ERYTHROPHAGOCYTOSIS

Efficient phagocytosis requires phagocytes to digest multiple cells continuously, especially in acute inflammation where the apoptotic cells-to-phagocytes ratio is high (Doran et al., 2020). The significance of erythrophagocytosis in this context also depends on whether the M φ can remove such a tremendous amount of erythrocyte before irreversible hemolytic-induced brain injury commences (Bulters et al., 2018). Of relevance are microenvironmental factors, including neuroinflammation and oxidative stress, that may alter phagocytic function and therefore serve as viable therapeutic targets.

Inflammatory Mediators

The correlation between M φ phenotypes and phagocytosis is complex. Erythrophagocytosis skewed M φ toward the M2 phenotype, which reciprocally facilitated the removal of dying cells (Bensinger et al., 2009; Roszer, 2017; Chang C. F. et al., 2018). It possibly explains for the protective roles of M2-M φ observed in both preclinical and clinical studies of stroke (Chernykh et al., 2016; Min et al., 2016; Bai et al., 2020). The cytokines involved in M1- and M2-M φ activation could modulate erythrophagocytosis in ICH.

Interleukin-4 (IL-4) is the canonical activator of signal transducer and an activator of transcription 6 (STAT6), which is essential to the activation of M2 phenotype (Lawrence and Natoli, 2011). In ICH, exogenous IL-4 activated STAT6 and enhanced erythrophagocytosis in animal after ICH (Xu et al., 2020). The article revealed two potential mechanisms. Firstly, IL-4/STAT6 was observed to upregulate CD36, the scavenger receptor initiating phagocytosis. This may result from the direct binding of STAT6 to the promotor regions of CD36 gene (Szanto et al., 2010). Secondly, the study proved IL-1 receptor like 1 (ST2) was required for IL-4/STAT6-mediated clearance of

erythrocyte. As ST2 promoted phagosome maturation (Buckley et al., 2011), it is likely that IL-4/STAT6 regulated phagosome infusion and thus enhancing phagocytosis in ICH. It is important to note that IL-4/STAT6 transcriptionally upregulates the anti-inflammatory cytokines, which is likely to contribute to IL-4/STAT6 mediated pro-phagocytosis effect. For example, Arg1 is required for STAT6-mediated pro-phagocytosis by M φ in ischemic brain (Cai et al., 2019). The mechanism may link to the enzymatic role of Arg1 in arginine metabolism, which increased the M φ communication and resulted in continual phagocytosis (Yurdagul et al., 2020).

In the contrary, cytokines involved in M1-M φ activation are potential to inhibit the removal of apoptotic cells. Amongst, TNF-α is the potential cytokine for targeting. TNF-α stimulates the M1 phenotype and is also regarded as a marker of M1-M φ (Lan et al., 2017). In ICH, TNF-α downregulated CD36 in microglia and impaired its function in erythrophagocytosis (Fang et al., 2014). What's more, TNF-α upregulated the "don't eat-me" signal CD47 in vascular smooth muscle cells and renders less phagocytosis (Kojima et al., 2016). Thus, reverse the inhibition of TNF-α in erythrophagocytosis may be a potential approach to promote hematoma clearance. It is important to note that TNF-α inhibitors have been approved in the treatment of many diseases, such as ankylosing spondylitis and Crohn disease (Gerriets et al., 2021). However, whether these inhibitors can facilitate erythrophagocytosis and perform therapeutic effects remain unproved in ICH.

Oxidative Stress

Oxidative stress is one of the major contributors toward pathological injury in ICH. The source of oxidative stress in ICH includes hemolytic products, mitochondria dysfunction, and M φ (Hu et al., 2016). Hemolytic products, particularly iron, catalyze a sequence of reactions known as the Haber-Weiss reaction, yielding highly reactive oxygen species (ROS) (Xiong et al., 2014). Mitochondria dysfunction allows abnormal leakage of electrons from electron transport chain, overwhelming antioxidant system and leading to accumulation of ROS. M φ also generates ROS by inhibiting oxidative metabolism (Zhou Y. et al., 2014) and processing large quantities by-products from the cell corpse (Splettstoesser and Schuff-Werner, 2002).

Oxidative stress have profound effects on M φ (Vernon and Tang, 2013). In M φ, ROS is essential for bactericidal effects, whereas it can kill the phagocytes when the levels becomes overwhelming (Morrow et al., 2007; van-Charvet et al., 2010). What's more, the sudden onset of oxidative loading allows the transcription nuclear factor (NF)-κB to transfer from cytoplasm to nucleus, initiating the transcription of proinflammatory mediators including TNF-α (Brigelius-Flohe and Flohe, 2011; Sivandzade et al., 2019). As previously discussed, TNF-α is potential to block phagocytosis. Moreover, oxidants may alter cell structures or destroy the signals required for phagocytes (Anderson et al., 2002; Schrijvers et al., 2005). In all, oxidative stress may be a detrimental factor dampening normal function of M φ and compromising the removal of apoptotic cells.

Thus, the restriction of oxidative stress serves as a potential approach to improve M φ viability and facilitate erythrophagocytosis in ICH. To achieve this, strengthening the self-defense ability of M φ is a reasonable direction. To counteract the oxidative stress, M φ has developed the self-defense mechanisms with the mainstay represented by Nrf-2 (Virag et al., 2019). Treating microglia with the activator of Nrf-2 showed faster erythrophagocytosis speed (Zhao et al., 2015a). The roles of other self-defense mechanisms warrants more investigation. Besides, some substances that could sequestrates iron, the source of ROS, are also potent targets in facilitating erythrophagocytosis, such as lactoferrin. As a glycoprotein of transferrin family, lactoferrin was found to limit oxidative stress and promote microglia-mediated phagocytosis (Actor et al., 2009; Zhao et al., 2021). Lastly, Arg1, the M2 marker which is essential for arginine metabolism, could alleviate the oxidative stress by competing with inducible nitric oxide synthase (iNOS) for the arginine substrate (Corraliza et al., 1995; Morris and Jr, 2007). Given the role of arginine metabolism in phagocytosis (Yurdagul et al., 2020), this somehow reveals the internal links among inflammation, metabolism and oxidative stress in modulating phagocytosis.

UPSTREAM REGULATORS FOR ERYTHROPHAGOCYTOSIS

To initiate the erythrophagocytosis, the scavenger receptors in M φ drive the cell-to-cell interaction. These receptors are under controlled by by liver X receptor (LXR) and PPARs (α, β/δ and γ isotypes) (Roszer, 2017). Amongst, PPAR γ is the most studied regulator in ICH. Moreover, modulating the microenvironment factors shapes the function of M φ and enhances the phagocytosis efficacy. Nrf-2, as a powerful regulator of oxidative stress, has shown great therapeutic value in facilitating hematoma resolution and treating ICH. Last, energy metabolism is a critical component of efficient phagocytosis (Jiang et al., 2013). The regulatory role of AMPK, the energy sensor, in phagocytosis was also reviewed.

PPAR γ

PPAR γ transcriptionally regulates genes that are critical to brain tissue repairment (Cai et al., 2018). Upon activation, PPAR γ heterodimerizes with retinoid X receptor (RXR) and subsequently engages with the conserved DNA sequences, namely peroxisome proliferator response elements (PPREs). PPREs is located in the promoter regions of cytoprotective genes, including the scavenger receptors and catalase, with the latter is essential to minimize oxidative injury (Cai et al., 2018). Thus, PPAR γ directly modulates phagocytosis and alleviates the oxidative stress, rendering it to be the most potent target in driving erythrophagocytosis and hematoma resolution in ICH.

In ICH, the activation of PPAR γ could upregulate the scavenger receptors and facilitate erythrophagocytosis. Generally, the scavenger receptors for clearing apoptotic cells are regulated by liver X receptor (LXR) and PPARs (α, β/δ and γ isotypes) with various combination patterns (Roszer, 2017): CD36 solely by PPAR γ, Axl by PPARs, and Mertk by PPARs and LXR. These patterns reflected the indispensable role of PPARs, especially

PPAR γ, in regulating phagocytosis, which was supported by a number of ICH studies. Zhao et al. were the first to demonstrate that PPAR γ agonist could upregulate CD36 in microglia, thereby facilitating erythrophagocytosis *in vitro* (Zhao et al., 2007b). This pioneering work further proved that PPAR γ activation enhanced hematoma resolution and functional recovery in ICH. It paved the way for targeting M φ-mediated hematoma resolution in ICH (Zhao et al., 2009). Subsequently, activation of PPAR γ was observed to upregulate other scavenger receptors, Axl and Mertk, and expedite hematoma resolution in ICH (Zhuang et al., 2020). Moreover, the effects of PPAR γ activation in erythrophagocytosis were also verified in other types of hemorrhagic stroke (Wu et al., 2011; Flores et al., 2016). By activating PPAR γ, several pharmacological agents were found to confer protective effects in experimental ICH (Wang Y. et al., 2018; Fu et al., 2020; Zhao et al., 2020; Zhuang et al., 2020). Taken together, PPAR γ activation is one of the mainstays of phagocytosis modulator in facilitating hematoma clearance after ICH.

In addition, PPAR γ activation also alleviates inflammation and oxidative stress in ICH. For instance, PPAR γ agonists reduce proinflammatory TNF-α and IL-1β expression (Zhao et al., 2007b). Mechanistically, PPAR γ could prevent their nuclear receptor corepressor from being cleaved from the genes of TNF-α and IL-1β (Ghisletti et al., 2007; Jennewein et al., 2008). For oxidative stress, PPAR γ reciprocally interacted with Nrf-2 and synergistically inhibited NFκB mediated-oxidative stress (Zhao et al., 2015c; Luo et al., 2021). Thus, PPAR γ activation may also alleviate the inflammation and oxidative stress, producing a phagocytosis-friendly microenvironment for M φ.

To magnify PPAR γ-mediated protective effects, both the endogenous and exogenous stimulators are viable targets. PPAR γ is initially activated by the cell corpse-derived lipids in M φ when phagocytosis commences (Kourtzelis et al., 2020). Interestingly, the PPAR γ activity of M φ could also be modulated by other engulfed elements in addition to apoptotic cells. For instance, M φ could uptake the functional mitochondria and humanin released by astrocytes, which in turn upregulated the expression of PPAR γ and its mediated phagocytosis in ICH (Jung et al., 2020). In addition, activating PPAR γ-targeted genes and PPAR γ-mediated hematoma resolution could also be achieved by activating its transcription partner, i.e., RXR (Chang et al., 2020; Ting et al., 2020). Lastly, a clinical trial using pioglitazone, a known PPAR-gamma agonist approved by FDA, has been conducted in ICH patients, aiming to reduce hematoma burden and improve outcomes (NCT00827892). Other nuclear receptors may also play a role in clearing apoptotic cells, such as LXR, retinoic acid receptor (RAR), and glucocorticoid receptor (GR) (Roszer, 2017). Their roles in ICH warrants further investigation.

Nrf-2

Nrf-2 is the principal transcriptional factor protecting cells from endogenous and exogenous stress (Kensler et al., 2007). Upon activation, Nrf2 heterodimerizes with the Maf family and binds to the antioxidant response elements (AREs) located in the regulatory regions of cytoprotective genes. These genes include antioxidant agents, such as catalase, HO-1, SOD, etc. (Zhao and Aronowski, 2013). Growing evidence supports the beneficial roles of Nrf2 in ICH. For instance, the absence of Nrf2 in rodents resulted in worse oxidative injury and more neurological deficits while the activation of Nrf2 reversed these effects in ICH (Wang et al., 2007; Zhao et al., 2007a; Iniaghe et al., 2015; Cheng et al., 2021).

Nrf-2 activation has also been proved to enhance the erythrophagocytosis by microglia and hematoma clearance in ICH (Zhao et al., 2015a). The effect of Nrf2 in promoting phagocytosis is partly CD36-dependent. The modulation of Nrf2 on CD36 may be PPAR γ-dependent or PPAR γ -independent, but this has not yet been confirmed (Ishii et al., 2004; Zhao et al., 2015c). Moreover, Nrf-2 regulates the genes responsible for detoxifying the blood products, including genes of ferritin, hemopexin and haptoglobin (Zhao and Aronowski, 2013; Deng et al., 2020; Liu et al., 2021) Nrf2 also regulates NF-κB pathway and alleviates inflammation in ICH, rendering targeting Nrf2 as a highly promising approach to augmenting hematoma resolution (Cheng et al., 2021). Indeed, agonists of Nrf2, such as sulforaphane, dimethyl fumarate and others, have been shown to accelerate hematoma reduction (Zhao et al., 2015a,b).

AMP-Activated Protein Kinase

The reprograming of energy metabolic pathways is an emerging hallmark of anti-inflammatory (M2) M φ (Devanney et al., 2020). Particularly, these anti-inflammatory cells exhibit increased mitochondria oxidative metabolism with much lower level of glycolysis, pinpointing the demand for efficient energy production for inflammation resolution and tissue repair. AMP-activated protein kinase (AMPK) attracts particular interest in fulfilling this energy demand of M φ (Jiang et al., 2013). As an intracellular sensor of the "fuel status", AMPK is activated by the drop of ATP-to-ADP ratio, which occurs in conditions like mitochondria inhibition, exercise, and starvation (O'Neill and Hardie, 2013; Herzig and Shaw, 2018). To counteract energy insufficiency, AMPK switches on catabolic activities to generates energy more efficiently while dampening anabolic process that consumes ATP. Given the intimate connection between cellular metabolism and immune response, the role of AMPK in macrophage functions has been reviewed (O'Neill and Hardie, 2013).

AMP-activated protein kinase activation has been reported to skew macrophage activation toward the M2 phenotype and enhance hematoma resolution in ICH (Vaibhav et al., 2018). Several *in vitro* studies also demonstrated that AMPK activation contributed to the M2 M φ polarization and phagocytosis (Wan et al., 2013; Jin et al., 2014; Kaiser et al., 2020; Seneviratne et al., 2020). Consistently, the absence of AMPK- α1, the sole isoform expressed in M φ, impaired the anti-inflammatory and prophagocytic effect of M φ in various brain disorders (Vaibhav et al., 2018; Lv et al., 2020).

The mechanisms underlying AMPK's pro-phagocytosis effect are complex. On the one hand, AMPK cooperates with the transcriptional regulators (PPAR γ and Nrf-2), modulating their downstream scavenger receptors including CD36 and Mertk

(Wan et al., 2013; Galvan et al., 2014; Wang Y. J. et al., 2018; Kaiser et al., 2020; Lv et al., 2020). On the other hand, AMPK promotes the organization of cytoskeleton and the acidification of endosomal-lysosomal system, facilitating the ingestion and digestion of apoptotic cells (Labuzek et al., 2010; Bae et al., 2011). In sum, AMPK activation favors inflammation resolution and might enhance apoptotic cells clearance in ICH.

Pharmacological agents, such as AdipoRon and CTRP3, also showed potential in driving AMPK-mediated augmentation on phagocytosis or inflammation resolution and functional recovery in preclinical ICH studies (Wang et al., 2016; Zheng et al., 2019). Several clinical trials on RIC-mediated AMPK activation and hematoma clearance are ongoing: NCT04757597, NCT04657133, NCT03930940 and NCT03481777. However, the direct evidence on the pro-phagocytotic effects of AMPK activation warrants verification in ICH.

FUTURE DIRECTIONS

Given the limited evidence available on ICH in this context, studies in other types of stroke could inspire the future research direction in ICH. Ischemic stroke is the main type of stroke and therefore has drawn greater scientific interest than other types of strokes. Regarding the phagocytosis of apoptotic cells (including injured RBCs and neurons), the underlying mechanisms appear to be similar and comparable between the two conditions. For example, the scavenger receptor CD36, which was widely studied in ICH, has also been proved to mediate phagocytosis in ischemic stroke (Woo et al., 2016). The phagocytosis effects of other molecules, such as Arg-1 and PPAR γ, were also reported in ischemic strokes (Cai et al., 2019; Zhang et al., 2019).

TABLE 2 | Merits and Demerits of M φ-mediated phagocytosis.

Effects of phagocytosis	Targets	Details of effects
Merits	Red blood cells	Reduce clot toxicity and Support the functional recovery Zhao et al., 2009; Chang C. F. et al., 2018
	Neurons	Reduce inflammatory cytokines Lecca et al., 2018; favor neurogenesis Sierra et al., 2010.
	Synapse	Favor remyelination Natrajan et al., 2015.
Demerits	Red blood cells	Ferroptosis of phagocytes Youssef et al., 2018
	Neurons	Neuron death Brown and Neher, 2014; delayed neuron loss Neher et al., 2013; degeneration of dopaminergic neurons Barcia et al., 2013;
	Synapse	Synapse loss Hong et al., 2016; demyelination Han et al., 2012; worse functional outcome Shi et al., 2021.

Lastly, metabolic modulation, the phagocyted targets and the dynamic picture of pathophysiologic response after initial erythrophagocytosis are also important areas for future investigations.

Better Understanding of the Metabolic Modulation in Erythrophagocytosis

One of the priorities of future studies was to explore the basic mechanisms of erythrocytes removal in the hemorrhagic brain. Although the basic biology of efferocytosis has been deeply studied, whether it could be applied to post-ICH erythrophagocytosis needs more investigation (Doran et al., 2020; Kourtzelis et al., 2020). Amongst, this review underlines the significance of defining the metabolic adaption of M φ which contributes to ongoing phagocytosis. Then, we could explore the potential targets for modulating this adaption to achieve efficient phagocytosis in ICH. Interestingly, the roles of metabolic modulation in phagocytosis might explain the neuroprotective effects of some approved agents. For instance, uncoupling protein 2 (UCP2), a mitochondria membrane protein, has been observed to improve neurological outcomes in stroke (Mattiasson et al., 2003; Nakase et al., 2007; Mehta and Li, 2009; Zhao et al., 2019). This protein maintained the membrane potential and supported continuous phagocytosis (Krauss et al., 2002, 2005; Park et al., 2011). However, whether UCP2 plays a role in facilitating phagocytosis in ICH is undefined.

Better Understanding of the Phagocyted Targets

Erythrophagocytosis shares similar mechanisms in phagocytosing different apoptotic cells. Thus, enhancing the erythrophagocytosis signals can also eliminate other apoptotic cells nearby (Galloway et al., 2019). In ICH, the hematoma usually contains unvital neurons due to mechanical force and/or neurotoxicity of hematoma (Xi et al., 2006). Historically, eliminating the dying brain cells and synapses was regarded as a beneficial process since it would prevent self-antigen exposure and establish new homeostasis in the brain (review by Galloway et al. (2019)). However, the adverse effects of eliminating apoptotic neurons have recently been demonstrated in several brain disorders, including Alzheimer's disease, multiple sclerosis, and strokes (Neher et al., 2013; Hong et al., 2016; Werneburg et al., 2020). The phagocytosis of "stressed-but-viable" neurons by M φ results in cell death, called "phagoptosis," which could lead to delayed neuron loss (Brown and Neher, 2012; Neher et al., 2013). In ICH, a recent study demonstrated that the Mertk-dependent phagocytosis of synapse worsened the neurological outcome in animals after stroke (Shi et al., 2021). **Table 2** outlines other adverse effects of phagocytosis which may be potentially contradictory to this review's central tenet and suggest the need for a more specific phagocytosis system. Moreover, a systemic upregulation of erythrophagocytosis might disturb the physical removal of RBCs by splenic red pulp macrophage, since it involves the same phagocytosis-relevant receptors (Klei et al., 2017). Thus, future studies need to search for a pro-phagocytotic drug with localized specificity.

CONCLUSION: ERYTHROPHAGOCYTOSIS AS A SPATIOTEMPORALLY DEVELOPING STORY

The foregoing review suggests that it is necessary to consider erythrophagocytosis in ICH as a spatiotemporally progressing event. There are mainly two concerns. Firstly, how could the M φ completely remove the damaged RBCs in a situation where the latter vastly outnumbers the former? Moreover, the population of M φ might be further reduced by the primary injury or the intracellular iron toxicity (van-Charvet et al., 2010; Youssef et al., 2018). To prevent the overwhelming of phagocytotic machinery, the number of M φ must be regulated to obtain sufficient phagocytotic capacity (Morioka et al., 2019). Therefore, therapies aimed at maximizing the number of functional phagocytes could improve RBC removal and should become a focus of future research. Inspiringly, this idea of introducing more functional M φ has recently been found to facilitate animal recovery from traumatic brain injury (Li Z. et al., 2021). Another layer of complexity lies in the fact that these immune cells tend to execute time-dependent effects in cerebrovascular injury, i.e., worsening the brain injury at acute phase but repairing the tissue at later stage (Mastorakos et al., 2021). One should therefore also take into consideration the optimal therapeutic time window when attempting to bring these cells into the brain. Secondly, what is the fate of M φ after engulfing RBCs in ICH? Although M φ could digest RBCs into the degradation products within themselves, could they process the blood components, especially iron, in a non-toxic way? Microglia contains the system for transporting and storing the iron in the normal brain, which is critical to the brain iron cycle and homeostasis (Winn et al., 2020). However, it remains unknown whether microglia could maintain its own homeostasis rather than undergoing iron-dependent cell death, namely "ferroptosis" in ICH (Youssef et al., 2018). Further studies are needed to achieve a better understanding of erythrophagocytosis and translate its therapeutic value to clinical practice.

AUTHOR CONTRIBUTIONS

JL and ZZ wrote the manuscript. GL supervised the drafting and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Neuroprotective Effect of Physical Activity in Ischemic Stroke: Focus on the Neurovascular Unit

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Cerebral ischemia is one of the major diseases associated with death or disability among patients. To date, there is a lack of effective treatments, with the exception of thrombolytic therapy that can be administered during the acute phase of ischemic stroke. Cerebral ischemia can cause a variety of pathological changes, including microvascular basal membrane matrix, endothelial cell activation, and astrocyte adhesion, which may affect signal transduction between the microvessels and neurons. Therefore, researchers put forward the concept of neurovascular unit, including neurons, axons, astrocytes, microvasculature (including endothelial cells, basal membrane matrix, and pericyte), and oligodendrocytes. Numerous studies have demonstrated that exercise can produce protective effects in cerebral ischemia, and that exercise may protect the integrity of the blood-brain barrier, promote neovascularization, reduce neuronal apoptosis, and eventually lead to an improvement in neurological function after cerebral ischemia. In this review, we summarized the potential mechanisms on the effect of exercise on cerebral ischemia, by mainly focusing on the neurovascular unit, with the aim of providing a novel therapeutic strategy for future treatment of cerebral ischemia.

Keywords: exercise, cerebral ischemia, neurovascular unit, blood-brain barrier, mechanism

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INTRODUCTION

Research on neuroprotective therapy for cerebral ischemia has been a hot topic worldwide. Ischemic tolerance refers to a short period when patients experience cerebral ischemia before the onset of a severe ischemic stroke, thus enhancing the tolerance of brain tissue to the subsequent ischemic injury (Wang et al., 2015; Hao et al., 2020; Daniele et al., 2021). This suggests that preischemic interventions can induce tolerance to secondary severe injury after cerebral ischemia. Triggers of various types of mild stressors or stimuli (i.e., surgical, remote ischemic preconditioning, exercise, acupuncture, and pharmacological methods) induce adaptive endogenous tolerance to ischemia injury by activating a multitude cascade of molecules (Thushara Vijayakumar et al., 2016; Vinciguerra et al., 2018; Hao et al., 2020). As a preventive method, exercise is accepted relatively easily by patients. As a safe preintervention approach, exercise actually has less adverse effects, which allows it to be more suitable for clinical application. A large number of studies have demonstrated that exercise before cerebral ischemia, also known as exercise preconditioning, can induce ischemic tolerance, thereby effectively alleviating brain injury and promoting functional recovery after an ischemic stroke (Zhang et al., 2011; Dornbos and Ding, 2012;

Sakakima, 2019). Therefore, elucidating the neuroprotective mechanisms and benefits of exercise and exercise intervention, pre- and post-stroke, will encourage patients with high-risk factors of ischemic stroke to actively exercise for prevention and treatment of stroke. The close connection between microvascular endothelial cells and surrounding astrocytes plays an important role in maintaining an intact neurovascular coupling. Regulation of the microvascular basal matrix membrane, activation of endothelial cells, and changes in astrocyte adhesion can directly affect the transmission of information between the microvasculature and neurons.

A neurovascular unit (NVU), which was initially proposed by Lo et al. (2003) in the early 2000s, is a structural and functional unit of the central nervous system. It emphasizes the dynamic interactions among endothelial cells, astrocytes, pericytes, basal membrane, microglia, neurons, and the extracellular matrix and the importance of such interactions in the pathophysiology of stroke (Steliga et al., 2020; Moon et al., 2021; Naranjo et al., 2021; Wang et al., 2021). The concept of NVU not only provides a platform for understanding central nervous system injury, but also provides a possibility for both clinically successful and timely intervention of brain injury (Yu et al., 2020; Zhou et al., 2020). Exercise, as a preconditioning approach, has been discovered to play a neuroprotective role by interfering with NVU (Dornbos and Ding, 2012; Murugesan et al., 2012; Wang et al., 2014; Laitman and John, 2015). This review is a summary of the effect and potential mechanism of exercise on NVU, to provide a theoretical basis for elucidating the neuroprotective mechanism of exercise, and its further application in cerebral ischemia.

CEREBRAL ISCHEMIA AND NEUROVASCULAR UNIT INJURY

Cerebral Ischemia and Neuronal Injury

The mechanism of neuronal injury caused by cerebral ischemia is complex. Neuronal apoptosis, after cerebral ischemia, is regulated by many different events and may develop by initiating the internal cellular death mechanism. Many studies have discovered that cerebral ischemia can induce cell apoptosis, and most apoptotic cells are neurons, with a few glial cells and vascular endothelial cells (Chan, 2004; Peng et al., 2004; Radak et al., 2017). Delayed neuronal apoptosis is a process that is different from necrosis, causing neuronal loss and occurring after cerebral ischemia (Huang et al., 2021; Kang et al., 2021). Regional expression of apoptosis-related proteins, such as caspase family, Bcl-2, and Bax, are involved in the occurrence of neuronal apoptosis after the onset of cerebral ischemia (Zhou et al., 2003; Long et al., 2022). It has been reported that brain ischemia for 10 min not only causes neuronal apoptosis in rats, but permanent cerebral ischemia for 60 min causes neuronal apoptosis (O'Sullivan et al., 2007). The number of apoptotic neurons fluctuates in a curvilinear manner over time, indicating that, during the process of cerebral ischemia, the main factors that determine neuronal apoptosis are the severity of ischemia and susceptibility of neurons. Ischemia causes energy failure, leading to increased intracellular sodium and calcium, cell lysis, and neuronal apoptosis. Studies have shown that there are many apoptotic neurons within the ischemic area at 0.5 h after the development of cerebral ischemia (Radak et al., 2017). Apoptotic cells are scattered in the preoptic region, striatum, and cerebral cortex. The number of apoptotic neurons reached a peak at 12 h after reperfusion, and various morphologies of apoptotic cells were observed (Nakka et al., 2008; Li et al., 2017a). The results mentioned above indicate that the sensitivity of neurons to ischemia-reperfusion injury is different, and that neuronal apoptosis is a dynamic developmental process (Naito et al., 2020). The induced expression of many neuronal genes and proteins is initially an adaptive response to cerebral ischemia stress, but due to repeated or continuous occurrence of cerebral ischemia, they gradually alter to promote or inhibit apoptosis, thereby constituting the complex regulatory factor system of neuronal apoptosis within the body (Lehotský et al., 2009; García de la Cadena and Massieu, 2016; Jensen et al., 2019).

Necrosis is another important type of neuronal death. Studies have reported that the activation of receptor-interacting protein kinase (RIPK)-mixed lineage kinase domain-like protein (MLKL) signaling pathway is involved in the onset of neuronal necrosis after injury by cerebral ischemia (Deng et al., 2019; Zhang et al., 2019, 2020). The expression of p-RIPK1 and p-MLKL proteins was found to be increased in the ischemic brain. Furthermore, use of RIPK1 inhibitors could hinder p-RIPK1 and p-MLKL protein expression, with reduced necrosis and infarct volume and promotion of neurological function (Deng et al., 2019; Liao et al., 2020). These data provide evidence that p-RIPK1 is involved in the initiation of RIPK3-/MLKL-induced necroptosis after cerebral ischemia. In addition, Pellino3, which is a ubiquitin E3 ligase, inhibits the formation of the death-induced signaling complex in response to tumor necrosis factor-α (TNF-α) by targeting RIPK1. Furthermore, it has been shown to have an effect in counteracting necroptosis *via* RIPK1 ubiquitination, and neuronal necroptosis could be reduced by upregulation of Pellino3 (Zhang et al., 2021). Zhang et al. (2020) found that knockout of Rip3 or Mlkl had a neuroprotective effect in acute ischemic stroke mice, and that necroptosis loss-of-function mice have attenuated inflammation in the brain infarct tissue. As an efficient and specific inhibitor of RIPK3, Gsk-872 inhibited hypoxia-induced phosphorylation of RIPK1/3 and MLKL proteins, reduced neuronal death, and alleviated brain injury (Yang et al., 2017).

The autophagy signaling pathway in cerebral ischemia is complex, is connected to necrosis and apoptosis, and affects the degree of brain injury (Kim K. A. et al., 2018; Wang et al., 2018; Xu et al., 2021). Su et al. (2014) demonstrated that the autophagy inhibitor 3-methyladenine (3-MA) reversed the neuroprotective effects that were induced by remote ischemic preconditioning against cerebral ischemia-induced injury, whereas the autophagy inducer rapamycin ameliorated neurological dysfunction post-stroke. This indicates that the activation of autophagy is involved in remote ischemic preconditioning-induced neuroprotection in cerebral ischemia (Su et al., 2014). Autophagy activation is common after the onset of brain ischemic/reperfusion injury. However, autophagy plays a beneficial or detrimental role in neuronal death, depending on injury stage and degree of

autophagy (Wei et al., 2012; Chen et al., 2014; Shi et al., 2021). Guo et al. (2021) conducted rat models of transient middle cerebral artery occlusion (tMCAO) and permanent MCAO (pMCAO), respectively. Then, the dynamic changes of autophagy activity, following tMCAO or pMCAO, were measured. The results demonstrated that both Beclin1 and LC3 expression levels, as indicators of autophagy, were significantly altered at different time points within seven days after tMCAO or pMCAO. Interestingly, autophagy induction elicited overt neuroprotection after tMCAO, and this effect was related to increased autophagy flux, indicating that autophagy plays a neuroprotective role, mainly at the subacute phase of tMCAO, but has few effects after pMCAO.

Cerebral Ischemia and Disruption of Blood-Brain Barrier

After cerebral ischemia, the blood-brain barrier (BBB) not only has abnormal function but also suffers from structural damage (Abdullahi et al., 2018; Li et al., 2019; Sarvari et al., 2020). The microvascular basement membrane is interrupted or lost, as the components of the basement membrane (i.e., laminin fibronectin and type IV collagen) are significantly reduced. In addition, damage to the BBB is not only a destruction of anatomical structure, but also a selective compensatory mechanism (Sheikh et al., 2022). The connection between neurons and microvessels is not only related to regulation of blood flow but also to the permeability of BBB. Previous studies have demonstrated that the necessary anatomical structure for BBB permeability is the close connection between microvascular endothelial cells and astrocytes. Furthermore, the direct regulation of astrocytes on blood vessels is realized by promoting the expansion or contraction of blood vessels (Ito et al., 2011, 2013). Other studies have discovered that pericytes can migrate rapidly from microvessels during cerebral ischemia, suggesting that pericytes can play a similar role to glial cells in maintenance of BBB formation and function (Gonul et al., 2002; Duz et al., 2007; Al Ahmad et al., 2011). In addition, an increase of BBB permeability under pathological conditions is significantly correlated with destruction of the extracellular matrix (Yang et al., 2015; Michalski et al., 2020).

Matrix metalloproteinases (MMPs) are a group of zinc-dependent enzymes that have the ability to degrade the extracellular matrix. Under physiological conditions, MMPs are known to be involved in embryo development, angiogenesis, nerve development, and regulation of tissue remodeling (Parks, 1999; Vu and Werb, 2000; Page-McCaw et al., 2007). After the onset of cerebral ischemia, MMPs can be rapidly produced by neurons, astrocytes, microglia, and endothelial cells, leading to degradation and destruction of the matrix layer mucin, collagen fiber IV, and cellular fibronectin (Candelario-Jalil et al., 2009; Chaturvedi and Kaczmarek, 2014). Animals and patient studies have discovered that cerebral ischemia can induce expression of MMPs, particularly, the increased activity of MMP-2 and MMP-9, which is closely related to increased cerebral microvascular permeability, BBB destruction, inflammatory cell invasion, and brain edema (Rosell and Lo, 2008; Kurzepa et al., 2014; Yang and Rosenberg, 2015). In conclusion, MMPs degrade the major components of the extracellular matrix and cause damage to BBB, resulting in cerebral edema and transformational hemorrhage after ischemic stroke (Jin et al., 2010; Seo et al., 2012).

EXERCISE AND RESTORATION OF NEUROVASCULAR UNIT

The pathological process after the onset of cerebral ischemia is complex, and the substances and factors that are released post-stroke have different roles. Each component of the NVU becomes damaged after stroke and maintains the microvascular-neuronal connection. The integrity of NVU is key to the prevention and treatment of ischemic stroke. Exercise, as a neuroprotective method, has been validated to be involved in protecting NVU during ischemic stroke (Wang et al., 2014).

Exercise and Blood-Brain Barrier Protection

After cerebral ischemia, a series of cascade reactions cause BBB dysfunction and an increase in cerebrovascular permeability, leading to brain edema. MMP-9 degrades the extracellular matrix, which is directly related to BBB dysfunction. Previous studies have indicated that MMP-9 reaches its peak at 48 h after ischemia, causing irreversible damage to BBB (Yang and Rosenberg, 2011). Inhibition of MMP-9 can alleviate dysfunction of the BBB, thereby reducing the degree of cerebral edema after cerebral ischemia (Chaturvedi and Kaczmarek, 2014; Kurzepa et al., 2014). Exercise can improve BBB function and integrity of the basement membrane after ischemia by inhibiting MMP-9 overexpression and upregulating tissue inhibitors of MMP (TIMP) (Guo et al., 2008a). Davis et al. (2007) discovered that exercise preconditioning ameliorates ischemic stroke-induced BBB dysfunction by strengthening the basal lamina with the involvement of MMP-9. Treadmill exercise can provide a protective effect on BBB disruption by degrading occludin, zonula occludens-1 (ZO-1), and an increase of MMP-9 after the onset of chronic cerebral hypoperfusion (Lee et al., 2017). Teymuri Kheravi et al. (2021) assessed the effects of two different exercises (i.e., swimming and treadmill training) prior to stroke induction. The results demonstrate that MMP-2 expression was increased after both exercise types, with reduced brain damage. In addition, exercise also alleviates BBB dysfunction by upregulating TNF-α levels. Furthermore, exercise training significantly increases the expression of TNF-α and alleviates BBB dysfunction, through extracellular-regulated protein kinase (ERK) 1/2 (Guo et al., 2008b; Chaudhry et al., 2010; Curry et al., 2010). Aquaporin (AQP)-4, an important regulator of cerebral edema formation, functions to alleviate cerebral edema in exercise preconditioning. In the study conducted by He et al. (2014), MRI was utilized to evaluate the dynamic impairment of cerebral edema after cerebral ischemia. The results demonstrate that treadmill pretraining improved the relative apparent diffusion

coefficient (rADC) loss after cerebral ischemia, and that T2W1 values of the ipsilateral cortex and striatum decreased within 2 days after stroke, while the brain water content and expression of AQP4 were decreased at 2 days after ischemia following pretraining.

Exercise and Neuronal Death

After the occurrence of an ischemic stroke, cell death, especially neuronal death, occurs in the core area of ischemia. Furthermore, the extent of infarction is related to the degree of interruption of blood supply. Reducing neuronal apoptosis can help improve the neurological function after the onset of cerebral ischemia (Memezawa et al., 1992; Li et al., 1998; Radak et al., 2017; Uzdensky, 2019).

Extracellular-regulated protein kinase 1/2 functions in the neuroprotective mechanism of exercise preconditioning. Lee et al. (2020) indicate that treadmill exercise improved short-term memory by inhibiting apoptosis in the hippocampus of the ischemia gerbils, which may be associated with the activation of the ERK-Akt-cAMP response element-binding protein (CREB)-brain-derived neurotrophic factor (BDNF) pathway. Similarly, swimming preconditioning improves the neurological outcome of cerebral ischemia in long-term ovariectomy rats, which is related to the activation of the ERK1/2/CREB/BDNF signaling pathway (Zhang et al., 2018). Zhou et al. (2018) discovered that the improvement of neurobehavioral performance by Willed movement training, following tMCAO, has been suggested to be involved in the ERK/CREB pathway. Notably, there was a region-specific discrepancy between ERK and CREB phosphorylation in a swim stress study (Shen et al., 2004). Exercise may also enhance the proliferation and differentiation of endogenous neural stem cells in the hippocampus of ischemia rats by enhancing phosphorylation of ERK. Subsequently, treatment with U0126 (an inhibitor of ERK) reversed the beneficial effects of exercise (Liu et al., 2018).

After the occurrence of ischemic stroke, heat shock protein (HSP)-70 (HSP-72) can protect neuronal apoptosis, caused by ischemia and hypoxia, and play an important role in cell survival and recovery. The increased synthesis of HSP-70 (HSP-72) can be utilized as an indicator of active repair and compensate for nerve reconstruction; it can also promote the recovery of neurological dysfunction after ischemia (Shevtsov et al., 2014; Kim J. Y. et al., 2018; Kim et al., 2020; Demyanenko et al., 2021). Recent studies have discovered that exercise can exert a neuroprotective effect on cerebral ischemia by regulating HSP-70 (HSP-72). Wang Y. L. et al. (2019) demonstrated that the percentage of HSP-72-containing neurons are tightly associated with a degree of ischemic stroke-induced brain injury, and exercise preconditioning can help improve neurological function post-stroke by preserving both the old and newly formed HSP-72-containing neurons. Liebelt et al. (2010) found that the expression of Bax and apoptosis-inducing factor (AIF) were reduced, while levels of Bcl-x(L) were increased in response to stroke after exercise. Additionally, inhibiting HSP-70 or pERK 1/2 reversed this resultant increase or decrease, leading to better neurological outcomes. However, inhibitors of phosphorylated ERK1/2 reduce brain injury, but do not reduce the expression of HSP-70, suggesting that ERK1/2 is not an upstream regulatory protein of HSP-70 after exercise preconditioning. A study of cerebral ischemia model induced by heat stroke showed that 3 weeks of pretreatment with progressive exercise-induced upregulation of HSP-72 and conferred neuroprotection against cerebral ischemia with reduced neuronal damage (Chen et al., 2007). Interestingly, poststroke exercise, if too early, can cause an elevated degree of cellular stress with increased expression of cell stress indicators, such as HSP-70 and hypoxia-inducible factor (HIF)-1α (Li et al., 2017b). The exacerbation of cell stress can be further aggravated during secondary brain injury post-cerebral ischemia, which indicates the importance of choosing an initiation time point of exercise and may have an effect on stroke recovery and rehabilitation (Li et al., 2017b).

Since the discovery of BDNF (Barde et al., 1982), its role in promoting neuronal regeneration and improving neurological functions has been widely studied. BDNF is mainly expressed in the hippocampus and cortex, and increasing the levels of BDNF to promote neuroregeneration is an important mechanism associated with the improvement of neurological function, after the onset of cerebral ischemia (Zhu et al., 2019, 2021; Tan et al., 2021). Studies have indicated that exercise can increase mRNA expression of BDNF in hippocampal regions of experimental animals, as well as improve hippocampal cell proliferation and cognitive function (Stagni et al., 2017; Liu and Nusslock, 2018). Many studies have sought to explore the mechanism underlying BDNF regulation of exercise-induced neuroprotection in ischemic stroke. Lee et al. (2020) demonstrated that treadmill exercise can attenuate memory impairment in cerebral ischemia gerbils by activating the ERK-Akt-CREB-BDNF signaling pathway. Another study that used swimming as the exercise method observed a similar phenomenon (Zhang et al., 2018). Caveolin-1/VEGF/BDNF and BDNF/TrkB signaling pathways are also involved in the recovery of motor and cognitive function after MCAO (Shi et al., 2016; Chen et al., 2019).

Numerous clinical and animal studies have suggested that regular aerobic exercise can increase the neuroprotective effect by enhancing the secretion of neurotrophins, including BDNF and insulin-like growth factor (IGF)-1, in the brain. Low concentrations of IGF-1 in the serum are found among chronic hemiparesis patients after they suffer from stroke (Silva-Couto Mde et al., 2014). In a randomized controlled trial, aerobic exercise, combined with cognitive training, improved the fluid intelligence of stroke patients. Upregulation with the higher serum IGF-1 expression was related to more robust improvements in cognition of patients, indicating that IGF-1 may participate in behaviorally induced plasticity (Ploughman et al., 2019). Ploughman et al. (2005) demonstrate that modest exercise can increase the IGF-1 expression, thereby contributing to the promotion of synaptic plasticity, resulting in an improvement of motor function after ischemic stroke. Exercise-induced IGF-1 can help protect cultured hippocampal cells against N-methyl-D-aspartate (NMDA)-mediated excitotoxicity by the negative feedback of NMDA receptor subunit NR2B (Li et al., 2017c). Zheng et al. (2014) discovered that an increase in physical exercise directly increases the number of neural progenitor cells by

activating the IGF-1/Akt signaling pathway, thereby contributing to recovery of neurological function post-stroke. Similar results were observed by Chang et al. (2011).

Exercise and Vascular Protection

Studies have demonstrated that exercise can protect the cerebrovascular system. Bullitt et al. (2009) discovered that aerobically active exercise contributes to the reduction of vessel tortuosity and elevation of small vessels, using the method of magnetic resonance angiography (MRA). A series of animal experiments reported that exercise led to an increase in the density of microvasculature and improvement in the blood supply of the brain. Monkeys, which are the closest human primates, show that 5 months of exercise can increase the number of blood vessels in the cerebral cortex. However, after 3 months of rest, the vascular density decreased to the baseline level, suggesting that only continuous exercise training maintains the promoting effect of angiogenesis (Rhyu et al., 2010). Ding et al. (2004) demonstrated that cerebral vascular integrity in the striatum region of middle-aged rats significantly improved after undergoing treadmill exercise training. Another study from the team discovered that integrins enhancement during exercise increased the neurovascular integrity post-stroke (Ding et al., 2006). Different durations of exercise have a similar effect. Four weeks of exercise increased the capillary distribution density and angiogenesis within the cerebellar region of rats (Isaacs et al., 1992). Additionally, 26 weeks of wheel running increased capillary and arteriole surface area densities and improved arteriolar reactivity and vasodilation in the motor cortex in response of exercise animals to hypercapsaic-hypoxic conditions (Stevenson et al., 2020a). Four weeks of voluntary wheel running demonstrated that sprouting angiogenesis is the main form of structural vascular plasticity that is detected in the motor cortex of rats, which is measured by vascular corrosion casts and resin replicas of the brain vasculature. In addition, increased capillary diameter and expanded endothelial cell nuclei diameters are observed in rats after exercise (Stevenson et al., 2020b).

Nitric oxide (NO) can help relax vascular smooth muscle cells and inhibit smooth muscle cell proliferation, which has an important function in inhibiting elevated blood pressure and vascular remodeling. Exercise can help stimulate phosphorylation and activation of AMP-activated protein kinase (AMPK) in vascular endothelial cells. This can promote phosphorylation of endothelial nitric oxide synthase (eNOS), increase the release of NO, improve endothelium-dependent relaxation function, and can be activated by shear force during exercise. Therefore, eNOS can increase NO production and enhance endothelium-dependent relaxation function (Lee-Young et al., 2009; Barr et al., 2017). Nitric oxide can be both protective and harmful in cerebral ischemia. NO is synthesized by nitric oxide synthase (NOS). Nitric oxide synthase can be divided into neuronal nitric oxide synthase (nNOS) and eNOS, both of which are expressed in the normal state, and inducible nitric oxide synthase (iNOS), which is expressed after injury. Studies have shown that eNOS has cerebral ischemia protection properties (van Faassen et al., 2009; Barr et al., 2017). Gertz et al. (2006) discovered that continuous exercise increases the levels of eNOS in the vasculature and improves the number of endothelial progenitor cells (EPCs) within the spleen and bone marrow. This boosts the circulating EPCs in the blood, thereby promoting neovascularization. Furthermore, animals that exercise have more newly generated cells in the vasculature, as well as a higher density of perfused microvessels, and increased blood supply of the brain in the ischemic region. However, after administration of NOS inhibitors or the antiangiogenic compound endostatin, the neuroprotective effect of exercise on angiogenesis and revascularization, as mentioned above, is reversed. This confirms that the improvement of outcomes and vascular function by exercise after stroke is related to the enhancement of angiogenesis and cerebral blood flow, partially through the eNOS-dependent signaling pathway. Exercise reduced brain injury following cerebral ischemia and restored impaired eNOS- and nNOS-dependent vascular function in type 1 diabetic rats (Arrick et al., 2012). Sun et al. (2019) indicated that exercise and exhaustive exercise animals both had higher expression of NO, increased NOS activity, and elevated expression of eNOS, nNOS, and iNOS. Interestingly, levels of eNOS are much higher within the exercise group, while iNOS has a significant increase in the exhaustive exercise group, which suggests that exercise can exert a neuroprotective effect by promoting levels of eNOS but not iNOS.

Vascular endothelial growth factor (VEGF) plays an important role during the process of exercise-induced angiogenesis. Exercise can induce the gene and protein expression of VEGF (Tang et al., 2010) and decreased brain infarct volume after the onset of cerebral ischemia (Ke et al., 2019). Measurement of the peri-infarct area of brain tissue shows an elevated expression of endothelial or angiogenesis markers (e.g., VEGF, VEGFR-2, and Ang-2), as well as endothelial progenitor cell marker (CD34+) after exercise (Pianta et al., 2019). The studies from Xie et al. (2019) indicated that exercise can alleviate neurological dysfunction after cerebral ischemia by promoting dendritic modification and synaptic plasticity, which is related to activation of caveolin-1/VEGF signaling pathway (Chen et al., 2019; Xie et al., 2019).

Infusion of endothelin-1 (ET-1), as a potent vasoconstrictor, into the animal's hippocampus using stereotaxic surgery is a method that is frequently used to develop an ischemia model (Joo et al., 2012; Farokhi-Sisakht et al., 2020). A clinical study including 27 patients has shown that ET-1 expression increases in exercise-induced ischemia and has a prognostic value as a marker of ischemia severity (Lubov et al., 2001). In an endurance exercise study, athletes that exercised at an intensity of 130% of individual ventilatory threshold, and the plasma levels of ET-1 and ET-3, were measured. Interestingly, ET-3 increased faster than ET-1 after exercise (Maeda et al., 1997). Exercise preconditioning can increase levels of HIF-1α, trigger expression of ET-1, and elevated the release of brain natriuretic peptide (BNP) to cause vasodilation. This vasodilation involved the use of some other factors, including VEGF, which led to a restoration of brain blood flow and attenuation of ischemic injury (Wang H. et al., 2019). Furthermore, Zhang et al. (2014) also discovered that preconditioning exercise protected ischemic-induced injury by improving cerebral blood flow and regulating ET-1 expression.

CONCLUSION

Exercise is an important method of preventing and rehabilitating cerebral ischemia, as it can not only reduce the incidence of stroke, but also play a protective role in the development of stroke, thereby reducing the severity and improving stroke outcome. Based on the current review, exercise can alleviate the damage of NVU after cerebral ischemia by inhibiting neuronal apoptosis, reducing BBB dysfunction, and promoting angiogenesis and synaptic plasticity. Elucidating the neuroprotective mechanism of exercise will be helpful in improving people's understanding of exercise, and encourage patients that are at high risk of stroke to actively participate in exercise. They will also provide new therapeutic strategies and potential drug targets for the prevention and treatment of cerebral ischemia in the future. Many challenges and issues remain to be solved and emphasized in the further study, including, but not limited to, exploring optimal duration, initiating time, intensity of exercise, varying responses of different brain regions and cell types to exercise, and the discrepancy of gender and age in response to exercise. Another existing difficulty is the low long-term compliance of patients. Therefore, it is necessary to strengthen health education for the target high-risk population and improve their understanding and attention to the significance of exercise intervention.

AUTHOR CONTRIBUTIONS

HZ designed and conceptualized this review. JH drafted and revised the manuscript. HZ and QX edited the manuscript. All authors contributed to the manuscript and approved the final version.

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L-F001, a Multifunctional Fasudil-Lipoic Acid Dimer Prevents RSL3-Induced Ferroptosis *via* Maintaining Iron Homeostasis and Inhibiting JNK in HT22 Cells

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Ferroptosis, an iron-dependent form of non-apoptotic cell death, plays important roles in cerebral ischemia. Previously we have found that L-F001, a novel fasudil-lipoic acid dimer with good pharmacokinetic characters has good neuroprotection against toxin-induced cell death *in vitro* and *in vivo*. Here, we investigated the protective effects of L-F001 against a Glutathione peroxidase 4 (GPX4) inhibitor Ras-selective lethality 3 (RSL3) -induced ferroptosis in HT22 cells. We performed MTT, Transmission Electron Microscope (TEM), Western blot, and immunofluorescence analyses to determine the protective effects of L-F001 treatment. RSL3 treatment significantly reduced HT22 cell viability and L-F001 significantly protected RSL3-induced cell death in a concentration-dependent manner and significantly attenuated Mitochondrial shrinkage observed by TEM. Meanwhile, L-F001 significantly decreased RSL3-induced ROS and lipid peroxidation levels in HT22 cells. Moreover L-F001could restore GPX4 and glutamate-cysteine ligase modifier subunit (GCLM) levels, and significantly deceased Cyclooxygenase (COX-2) levels to rescue the lipid peroxidation imbalance. In addition, FerroOrange fluorescent probe and Western blot analysis revealed that L-F001 treatment decreased the total number of intracellular Fe^{2+} and restore Ferritin heavy chain 1 (FTH1) level in RSL3-induced HT22 cells. Finally, L-F001 could reduce RSL3-induced c-Jun N-terminal kinase (JNK) activation, which might be a potential drug target for LF-001. Considering that L-F001 has a good anti-ferroptosis effect, our results showed that L-F001 might be a multi-target agent for the therapy of ferroptosis-related diseases, such as cerebral ischemia.

Keywords: L-F001, ferroptosis, iron homeostasis, lipid peroxidation, c-Jun N-terminal kinase

INTRODUCTION

Cerebral ischemia, occurring as the result of cardiac arrest, brain injury, shock, and age-related diseases, and then leading to neuronal death and neurological deficits, is a leading cause of disability and death worldwide (Madl and Holzer, 2004). Clinically, there is almost no effective therapy except to restore the blood flow either by pharmacological or mechanical thrombolysis till now

(Sarkar et al., 2019). However, only less than 10% of cerebral ischemia patients are eligible for tissue plasminogen activator therapy, and half of those patients fail to demonstrate clinical improvement (DeSai and Hays Shapshak, 2021). So, discovering new drugs against cerebral ischemia is still an urgent issue.

Ferroptosis, a newly discovered iron-associated programmed cell death was reported the play important role in cerebral ischemia. In terms of morphology, the density of mitochondria in ferroptosis is lower, and mitochondrial crests are reduced or disappeared and the outer mitochondrial membrane is ruptured (Dixon et al., 2012; Xie et al., 2016). The biochemical signs of ferroptosis are lipid peroxidation and intracellular elevated Fe^{2+} level (Xie et al., 2016). Meanwhile, the reduced Glutathione (GSH), Glutathione peroxidase 4 (GPX4) inactivation and COX-2 over activation also predict the occurrence of ferroptosis (Cao and Dixon, 2016).

Growing evidence indicates that iron is a risk factor in the development of cerebral ischemia and ferroptosis is involved in the cerebral ischemia (She et al., 2020). The intracellular elevated Fe^{2+} level is increased in ischemic brains and iron chelation reduces ischemia-induced brain injury (Abdul et al., 2021). Moreover, several studies elucidated that the activation of ferroptosis following excitatory toxicity in cerebral ischemia induced by the lipid peroxidation mediated by the degradation of GPX4 and overexpression of Acyl-CoA Synthetase Long-Chain Family Member 4 (She et al., 2020; Cui et al., 2021). Meanwhile, the pathology of cerebral ischemia can be effectively alleviated by lipophilic ferroptosis inhibitors such as ferrostatin-1 and liproxstatin-1 (Alim et al., 2019; Guan et al., 2021). In addition, iron metabolism plays an important role in ferroptosis's pathogenesis, which is also a potential target for cerebral ischemia and ischemic stroke (Dixon and Stockwell, 2014; Doll and Conrad, 2017; Chen et al., 2021). When iron homeostasis is broken, the iron redox couple (Fe^{3+}/Fe^{2+}) could trigger a Fenton reaction which causes overwhelming lipid peroxidation and definitively induces ferroptosis. And using the iron-chelating agent deferoxamine pretreatment can protect against both ferroptosis and cerebral ischemia (Li et al., 2008). Additionally, c-Jun N-terminal kinase (JNK), one member of the mitogen-activated protein kinase (MAPK) plays important role in regulating iron homeostasis through JNK/Sp1 and Stat4/Sp1 signal (Qiu et al., 2020).

L-F001 (**Figure 1A**), a fasudil-lipoic acid derivative synthesized by our lab has neuroprotection with multifunctional effects such as Rho-associated kinase (ROCK) inhibition. Our previous studies confirmed that L-F001 could prevent cell death induced by paraquat through alleviating endoplasmic reticulum stress and mitochondrial dysfunction in PC12 cells (Shen et al., 2015). In addition, L-F001 also could effectively reduce the cytotoxicity induced by 6-Hydroxydopamine hydrobromide (6-OHDA) *in vitro* and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopamine neuron toxicity in mice and improve the levels of p-Akt (Ser473), p-GSK3β(Ser9) to exert a neuroprotective effect (Luo et al., 2017). Moreover, L-F001 can significantly inhibit neuroinflammation induced by LPS and reduce microglial activation *in vitro* and *in vivo* (Chen et al., 2017). Considering ferroptosis plays important roles in these PD models (Tian et al., 2020; Mahoney-Sánchez et al., 2021) and L-F001 has anti-PD effects, we hypothesized that L-F001 might be benefit for ferroptosis to rescue other diseases, which might be a target for the therapy of cerebral ischemia. RSL3 is a GPX4 inhibitor which can block the ability for GPX4 that catalyzes GSH to oxidized glutathione (GSSG) and then reduces toxic peroxide to nontoxic hydroxyl compound to indirectly inhibits lipid peroxidation (Yang et al., 2014). Here we investigate the effects of L-F001 on RSL-3-induced ferroptosis in HT22 cells, a mice hippocampal cell line. Our data showed that L-F001 could inhibit RSL3-induced lipid peroxidation, maintain iron homeostasis, and inhibit the JNK pathway to attenuate ferroptosis. And L-F001 might be a multi-target agent for the therapy of ferroptosis-related diseases, such as cerebral ischemia.

MATERIALS AND METHODS

Materials

L-F001 (purity>98%) was synthesized before, and the chemical characterization (mass spectroscopy, purity, etc) could be found over there (Chen et al., 2014). Dulbecco's Modified Eagle's Medium (DMEM) was from Gibco-BRL (NY, USA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA). A BCA protein assay kit was purchased from Thermo (Waltham, MA, USA). DMSO was obtained from Sigma-Aldrich Inc. (St Louis, MO, USA). Anti-GPX4 antibody, anti-FTH1antibody from Abcam (Cambridge, USA). Anti-COX-2, anti-JNK/p-JNK antibody from Cell Signaling Technology (Woburn, USA). Anti-GCLM antibody form ABclonal (Wuhan, Hubei, P.R.C.).

Cell Culture

The sources of the cells are described in previous articles (Lu et al., 2020). HT22 cells were cultured in DMEM (10% FBS) under 5% CO_2 and 37°C. And cells were subcultured every 2 days. Morphological change of induced cells was observed by phase-contrast microscopy (Olympus, Tokyo, Japan).

MTT Assay

MTT assay was prepared according to our previous article to determine cell viability (Peng et al., 2021). HT22 cells were cultured in 96-well plates at 5×10^3 cells per well and cultured 24 h before administration. Ten microliter of MTT (5 mg/ml) was then added to each well, and the mixture was incubated for 2 h at 37°C. MTT reagent was then carefully replaced with DMSO (100 μl per well) to dissolve formazan crystals. After the mixture was shaken at room temperature for 10 min, absorbance was determined at 490 nm using a microplate reader (Bio-Tek, USA). Cell survival assays were performed in triplicate.

Transmission Electron Microscope

Transmission electron microscope protocol was performed as previously described (Peng et al., 2021). HT22 cells were grown on a 60 mm dish at 6×10^5 cells per dish and cultured 24 h before administration. Then cells were treated with N2L and RSL3. A transmission electron microscope observed the ultrastructure

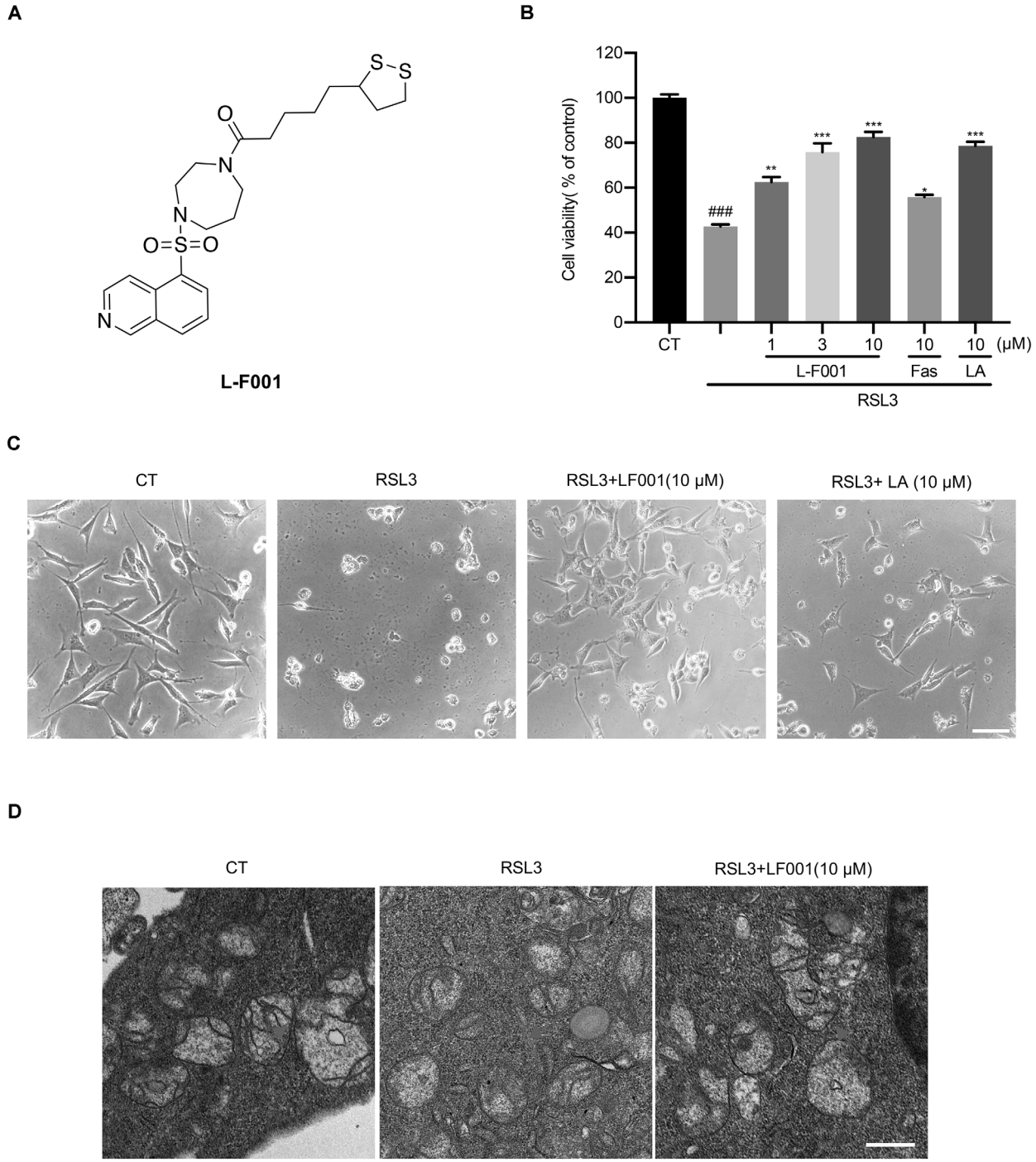

FIGURE 1 | L-F001 reduces RSL3-induced cytotoxicity in HT22 cells. **(A)** Chemical structure of L-F001. **(B)** HT22 cells were treated with 0.1 μM RSL3 alone, and then added L-F001 (1–10 μM), fasudil (10 μM), and LA (10 μM) at 37°C for 24 h. MTT assays were used to determine cell viability ($n = 5$). **(C)** The morphology of HT22 cells (scale bar = 25 μm). **(D)** Transmission electron microscopy pictures of mitochondrial in cells from control, RSL3, and RSL3 + L-F001 group (Scale bar = 1,200 nm). $^{###}P < 0.001$ compared with the control group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the RSL3-induced group.

of mitochondria. Briefly, HT22 cells were fixed following drug treatment with 4°C pre-cooled 2.5% glutaraldehyde for 24 h and with 1% osmium tetraoxide for 1 h. The cells were then dehydrated for 15 min at a series of acetone concentrations (50%, 70%, 80%, 90%, and 100%) and embedded in resin. The samples were sliced and double-stained with uranyl acetate and lead citrate, and representative images were obtained using a JEM-1400 electron microscope (JEOL Ltd., Japan).

Measurement of ROS

Measurement of ROS was performed as previously described (Peng et al., 2021). The DHE probes were diluted 1:1,000 with serum-free medium to a final concentration of 10 μM. The serum-free cell culture medium was removed, and an appropriate volume of diluted DHE was added. The appropriate volume to cover the cells was added and the cells were incubated for 20 min in a 37°C cell incubator. The cells were washed three times with serum-free cell culture to adequately remove DHE that did not enter the cells. The high-content screening (HCS) system (Thermo, Waltham, MA, USA) was used to take fluorescence images and analyze the data (excitation wavelengths = 549 nm).

Measurement of Lipid Oxidation Level

Measurement of lipid oxidation level was using BODIPY 581/591 C11 probe (Glpbio, Guangzhou, China). HT22 cells were grown on a 20 mm glass-bottom dish at 5×10^4 cells per dish and cultured 24 h before administration. Cells were cultured with 2.5 μM C11-BODIPY581/591 for 30 min after drug treatment. The excitation and emission band of oxidized type is pass of 460–495 nm and 510–550 nm, respectively. But the excitation and emission band of reduced type is pass of 565–581 nm and 585–591 nm, respectively. Then photos were taken under a laser confocal microscope (Olympus, Tokyo, Japan).

Measurement of Endogenous Hydroxyl Radicals

Rho-Bob is a gift from Prof. Fang Liu from Guangzhou University of Chinese Medicine, which detected the endogenous hydroxyl radicals in cells and the measurement was performed as previously described (Peng et al., 2021). Cells were cultured with 5 μM Rho-Bob for 30 min and observed under a confocal microscope. The excitation and emission band of the oxidized type is 532 nm. And the excitation and emission band of the reduced type is pass of 580–600 nm and 640–660 nm, respectively. Then photos were taken under a laser confocal microscope (Olympus, Tokyo, Japan).

Detection of Intracellular Ferrous Ion Content

Cells were cultured with 1 μM FerroOrange for 30 min and observed under a confocal microscope (Ex/Em: 561 nm/570–620 nm).To facilitate the observation and analysis, the fluorescence signal from FerroOrange was marked as orange. And cell images were taken using a laser confocal microscope (Olympus, Tokyo, Japan).

Immunofluorescence

Measurement of Immunofluorescence was performed as previously described (Chen et al., 2014). The cells were washed three times with PBS. After being fixed and blocked, the cells were stained overnight at 4°C with anti-COX-2 antibody (CST, 12282, 1:250 dilution) and anti-GPX4 antibody (Abcam, ab125066, 1:500 dilution), followed by Fluorescent secondary antibody (Abcam, ab150080, ab150077, 1:500). Finally, DAPI was used for nuclear staining and cell images were taken using a laser confocal microscope (Olympus, Tokyo, Japan).

Western Blotting Analysis

Western blotting analysis was performed as previously described (Chen et al., 2014). The primary antibodies are described in "Materials" Section.

Statistical Analysis

Data were shown as the mean ± S.E.M. for 3–5 times independent experiments. Western blotting and Fluorescence intensity was quantified through Image J software. Differences between groups were tested using one-way analysis of variance (ANOVA), followed by a Tukey-Kramer test as *post-hoc* comparison using the software Prism 8 (Chicago, USA). Differences were considered statistically significant if $^*P < 0.05$.

RESULTS

L-F001 Reduces RSL3-Induced Cytotoxicity in HT22 Cells

In our previous research, L-F001 could prevent 6-OHDA-induced cytotoxicity, and 6-OHDAwas a good ferroptosis inducer that could degrade ferritin and damage the iron homeostasis (Sun et al., 2020; Tian et al., 2020). L-F001 (1–10 μM) was incubated with RSL3 in HT22 cells to evaluate protective effects. Our results show that L-F001 reduced RSL3-induced HT22 cells' cytotoxicity in a dose-dependent manner, and among them, 10 μM L-F001 could almost completely reverse RSL-3-induced HT22 cell injury and restore cell morphology. lipoic acid (LA) had a similar protective effect, but fasudil (Fas) had a slight protective effect (**Figures 1B,C**). Therefore, the ROCK inhibition of L-F001 was hardly related to the anti-ferroptosis effect. And LA group, which has the function of resisting ferroptosis (Zhang et al., 2018; Liu et al., 2020), plays an important role in the anti-ferroptosis of L-F001. Electron microscope investigation showed the shrunken mitochondria and absence of mitochondria cristae in RSL3-treated HT22 cells, and obvious improvement of mitochondrial morphology could be observed after treatment with L-F001 (**Figure 1D**).

L-F001 Reduces RSL3-Induced ROS and Lipid Peroxidation in HT22 Cells

We measured the intracellular ROS and lipid oxidation levels by dihydroethidium and C11-BODIPY 581/591 probes. ROS and lipid oxidation levels in HT22 cells dramatically increased under the RSL3 treatment. Pretreatment with 10 μM L-F001 significantly decreased ROS (**Figures 2A,B**) and lipid peroxidation levels in RSL3-induced HT22 cells (**Figure 2C**). Those results showed L-F001 could effectively improve the lipid metabolism process in ferroptosis.

L-F001 Affects the Levels of Lipid Peroxidation-Related Proteins in HT22 Cells

RSL3 pretreatment resulted in obvious shifts of lipid peroxidation-related proteins in HT22 cells, including cyclooxygenase-2 (COX-2; Yang et al., 2014), GCLM, and

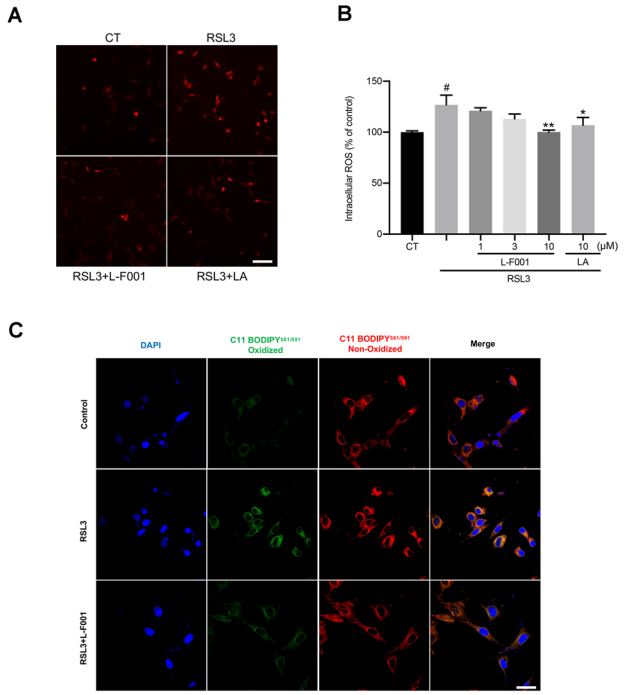

FIGURE 2 | L-F001 reduces RSL3-induced ROS production and lipid peroxidation in HT22 cells. **(A)** HT22 cells were treated with 0.1 μM RSL3 alone, and then added L-F001 (1–10 μM) and LA (10 μM) at 37°C for 24 h. ROS formation was measured with a fluorescence microscope (scale bar = 50 μm). **(B)** ROS levels were measured with a fluorescence plate reader (n = 3). **(C)** The lipid ROS formation was monitored by a laser confocal microscope using a C11-BODIPY581/591 probe (Scale bar = 25 μm). $^{\#}P < 0.05$ compared with the control group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the RSL3-induced group.

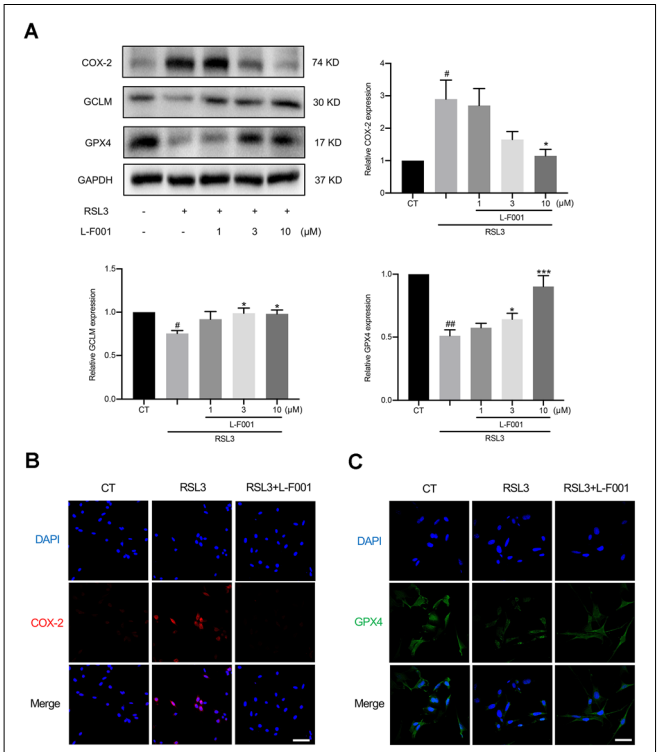

FIGURE 3 | L-F001 affects the levels of lipid peroxidation-related protein proteins in HT22 cells. HT22 cells were treated with 0.1 μM RSL3 alone, and then added L-F001 (1–10 μM) at 37°C for 24 h. **(A)** The levels of COX-2, GCLM, and GPX4 were measured by Western blot, and the amount of COX-2, GCLM, and GPX4 were estimated by densitometric analysis of each protein band (n = 3). **(B,C)** Cells were immunofluorescence stained with anti-COX-2 and GPX4 antibodies. Nuclear counterstaining was done with DAPI (Scale bar = 100 μm). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the control group. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with the RSL3 group.

GPX4 protein levels (Seibt et al., 2019) 3 and 10 μM L-F001 pretreatment resulted in obviously reduced levels of COX-2 and restored levels of GPX4 and GCLM inRSL3-induced HT22 cells (**Figure 3A**). Immunofluorescence results confirmed those changes (**Figures 3B,C**). These data suggested that L-F001 could restore GCLM and GPX4 levels to inhibit ferroptosis in HT22 cells.

L-F001 Reduces RSL3-Induced Impairment of Iron Homeostasis in HT22 Cells

Excess intracellular Fe^{2+} is a marker of ferroptosis, which triggers Fenton's reaction to lead to membrane lipid peroxidation. We determined the Fe^{2+} by FerroOrange fluorescent probe and found that 10 μM L-F001 significantly reduced Fe^{2+} levels under RSL3 treatment in HT22 cells (**Figure 4A**). Meanwhile, hydroxyl radicals which were produced in Fenton's reaction dramatically increased under the RSL3 treatment in HT22 cells. Pretreatment with 10 μM L-F001 significantly decreased hydroxyl radicals' production in RSL3-induced HT22 cells (**Figure 4B**). To measure the iron storage function, we detected the level of FTH1. And Pretreatment with 10 μM L-F001 could significantly restore the FTH1 level under RSL3 treatment in HT22 cells (**Figure 4C**).

L-F001 Inhibits RSL3-Induced JNK Activation in HT22 Cells

Lipid peroxidation also induces phosphorylation of JNK, which is involved in cellular responses to environmental stresses. To explore the potential mechanism of L-F001 inhibiting RSL3-induced ferroptosis, we detected p-JNK/JNK protein levels. p-JNK/JNK increased upon the treatment with RSL3 in HT22 cells, and 10 μM L-F001 pretreatment hindered the increase of JNK phosphorylation level (**Figure 5**).

DISCUSSION

Ferroptosis, a new type of programmed cell death is associated with iron ions and plays an important role in cerebral ischemia (Datta et al., 2020; Liu et al., 2022). L-F001 is a good neuroprotective agent with multifunctional effects such as anti-inflammation and anti-oxidative stress (Shen et al., 2015; Chen et al., 2017; Luo et al., 2017). We found that L-F001 could reduce the lipid peroxidation, impairment of iron homeostasis, and JNK activation in the RSL3-induced HT22 hippocampal neuronal cell line, providing a potential

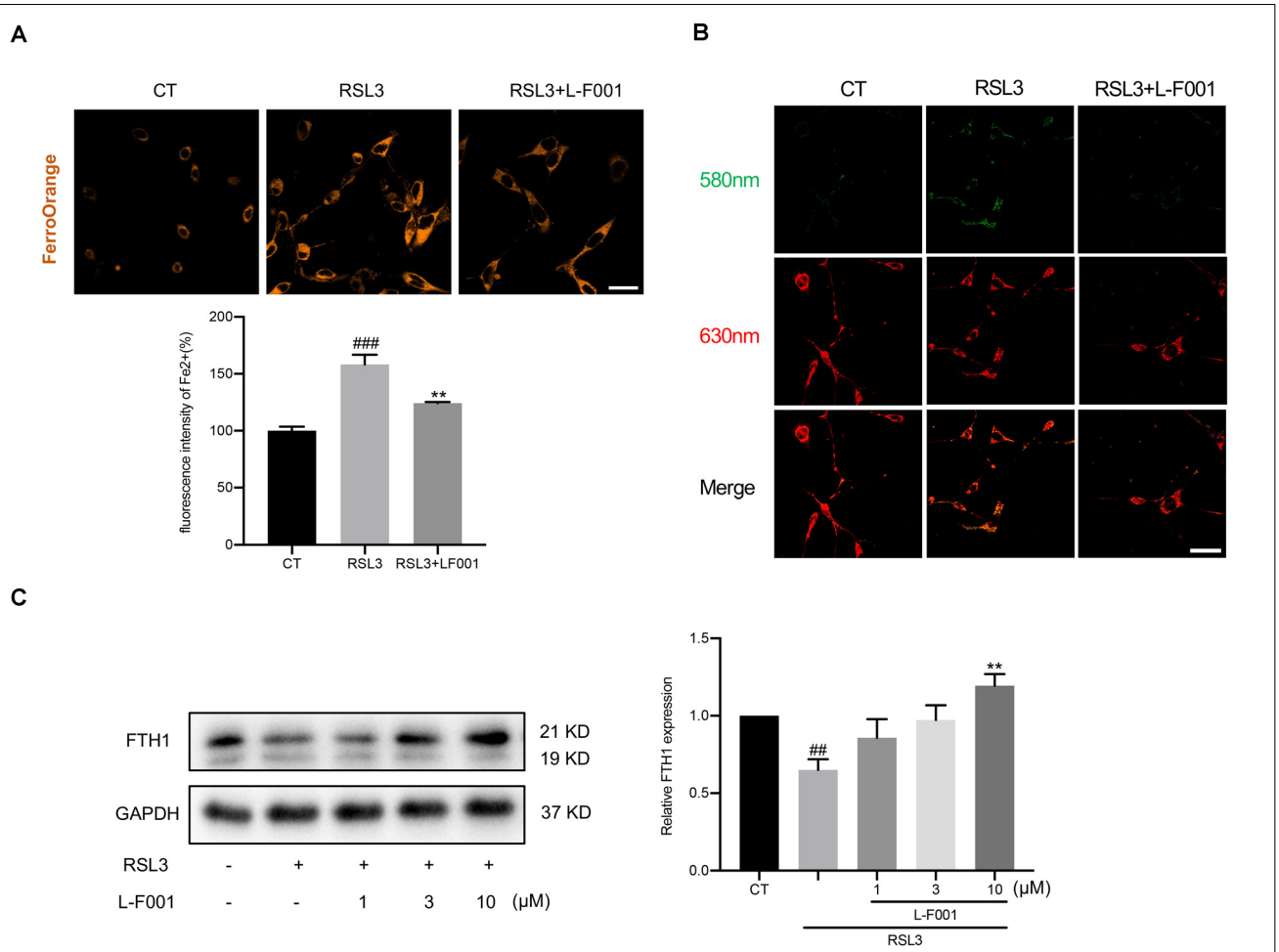

FIGURE 4 | L-F001 reduces RSL3-induced impairment of iron homeostasis in HT22 cells. HT22 cells were treated with 0.1 μM RSL3 alone, and then added L-F001(10 μM) at 37°C for 24 h. **(A)** Intracellular Fe^{2+} level in HT22 cells were detected by FerroOrange probe (scale bar = 25 μm). The fluorescence intensity of the CT group was defined as 100% (n = 5). **(B)** Endogenous hydroxyl radicals' level in HT22 cells were detected by Rho-Bob probe (Scale bar = 25 μm). **(C)** Protein level and analysis of FTH1 in cells (n = 3). $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with the control group. $^{**}P < 0.01$ compared with the RSL3-induced group.

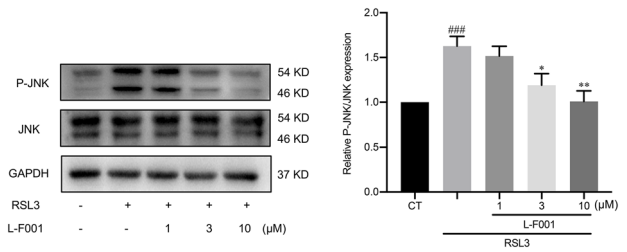

FIGURE 5 | L-F001 inhibits RSL3-induced JNK activation in HT22 cells. HT22 cells were treated with 0.1 μM RSL3 alone, and then added L-F001(1–10 μM) at 37°C for 24 h. The levels of P-JNK and JNK were measured by Western blot, and the amount of P-JNK and JNK were estimated by densitometric analysis of each protein band (n = 3). $^{\#\#\#}P < 0.001$ compared with the control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the RSL3 group.

strategy apart from ROCK inhibition for treating ferroptosis-related cerebral ischemia.

MTT assay was used to evaluate cell viability in our previous studies on ferroptosis (Peng et al., 2021) and the neuroprotective effect of L-F001. L-F001 significantly rescued cell viability and showed a similar effect with LA in the RSL3-induced HT22 cells. Still, fasudil had little effect to protect RSL3-induced cell death, indicating that the protection of L-F001 was almost related to the LA group (**Figures 1B,C**). LA belongs to a group of B vitamins, which plays the role of coenzyme in the oxidative respiratory chain and has antioxidant activities. In recent years, it has been found that lipoic acid has an anti-ferroptosis effect, which can not only alleviate the ferroptosis of PC12 cells induced by MPP^+ by activating PI3K/Akt/Nrf2 (Liu et al., 2021), but also alleviate the ferroptosis induced by cobalt nanoparticles (Liu et al., 2020). At the animal level, lipoic acid can significantly reduce iron overload and lipid peroxidation in P301S Tau transgenic mice (Zhang et al., 2018). Compared to LA, L-F001 has a good function of ROCK inhibition and anti-inflammation effect which were often therapeutic targets

for cerebral ischemia. At the same time, L-F001 significantly recovered mitochondrial damage (**Figure 1D**). These findings indicated L-F001 could inhibit ferroptosis and the effect of L-F001 on ferroptosis is largely attributed to the lipoic acid group.

Lipid peroxidation is a bridge between ferroptosis and cerebral ischemia. Ferroptosis is characterized by lipid peroxidation, and the levels of lipid hydroperoxides were significantly higher in ischemic stroke patients (Zeiger et al., 2009; Yang and Stockwell, 2016). L-F001 not only reduced ROS production and lipid peroxidation (**Figures 2A,B**) but also affects the levels of lipid peroxidation-related protein. Meanwhile, we have found that the ROS production and cell viability after L-F001 treatment is similar with LA in RSL3-induced HT22 cells, and there was no statistical difference between the two groups in 10 μM. Therefore, we thought the LA group is the functional part of L-F001 to resist ferroptosis. And LA has been proved that it had a good 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging ability which represented direct scavenging ability on free radicals (Zhao and Liu, 2011). For this reason, L-F001, as a derivative of LA, might be inferred that it would also have good DPPH scavenging ability same as LA. COX-2 is a key enzyme of lipid peroxidation involved in synthesizing prostaglandins and studies have shown that COX-2 is markedly upregulated during ferroptosis, which is a downstream and suitable marker for the lipid peroxidation that occurs during GPX4-regulated ferroptosis (Yang et al., 2014). Knocking out COX-2 can reduce neuron ferroptosis and lipid peroxidation (Xiao et al., 2019). Interestingly, we found that L-F001 could decrease the level of COX-2, proving that lipid peroxidation has gone (**Figures 3A,B**). GCLM is a part of the first rate-limiting enzyme of glutathione synthesis, which can produce GSH, a substrate of GPX4, to resist ferroptosis (Kang et al., 2021). GPX4 is a selenium-containing membrane lipid repair enzyme, which can suppress the activation of lipoxygenase and cyclooxygenase at the nucleus and endoplasmic reticulum by reducing fatty acid hydroperoxide as activators of lipoxygenase and cyclooxygenase (Imai et al., 2017). The GPX4 inhibitor RSL3 promotes lipid peroxidation and ferroptosis (Franklin et al., 2009), which leads to GPX4 and GCLM degradation. L-F001 pretreatment could rescue RSL3-induced decrease of GPX4 and GCLM levels (**Figures 3A,C**). These results confirmed that L-F001 could reduce lipid peroxidation to play an anti-ferroptosis role.

Iron homeostasis is a complex process and relies on the coordination of multiple iron metabolism proteins, including the heavy and light subunit of ferritin (FTH1 and FTL), which is a protein complex that safely concentrates intracellular iron in a mineralized, redox-inactive form; transferrin, an iron-binding serum protein; ferroportin, the only known cellular iron efflux pump (Bogdan et al., 2016). Excessive Fe^{2+} is a hallmark of ferroptosis, which reacts with H_2O_2 to produce many hydroxyl radicals (OH•), and OH• promotes the oxidation of polyunsaturated fatty acids to hydroperoxide derivatives of lipids (LOOH) on the cell membrane (Li et al., 2008). FTH1 plays a vital role in the efflux and storage of Fe^{3+}/Fe^{2+} in cells. Several previous studies elucidated that treating iron chelator deferoxamine (Van der Loo et al., 2020; Abdul et al., 2021) and promoting iron export could prevent ferroptosis damage after ischemic stroke. In the RSL3-treated HT22 cells, we observed the excessive Fe^{2+} and the downregulation of FTH1, and L-F001 can reduce excessive Fe^{2+} and restored FTH1 levels (**Figures 4A–C**). These results indicated that L-F001 could maintain intracellular iron homeostasis and decrease Fenton's reaction to playing an anti-ferroptosis role.

The activation of the MAPK pathway is one of the important signs of ferroptosis (Stockwell et al., 2017), in which JNK is an important member. JNK responds to environmental stress, and cerebral ischemia can also induce the robust activation of JNK cascades, ultimately resulting in neuron death (Nozaki et al., 2001). In ferroptosis, excess lipid peroxidation products produced during the oxidation of the plasma membrane can remain active in the JNK and enhanced phosphorylation (Nagase et al., 2020; Qiu et al., 2020). And using JNK specific pharmacological inhibitors can alleviate not only ferroptosis (Fuhrmann et al., 2020) and cerebral ischemia (Sun et al., 2015; Zheng et al., 2020). L-F001 significantly reduced JNK phosphorylation (**Figure 5**), whose effect is reduced intracellular ROS, thus reducing the cell damage caused by JNK hyperphosphorylation. As for the other two MAPKs signaling (ERK and P38), they can all be activated by the same stimulation factors such as cytokines, neurotransmitters, hormones, cellular stress, and so on, and undergo the same changes. Many articles related to ferroptosis only detect one of them to characterize the change of ERK and P38 (Yang et al., 2021; Wang et al., 2022). In this study, we focused on the protective effects of L-F001 on ferroptosis and tentatively evaluated the JNK pathway. On the other side, our previous results indicated that L-F001 could activate Nrf-2/HO-1 signaling (Luo et al., 2017), we also found that Nrf2/HO-1 signaling is activation under RSL-3 treatment rather than downregulation (Peng et al., 2021). Furthermore, some research demonstrated that many active compounds exhibit protection through further upregulation of Nrf2/HO-1 signaling (Gou et al., 2020; Wei et al., 2021; Fu et al., 2022), compared to the already increased Nrf2 signaling. Therefore Nrf2/HO-1 signaling activation might be an important part in L-F001 anti-ferroptosis role. And in a future study, we will deeply explore the signaling pathway mechanism of L-F001 against ferroptosis such as Nrf2/HO-1, ERK, and P38.

In conclusion, due to the lipoic acid group, L-F001 is a good antioxidant and ferroptosis inhibitor, which can significantly restore RSL3-induced broken iron homeostasis, reduce lipid peroxidation, and JNK overactivation in HT22 cells. Consequently, because of its anti-ferroptosis and ROCK inhibition effect, L-F001 can potentially treat ferroptosis-related cerebral ischemia without the hypotensive response of fasudil. However, there are multiple programmed cell deaths involved in cerebral ischemia apart from ferroptosis, and the animals and clinical application need to be further verified.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

WP: investigation, writing—original draft, and formal analysis. SW and JH: investigation and formal analysis. ZZ: investigation and writing—review. YY: investigation. RZ: formal analysis and validation. RP: writing—review and editing. YO: conceptualization, project administration, and supervision. All authors contributed to the article and approved the submitted version.

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REVIEW
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Novel Mechanisms and Therapeutic Targets for Ischemic Stroke: A Focus on Gut Microbiota

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Ischemic stroke is the most common type of stroke with limited treatment options. Although the pathological mechanisms and potential therapeutic targets of ischemic stroke have been comprehensively studied, no effective therapies were translated into clinical practice. Gut microbiota is a complex and diverse dynamic metabolic ecological balance network in the body, including a large number of bacteria, archaea, and eukaryotes. The composition, quantity and distribution in gut microbiota are found to be associated with the pathogenesis of many diseases, such as individual immune abnormalities, metabolic disorders, and neurodegeneration. New insight suggests that ischemic stroke may lead to changes in the gut microbiota and the alterations of gut microbiota may determine stroke outcomes in turn. The link between gut microbiota and stroke is expected to provide new perspectives for ischemic stroke treatment. In this review, we discuss the gut microbiota alterations during ischemic stroke and gut microbiota-related stroke pathophysiology and complications. Finally, we highlight the role of the gut microbiota as a potential therapeutic target for ischemic stroke and summarize the microbiome-based treatment options that can improve the recovery of stroke patients.

Keywords: ischemic stroke, gut microbiota, microbiome, mechanism, target, treatment

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INTRODUCTION

Ischemic stroke accounts for about 70–80% of all stroke patients (Feigin et al., 2003). The main cause of ischemic stroke is insufficient blood and oxygen supply to the brain. Embolus or thrombus forms could block cerebrovascular, which make blood supply of local brain tissue decrease, thereby causing brain tissue damage (Dirnagl et al., 1999). Currently, there are two main therapies for ischemic stroke: thrombolysis and thrombectomy. However, their applications in clinical practice are still very limited due to the short treatment window (Fisher and Saver, 2015). In recent years, the pathological mechanisms and potential therapeutic targets of ischemic stroke have been comprehensively studied, including excitotoxicity, oxidative stress, neuroinflammation, apoptosis, and blood-brain barrier (BBB) disruption (Zhang Z. Y. et al., 2019). However, no effective therapies were translated into clinical practice. Therefore, it still needs our great attention to find new therapies to prevent or reduce neuronal injury after ischemic stroke.

Gut microbiota is a complex and diverse dynamic metabolic ecological balance network, including a large number of bacteria, archaea, and eukaryotes. Gut microbiota is formed at birth and retains maternal characteristics. After exposure to a complex microbiome, babies develop a largely stable gut microbiota by the time they are 1–3 years old (Mackie et al., 1999; Palmer et al., 2007). But it can also change due to the host's dietary habits, stress, antibiotic use, and

aging (Claesson et al., 2011; Wu and Hui, 2011; Shimizu, 2018). The composition, quantity and distribution in gut microbiota are associated with the pathogenesis of a wide variety of diseases, such as individual immune abnormalities, metabolic disorders, and neurodegeneration (Duvallet et al., 2017). At present, biphase associations between gut microbiota and many body organs have been identified, including gut-cardiac axis, gut-thyroid axis, and gut-liver axis (Koszewicz et al., 2021). The brain and gut microbiota can interact with each other not only through neuronal pathways but also through microbial metabolites, hormones, and the immune system, termed the gut-brain-microbiota axis (GBMAx) (El Aidy et al., 2015; Durgan et al., 2019). Ischemic stroke may lead to changes in the gut microbiota, which can affect surrounding or distant tissues and organs, causing serious damages to liver, kidney, lung, gastrointestinal tract, cardiovascular system, and so on. In turn, changes in the gut microbiota may be one of the risk factors for ischemic stroke and determine stroke outcomes (de Jong et al., 2016). The link between gut microbiota and stroke is expected to provide new perspectives for ischemic stroke treatment.

This article reviews the gut microbiota alterations during ischemic stroke, gut microbiota-related stroke pathophysiology and complications, as well as potential therapeutic strategies targeting gut microbiota for ischemic stroke.

Alterations of Gut Microbiota During Ischemic Stroke

Multiple clinical and animal studies have revealed the changes in gut microbiota following ischemic stroke. One case-control study showed that the gut microbiota was significantly disrupted in patients with ischemic stroke and transient ischemic attack compared to controls. The main manifestations were the increase of opportunistic pathogens and the decrease of commensal or beneficial genera (Yin et al., 2015). In another study, the gut microbiota of ischemic stroke patients had more short chain fatty acids producer compared to healthy controls. In addition, it was found that the genus Enterobacter was significantly correlated with good outcomes (Li et al., 2019). An animal experiment based on the mouse middle cerebral artery occlusion (MCAO) model showed that ischemic stroke resulted in reduced species diversity and bacterial overgrowth of Bacteroidetes in the gut (Singh et al., 2016). Another study found that the levels of Bacteroidetes phylum and Prevotella genus were significantly increased in the gut of cynomolgus monkeys after MCAO, while Firmicutes phylum as well as Faecalibacterium, Oscillospira, and Lactobacillus genera were decreased, Oscillobacter, and Lactobacillus were decreased. In addition, intestinal mucosal damage was also observed (Chen et al., 2019c).

In addition to causing gut microbiota dysbiosis, ischemic stroke may also facilitate the translocation and dissemination of selective strains of bacteria that originated from the host gut microbiota. Infection is usually more likely to be observed after an ischemic stroke. Stanley et al. (2016) demonstrated that the microbial community in the lungs of post-stroke mice were derived from the small intestine of the host using high-throughput 16S rRNA gene amplicon sequencing and bioinformatics analyses.

Changes in gut bacteria can also be a factor in ischemic stroke. Significant microbiological disorders have been detected in inflammatory bowel disease (including Crohn's disease and ulcerative colitis) and chronic kidney disease, all of which were found to be risk factors for ischemic stroke (Lee et al., 2010; Kristensen et al., 2014; Xiao et al., 2015). In addition, the composition of gut bacteria of people at high risk of stroke is also different from that of the normal population. Compared with the low-risk group of stroke, the levels of opportunistic pathogens among the people of high-risk group were found to be higher, and the difference of enterobacteriaceae was the most obvious. The people of low-risk group had higher concentration of butyrate-producing bacteria, such as Lachnospiraceae and Ruminococcaceae (Zeng et al., 2019). These findings may imply that disruption of microbial homeostasis in gut may precede the development of stroke. Therefore, it is feasible to predict and prevent stroke in advance by observing changes in intestinal flora.

There are also differences in gut microbiota among stroke patients of different ages. The incidence of stroke is closely related to age, with about 70–80% of ischemic strokes occurring in people over 65 years of age (Ovbiagele and Nguyen-Huynh, 2011), and age plays an important role in the development and prognosis of stroke (Yager et al., 2006; Manwani et al., 2011). Some pathophysiological processes are associated with aging, such as chronic inflammation and decreased immune function, can affect functional recovery after stroke and lead to poor prognosis in the elderly (Crapser et al., 2016; Ritzel et al., 2018). And On the other hand, the composition of gut microbes can be influenced by environment, disease and eating habits, as well as age and gender differences (Coman and Vodnar, 2020). The composition of the gut microbiota changes and the diversity diminishes as we get older. When the gut microbiome disorders, it has a detrimental effect on normal physiological activity and is also thought to affect age-related neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease (Mulak and Bonaz, 2015; Wasser et al., 2020; Escobar et al., 2022). Studies have shown that age plays an important role in the interaction between gut microbiota and stroke. Bacteroidetes and Firmicutes dominated the gut microbiota of both young and old adults. In older adults, the relative abundance of Firmicutes increased, and the content of SCFAs-producing bacteria and butyrate level decreased significantly (Biagi et al., 2010; Claesson et al., 2011), and intestinal permeability of the elderly was significantly higher than that of the young (Lee et al., 2020), which makes the older more susceptible to inflammatory response. And according to another study in mice, stroke outcomes can be improved in older mice by transplanting microbiota from younger mice. In contrast, after acquiring the microbiome of the older mice, the younger mice increased functional impairment after stroke (Spychala et al., 2018). In addition, age is an independent risk factor for post-stroke infection, the frequency and severity of infection after stroke were higher in the elderly. This may be related to the impaired integrity of the intestinal barrier, the entry of intestinal bacteria into peripheral tissues through the damaged barrier. And another possible explanation is intestinal

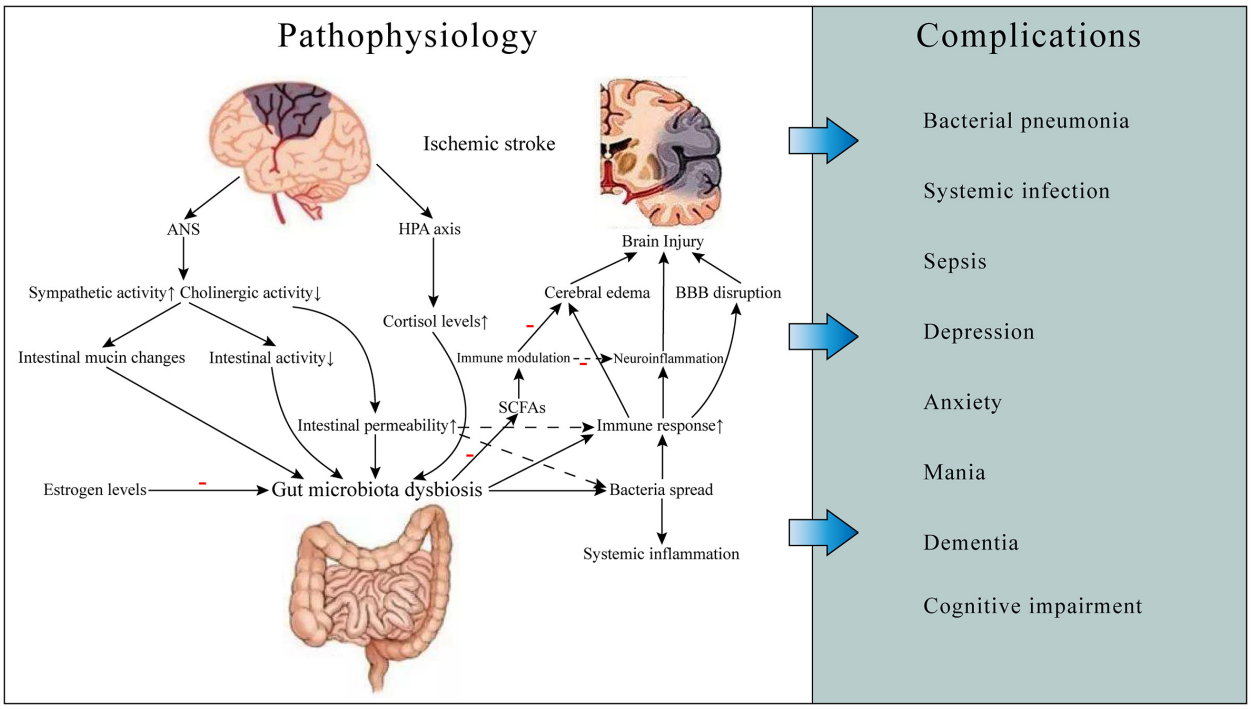

FIGURE 1 | Gut microbiota-related ischemic stroke pathophysiology and complications. Ischemic stroke can cause gut microbiota dysbiosis, which may result in increased gut permeability and worsening brain injury, thereby leading to some complications such as infections and neuropsychiatric disorders and poor prognosis. The mechanisms involved include neuroendocrine pathways, bacterial metabolite, and immune response. ANS, autonomic nervous system; HPA, hypothalamic-pituitary-adrenal; BBB, blood-brain barrier; SCFAs, short-chain fatty acids.

inflammation. Higher levels of pro-inflammatory cytokines were detected in older patients than in younger patients (Crapser et al., 2016; Spychala et al., 2018; Blasco et al., 2020).

In addition, differences in the performance of gut microbiota after stroke also exist between genders. As two common intestinal bacteria, Bacteroidetes and Firmicutes, there are more Firmicutes detected in the males' gut when they had a BMI of less than 33 compared with females. And when BMI is greater than 33, males have an advantage over females in the abundance of Bacteroidetes. In addition, the abundance of Lactobacilli in female is much higher than that in male (Haro et al., 2016). And there are also gender differences in post-stroke outcomes. In some studies, adult females have better recovery outcomes than males after stroke (Toung et al., 1998; Branyan and Sohrabji, 2020). And in middle age (45–55 years), male stroke patients have a higher mortality rate than female stroke patients (Redon et al., 2011). The prognosis of senile stroke women is proved significantly worse. This suggests that estrogen may play a protective role in the development of stroke. In addition, there were gender differences in the expression of bacterial metabolites after stroke. Fecal butyrate levels in male were significantly lower than in female after stroke (Ahnstedt et al., 2020), but LPS was found to be higher in male. After induced stroke, the male mouse model had greater intestinal permeability (Ahnstedt et al., 2020; El-Hakim et al., 2021). This suggests that male patients are more susceptible to intestinal microbiota translocation and post-stroke infection after stroke. There were also differences between male and female in inflammatory responses after stroke. Females expressed more Treg cells, while males had higher concentrations of CD8 + T cells (Jackson et al., 2019; Ahnstedt et al., 2020; Blasco et al., 2020). However, no more studies have clearly proved that gender can cause changes in the composition of intestinal microbiota in stroke patients, so the association between gender and stroke and intestinal microbiota needs further exploration.

Pathophysiological Mechanisms of the Interaction Between Gut Microbiota and Ischemic Stroke

The interaction between gut microbiota and ischemic stroke plays an important role in the occurrence, development and outcomes of stroke. We summarize the relevant pathophysiological mechanisms, including neuroendocrine pathways, bacterial metabolite, and immune response (**Figure 1**). The studies exploring this interaction and relevant mechanisms are listed in **Table 1**.

Neuroendocrine Pathways

Gut-brain-microbiota axis plays an important role in gut microbiota-related stroke pathophysiology. There are several neural pathways for GBMAx communication, such as spinal and vagal pathways, autonomic nervous system (ANS), enteric nervous system (ENS), and hypothalamic-pituitary-adrenal

TABLE 1 | Related studies exploring the relationship between gut microbiota and ischemic stroke.

Researchers and years	Studied species	Related study results	Findings
Caso et al., 2009	Fischer rats	Stress following ischemic stroke resulted in decreased intestinal activity, increased intestinal permeability, translocation of intestinal bacteria, and increased expression of intestinal inflammatory enzymes such as COX-2 and iNOS.	Ischemic stroke can cause increased intestinal permeability and bacterial dispersal through sympathetic activation.
Park et al., 2020	SD rats	Reproductive senescent females had significant gut dysbiosis at baseline and after ischemic stroke compared with adult females. Gut metabolites were differently affected by estrogen treatment in reproductive senescent females and adult females.	Microbial gut can be altered by reproductive senescence in female rats at baseline and after ischemic stroke and estrogen may impact stroke recovery differently in adult and reproductive senescent females due to an age-specific effect on gut microbiota and metabolites.
Chen et al., 2019b	SD rats	Oral administration of non-absorbable antibiotics reduced neurological impairment and the cerebral infarct volume, relieved cerebral edemas, and decreased blood lipid levels by altering the gut microbiota. Ischemic stroke decreased intestinal levels of SCFAs. Transplanting fecal microbiota rich in these metabolites was an effective means of treating the condition.	Interfering with the gut microbiota by transplanting fecal bacteria rich in SCFAs and supplementing with butyric acid were found to be effective treatments for cerebral ischemic stroke.
Chen et al., 2019a	SD rats	The combination of Puerariae Lobatae Radix and Chuanxiong Rhizoma protected the brain-gut barriers by increasing claudin-5 and ZO-1 levels, weakened the gut microbiota translocation by decreasing diamine oxidase, lipopolysaccharide and d-lactate, and effectively improved the neurological function after ischemic stroke.	Ischemic stroke can cause gut microbiota dysbiosis, increase intestinal permeability, disrupte the gut barrier and triggere gut microbiota translocation. The combination of Puerariae Lobatae Radix and Chuanxiong Rhizoma can reduce post-stroke brain damage through relieving the gut microbiota dysbiosis and brain-gut barriers disruption.
Chen et al., 2019c	Cynomolgus monkeys	The levels of the Bacteroidetes phylum and Prevotella genus were significantly increased, while the Firmicutes phylum as well as the Faecalibacterium, Oscillospira, and Lactobacillus genera were decreased after cerebral infarction. Gut-originating SCFAs were significantly decreased 6 and 12 months after cerebral infarction. The increases in plasma LPS, TNF-α, IFN-γ, and IL-6 after cerebral infarction coincided with overgrowth of the Bacteroidetes phylum.	Cerebral infarction induces persistent host gut microbiota dysbiosis, intestinal mucosal damage, and chronic systemic inflammation.
Stanley et al., 2018	C57BL/6 mice	Ischemic stroke induced changes in the gut microbiota in mice, including an increased abundance of Akkermansia muciniphila and an excessive abundance of clostridial species.	Ischemic stroke can induce far-reaching and robust changes to the intestinal mucosal microbiota.
Lee et al., 2020	C57BL/6 mice	Young fecal transplants contained much higher SCFAs levels and related bacterial strains. Aged stroke mice receiving young fecal transplant gavage had less behavioral impairment, and reduced brain and gut inflammation. SCFAs-producers supplement alleviated post-stroke neurological deficits and inflammation, and elevated gut, brain and plasma SCFAs concentrations in aged stroke mice.	The poor stroke recovery in aged mice can be reversed *via* post-stroke bacteriotherapy following the replenishment of youthful gut microbiome *via* modulation of immunologic, microbial, and metabolomic profiles in the host.
Patnala et al., 2017	C57BL/6 mice	Sodium butyrate mediated neuroprotection after ischemic stroke by epigenetically regulating the microglial inflammatory response, *via* downregulating the expression of pro-inflammatory mediators, TNF-α and NOS2, and upregulating the expression of anti-inflammatory mediator IL10, in activated microglia.	Sodium butyrate can epigenetically modify microglial behavior from pro-inflammatory to anti-inflammatory which could mitigate microglia-mediated neuroinflammation after ischemic stroke.
Schulte-Herbruggen et al., 2009	C57BL/6 mice	Peyer's patches in gut revealed a significant reduction of T and B cell counts after cerebral ischemia, while no differences in natural killer cells and macrophages were observed.	Cerebral ischemia may cause changes in intestinal immune cell populations.
Benakis et al., 2016	C57BL/6 mice	Antibiotic-induced alterations in the intestinal flora reduced ischemic brain injury in mice, an effect transmissible by fecal transplants. Intestinal dysbiosis altered immune homeostasis in the small intestine, leading to an increase in regulatory T cells and a reduction in interleukin (IL)-17-positive γδ T cells through altered dendritic cell activity. Dysbiosis suppressed trafficking of effector T cells from the gut to the leptomeninges after stroke.	Gut commensal microbiota may affect ischemic stroke outcome by regulating intestinal γδ T cells.
Sadler et al., 2020	C57BL/6 mice	Microbiota-derived SCFAs modulated post-stroke recovery *via* effects on systemic and brain resident immune cells. SCFAs, fermentation products of the gut microbiome, were potent and proregenerative modulators of post-stroke neuronal plasticity at various structural levels. This effect was mediated *via* circulating lymphocytes on microglial activation.	As a link along the gut-brain axis, SCFAs could be a potential therapeutic to improve recovery after ischemic stroke.

(Continued)

TABLE 1 | (Continued)

Researchers and years	Studied species	Related study results	Findings
Huang J. T. et al., 2021	C57BL/6 mice	Calorie restriction led to better long-term rehabilitation after ischemic stroke in comparison of normal control. Transplantation of gut microbiome from calorie-restriction-treated mice to post-stroke mice was eligible to obtain better long-term rehabilitation of stroke mice.	Calorie restriction conferred improvement effect on long-term rehabilitation of ischemic stroke *via* gut microbiota.
Akhoundzadeh et al., 2018	C57BL/6 mice	Pretreatment with probiotics significantly reduced infarct size by 52% in the mouse MCAO model. Administration of probiotics significantly decreased malondialdehyde content and TNF-α level in the ischemic brain tissue.	Probiotic supplements might be useful in the prevention or attenuation of brain ischemic injury in patients at risk of ischemic stroke.
Sun et al., 2016	C57BL/6 mice	Clostridium butyricum significantly improved neurological deficit, relieved histopathologic change, decreased MDA contents and increased SOD activities in the I/R injury mice. After Clostridium butyricum pretreatment, the expression of Caspase-3 and Bax were significantly decreased, the Bcl-2/Bax ratio was significantly increased, and butyrate contents in the brain were significantly increased.	Clostridium butyricum could exert neuroprotective effects against I/R injury mice through anti-oxidant and anti-apoptotic mechanisms, and reversing decrease of butyrate contents in the brain might be involved in its neuroprotection.
Spychala et al., 2018	C57BL/6 mice	The microbiota was altered after experimental stroke in young mice and resembled the biome of uninjured aged mice. Altering the microbiota in aged to resemble that of young increased survival and improved recovery following MCAO.	The gut microbiota can be modified to positively impact stroke outcomes from age-related diseases.
Wang et al., 2021	C57BL/6 mice	Stroke mice that received gut microbiota from sodium butyrate-treated mice had a smaller cerebral infarct volume than mice that received gut microbiota from NaCl-treated mice. This protection was also associated with improvements in gut barrier function, reduced serum levels of LPS, LPS binding protein, and proinflammatory cytokines, and improvements in the BBB.	The gut microbiota changes of mice aggravated brain injury after ischemic stroke and could be modified by sodium butyrate to afford neuroprotection against stroke injury.
Winek et al., 2016	C57BL/6 mice	When the antibiotic cocktail was stopped 3 days before surgery, microbiota-depleted mice with MCAO had significantly reduced survival compared to MCAO specific pathogen-free and sham-operated microbiota-depleted mice. All microbiota-depleted animals in which antibiotic treatment was terminated developed severe acute colitis. This phenotype was rescued by continuous antibiotic treatment or colonization with specific pathogen-free microbiota before surgery.	Conventional microbiota ensures intestinal protection in the mouse model of experimental stroke and prevents development of acute and severe colitis in microbiota-depleted mice not given antibiotic protection after cerebral ischemia.
Benakis et al., 2020b	C57BL/6 mice	Mice treated with a cocktail of antibiotics displayed a significant reduction of the infarct volume in the acute phase of stroke. The neuroprotective effect was abolished in mice recolonized with a wild-type microbiota. Single antibiotic treatment with either ampicillin or vancomycin, but not neomycin, was sufficient to reduce the infarct volume and improved motor-sensory function 3 days after stroke. This neuroprotective effect was correlated with a specific microbial population rather than the total bacterial density.	Targeted modification of the microbiome associated with specific microbial enzymatic pathways may provide a preventive strategy in patients at high risk for ischemic stroke.
Singh et al., 2016	C57BL/6 mice	Recolonizing germ-free mice with dysbiotic post-stroke microbiota exacerbated lesion volume and functional deficits after experimental stroke compared with the recolonization with a normal control microbiota. In addition, recolonization of mice with a dysbiotic microbiome induced a proinflammatory T-cell polarization in the intestinal immune compartment and in the ischemic brain. Moreover, therapeutic transplantation of fecal microbiota normalized brain lesion-induced dysbiosis and improved stroke outcome.	Acute brain lesions induced dysbiosis of the microbiome and, in turn, changes in the gut microbiota affected neuroinflammatory and functional outcome after brain injury through the brain-gut microbiota-immune axis.
Stanley et al., 2016	Human; C57BL/6 mice	The majority of the microorganisms detected in the patients who developed infections after having a stroke were common commensal bacteria that normally reside in the intestinal tracts. The source of the bacteria forming the microbial community in the lungs of post-stroke mice was the host small intestine.	Stroke promotes the translocation and dissemination of selective strains of bacteria that originated from the host gut microbiota.
Xu et al., 2021	Human; C57BL/6 mice	Enterobacteriaceae enrichment was an independent risk factor for patients with acute ischemic stroke in early recovery. Ischemic stroke induced rapid gut dysbiosis with Enterobacteriaceae blooming. Gut dysbiosis was associated with stroke-induced intestinal ischemia and nitrate production. Enterobacteriaceae exacerbated brain infarction by accelerating systemic inflammation. Inhibiting Enterobacteriaceae overgrowth alleviated brain infarction.	Ischemic stroke rapidly triggers gut microbiome dysbiosis with Enterobacteriaceae overgrowth that in turn exacerbates brain infarction.

(Continued)

TABLE 1 | (Continued)

Researchers and years	Studied species	Related study results	Findings
Tan et al., 2020	Human	TMAO levels showed no significant changes before and within 24 h of acute ischemic stroke treatment but decreased significantly thereafter. Elevated early TMAO levels were associated with poor outcomes of ischemic stroke patients.	TMAO levels decrease with time since stroke onset. Elevated TMAO levels at an earlier period portended poor stroke outcomes.
Zhang J. et al., 2021	Human	Plasma TMAO levels in patients with ischemic stroke were higher than those in controls. Patients with poor outcomes had significantly higher plasma TMAO levels at admission.	Plasma concentrations of gut microbial TMAO are higher in patients with ischemic stroke and related to poor functional outcomes.
Yamashiro et al., 2017	Human	By investigating the gut microbiota and concentrations of organic acids in ischemic stroke patients and normal individuals, it was found that ischemic stroke was independently associated with increased bacterial counts of Atopobium cluster and Lactobacillus ruminis, and decreased numbers of Lactobacillus sakei subgroup. In addition, ischemic stroke was associated with decreased and increased concentrations of acetic acid and valeric acid, respectively.	Gut dysbiosis in patients with ischemic stroke is associated with host metabolism and inflammation.
Zhu et al., 2020	Human	After controlling for potential confounders, multivariable logistic analysis showed that higher level of plasma TMAO was an independent predictor for cognitive impairment in post-stroke patients.	Increasing plasma level of TMAO may be associated with post-stroke cognitive impairment.
Yin et al., 2015	Human	The gut microbiome of stroke and transient ischemic attack patients was clearly different from that of the asymptomatic group. Stroke and transient ischemic attack patients had more opportunistic pathogens. This dysbiosis was correlated with the severity of the disease. The TMAO level in the stroke and transient ischemic attack patients was significantly lower than that of the asymptomatic group.	Stroke and transient ischemic attack patients showed significant dysbiosis of the gut microbiota, and their blood TMAO levels were decreased.
Li et al., 2019	Human	The gut microbiota of ischemic stroke patients had more short chain fatty acids producer than healthy controls.	Ischemic stroke patients show significant dysbiosis of the gut microbiota with enriched short chain fatty acids producer.

COX-2, Cyclooxygenase-2; iNOS, inducible Nitric Oxide Synthase; SCFAs, short-chain fatty acids; TNF, tumor necrosis factor; NOS2, Nitric Oxide Synthase 2; IL, interleukin; TMAO, trimethylamine N-Oxide; MCAO, middle cerebral artery occlusion; MDA, malondialdehyde; SOD, superoxide dismutase; I/R, ischemia/reperfusion; Bcl-2, B cell lymphoma-2; Bax, BCL-2-associated protein X; ZO-1, zonula occludens-1; LPS, lipopolysaccharide; BBB, blood-brain barrier; IFN, interferon.

(HPA) axis (Foster and McVey Neufeld, 2013; Carabotti et al., 2015). The function of gastrointestinal ANS will change after ischemic stroke. The production and release of norepinephrine is increased, and cholinergic activity is decreased, which results in altered intestinal mucin production, inhibiting intestinal activity and increasing intestinal permeability (Caso et al., 2009). This can affect the size and quality of the intestinal mucus layer. As the habitat of most intestinal microbiota, the change of the status of the mucus layer can affect the composition and function of the microbiota. Changes in intestinal permeability induced by stress would lead to the activation of glial cells and mast cells, increased production of interferon, and morphological changes of colonic epithelium. These changes are caused by the expression of reduced tight junction protein 2 and the occlusion of an important component of the intestinal tight junction (Demaude et al., 2006). Increased permeability of the intestinal epithelium will cause bacterial antigens to cross the intestinal epithelium and trigger an immune response, resulting in changes in intestinal flora and systemic effects (Yates et al., 2001). Thus, stroke-induced increases in intestinal permeability can be ameliorated after inhibition of β-adrenergic activity with β -blockers. It can also reduce the risk of bacteria spreading to surrounding organs (Stanley et al., 2016). HPA axis is also an important part of GBMAx and plays a role in enter-brain regulation. HPA axis regulates the body through the interaction of three endocrine glands, including hypothalamus, pituitary and adrenal, which can stimulate the release of steroid hormones such as cortisol under stress. Long-term elevation of serum cortisol can have toxic effects on the nervous system. Serum cortisol levels were found to be associated with stroke severity and post-stroke mortality (Christensen et al., 2004; Barugh et al., 2014). In addition, one study suggested that cortisol levels may be associated with gut microbiota diversity (Keskitalo et al., 2021). Interactions of gut microbiota and HPA axis may explain some severe mental disorders after ischemic stroke (Misiak et al., 2020).

Hormone levels are also thought to play a role in ischemic stroke. Deficiency of estrogen and other ovarian hormones was found to be a risk factor for ischemic stroke in post-menopausal women (Reeves et al., 2008). In animal experiments, different effects of estrogen on the prognosis and recovery of stroke rats were closely related to the age of the rats. Many studies demonstrated that neurological function in young female animals was less affected than in older animals after ischemic stroke. And in the same age groups, female animals showed better post-stroke neurological performance than males (Hall et al., 1991; Alkayed et al., 1998; Manwani et al., 2013). Estrogen can effectively prevent the growth of pathogenic bacteria, promote the growth and reproduction of beneficial bacteria, and maintain a reasonable composition of intestinal microbiota (Chen and Madak-Erdogan, 2016; Baker et al., 2017). There are significant differences in intestinal microbiome and metabolites between young and old women. Akkermansia muciniphila, for example,

is a bacterium that can affect energy regulation, metabolism, and cardiovascular function (Everard et al., 2013; Plovier et al., 2017). In healthy mice, levels of Akkermansia muciniphila were found to be lower in female mice than in male mice. But after a stroke, its levels were significantly elevated in male mice (Stanley et al., 2018; Park et al., 2020). Estrogen therapy in stroke patients can reduce lipopolysaccharide (LPS) production and increase short-chain fatty acids (SCFAs) levels, which helps to inhibit inflammatory response and reduce brain tissue damage after ischemic stroke (Blasco-Baque et al., 2012; Wu et al., 2021). Transplanting fecal microbiome from young women undergoing estrogen therapy to older women with stroke effectively improved their functional outcomes (Park et al., 2020). However, estrogen therapy, which has a protective effect in younger women, increased the risk and severity of stroke in post-menopausal women (Wassertheil-Smoller et al., 2003; Selvamani and Sohrabji, 2010a,b). These studies suggested that estrogen levels could regulate intestinal microbiome homeostasis after ischemic stroke and influence stroke outcomes. But more research is needed to explore the relationship between hormones, age, gut microbiota, and ischemic stroke.

Role of Bacterial Metabolite

Gut microbiota plays an important role in the production and secretion of over 100 metabolites. However, the role of these metabolites in neurological function after ischemic stroke has not been fully studied (Bostanciklioğlu, 2019). SCFAs are the main product that produced by gut microbiota through fermentation of dietary fiber, including acetate, propionate, and butyrate. Recent studies suggested that anaerobic bacteria, such as Firmicutes, produced a great amount of SCFAs by fermenting dietary fiber (Nakamura et al., 2017). SCFAs can also be produced by fermentation of proteins and amino acid, and about 1% of *E. coli* bacteria can produce branched SCFAs (such as isobutyric and isovaleric acids) through this pathway (Smith and Macfarlane, 1997; Louis and Flint, 2017). In addition, acetyl-CoA formed by glycolysis can also be converted to butyric acid by the action of Butyryl-CoA: Acetate-CoA transferase (Duncan et al., 2002). Moreover, SCFAs produced by different species of bacteria also varies. For instance, acetate is metabolized by enteric and acetogenic commensal bacteria (Maa et al., 2010). Propionate is the main metabolite of bacteroides and Firmicutes (Zapolska-Downar and Naruszewicz, 2009). Eubacterium, anaerobe and Faecalis are the main bacteria which produce butyrate (Zapolska-Downar et al., 2004).

Short-chain fatty acids have positive effects on human intestinal function, such as enhancing intestinal motility, reducing inflammatory cell level, and regulating intestinal hormones and neuropeptide levels (Silva et al., 2020). SCFAs are also important for cerebral development and maintenance of normal function of the central nervous system (CNS). They can cross the BBB, and are essential for several processes, such as microglial maturation, intestinal neuron stimulation of ANS, and mucosal serotonin secretion (Braniste et al., 2014; Erny et al., 2015). In addition, SCFAs also play an immunomodulatory role. They can induce T cells to differentiate into effector cells and regulatory cells according to the immune environment (Park et al., 2015).

In the early stage of ischemic stroke, the levels of acetic acid and propionic acid were found to be significantly lower than normal. The concentrations of isobutyric acid and isovaleric acid were increased in the early stage but low in the later stage. Butyric acid and valeric acid were deficient in both early and later stages of ischemic stroke (Chen et al., 2019b). This difference may be related to different metabolic pathways required to produce different SCFAs. Studies have demonstrated that SCFAs have a neuroprotective effect. The lower the concentration of acetic acid, valeric acid, especially butyric acid, in stroke patients, the greater the volume of cerebral infarction and the worse the neurological function score (Chen et al., 2019b). Oral infusion of SCFAs-producing bacteria and inulin reduced neurological deficits and improved post-stroke depression-like behavior in elderly mice (Lee et al., 2020). Butyrate has the function of reducing neurotoxicity, alleviating neuroinflammation, and relieving behavioral disorders. Intestinal butyrate supplementation can improve the level of neurological recovery after brain injury and reduce the volume of cerebral infarction. It was also effective in reducing cerebral edema, lowering blood lipid levels, and reducing the risk of thrombosis (Sharma et al., 2015; Patnala et al., 2017). Butyrate can regulate immune function by inhibiting histone deacetylase (HDAC) and mammalian target of rapamycin (mTOR) signal in circulating leukocytes. Studies have shown that higher concentrations of butyrate in feces or intravenous butyrate solution can enhance the antimicrobial activity of monocytes and macrophages and increase the body's resistance to pathogens (Chakraborty et al., 2017; Haak et al., 2018).

Trimethylamine N-oxide (TMAO) is a kind of metabolic product of gut microbiota, mainly derived from the dietary nutrients rich in phosphatidylcholine, choline, and L-carnitine. First, Gut microbes metabolize foods such as eggs and beef to produce the intermediate trimethylamine by the activity of trimethylamine (TMA) lyases. In the second step, TMA is oxidized to TMAO by hepatic flavin-containing monooxygenases (Bennett et al., 2013). TMAO can induce atherosclerosis by increasing uptake of cholesterol in macrophages and promoting foam cell formation (Wang et al., 2011), enhance platelet hyperresponsiveness and increases the risk of thrombosis by changing stimulus-dependent calcium signal (Zhu et al., 2016). Higher concentrations of TMAO were found to be associated with an increased risk of cardiovascular events (Wang et al., 2011; Koeth et al., 2014; Tang et al., 2014). In addition, high levels of plasma TMAO have been shown to reduce long-term survival in patients with chronic kidney disease (Tang et al., 2015). Notably, a study of TMAO and cardiovascular disease risk in hemodialysis patients showed a significantly higher risk of death in white patients than in blacks (Shafi et al., 2017). This suggests that the effects of TMAO may differ across racial and ethnic groups.

According to the current study, TMAO can aggravate brain injury after ischemic stroke through a variety of pathophysiological processes. In addition to accelerating atherosclerosis and enhancing thrombogenesis potential, it can also promote vascular inflammation and endothelial dysfunction

(Seldin et al., 2016; Boini et al., 2017; Li T. et al., 2017). TMAO also increases oxidative stress, enhances mitochondrial damage, and inhibits mTOR signaling, thereby impairing neural function (Chen et al., 2017; Li D. et al., 2018). TMAO is also a risk factor for hypertension and diabetes, which are linked to ischemic stroke. TMAO levels will rise and then decrease gradually over time after stroke onset. High concentrations of TMAO in patients with early onset are often associated with poor prognosis. Therefore, the measurement of plasma concentrations of gut microbial TMAO in stroke patients is helpful for us to predict the prognosis of patients (Tan et al., 2020; Zhang J. et al., 2019).

Immunological Mechanisms

As a gathering place of immune cells, gastrointestinal tract affects the growth and development of immune cells and plays an important role in regulating immune response (Li et al., 2019). After ischemic stroke, both local neuroinflammatory responses and peripheral immune responses can be activated (Chamorro et al., 2012). It is found that different kinds of immune cells can aggravate the injury or protect the damaged brain tissue, respectively.

After the occurrence of stroke, activation of cerebral resident immune cells such as microglia, astrocytes, neutrophils and macrophages increase the production of pro-inflammatory cytokines, chemokines, proteases, and adhesive proteins (Iadecola and Anrather, 2011; Yang et al., 2019). The activation of inflammatory cells destroys the integrity of BBB, increases the chemotaxis of inflammatory cytokines in cerebral ischemia area, aggravates the damage of brain tissue. Experimental studies have shown that endotoxins metabolized by microbiota, such as LPS, can exacerbate neuroinflammation either directly or by inducing migration of peripheral immune cells to the brain (Lukiw et al., 2018). And raising LPS levels in stroke mice can promote the production of inflammatory factors like interleukin (IL)-6 and tumor necrosis factor (TNF)-α, affects BBB function, increase the neurological impairment, aggravating cerebral edema and reduce life expectancy of the mice (Dénes et al., 2011). It also led to increased plasma levels of pro-inflammatory cytokines that may promote dysregulation of the gut microbiome (Yamashiro et al., 2017). The dysbiosis of intestinal microflora can further increase the production of peripheral inflammatory cytokines. These cytokines can cross the BBB and exacerbate brain ischemic injury (Liu et al., 2020). Rapid dysregulation of intestinal flora in the first 24 h after stroke can promote cerebral infarction through inflammatory response. By inhibiting the overgrowth of Enterobacteriaceae and other opportunistic pathogens in stroke patients, systemic inflammation and cerebral infarction can be effectively reduced (Xu et al., 2021).

Peripheral immune inflammatory cells are involved in the cerebral immune inflammatory response following ischemic stroke and play an important role in the process of brain injury and tissue repair. The main cells involved in the human immune system are B lymphocytes, T lymphocytes, MHC and effector cells (Flajnik and Kasahara, 2010). In ischemic stroke, impaired BBB promotes T infiltration and interferon (IFN)-γ accumulation (Kleinschnitz et al., 2010; Liesz et al., 2011). In animal stroke models, T cell and B cell counts in Peyer's patches decreased within 24 h, and activated T lymphocytes migrate from the Peyer patches of the small intestine or from the intestinal lamina propria to the brain within 2–3 days after stroke, where they primarily located in is leptomeninges (Schulte-Herbruggen et al., 2009). T cells can affect the secretion of cytokines IL-17 and IL-23 (Fan et al., 2020), lead to chemokine production and increased infiltration of cytotoxic cells (neutrophils and monocytes) into brain tissues, and then results in neurotoxic effects on ischemic lesions, resulting in increased infarct volume. Experiments have shown that inhibition of T lymphocyte invasion can effectively reduce the infarct size after stroke (Liesz et al., 2011). Conversely, upregulation of T regulates cell (Treg) level or increases IL-10 concentration can inhibit the production of proinflammatory mediators, thereby reducing the volume of cerebral infarction (Wei et al., 2011; Bodhankar et al., 2015). Similarly, regulatory B lymphocytes may also play a protective role in ischemic stroke by regulating anti-inflammatory factors such as IL-10 and transforming growth factor (TGF)-β (Doyle et al., 2015). Increasing B cell concentration in the brain can reduce infarct volume after stroke (Chen et al., 2012).

Intestinal microbiome dysregulation can reduce systemic anti-inflammatory cytokines such as TGF-β and IL-10 (Yan et al., 2009; Benakis et al., 2016). As a bacterial metabolite, SCFAs can act on immune cells by inhibiting histone deacetylase (HDAC) or by acting as a ligand for G-protein-coupled receptors. After stroke, SCFAs also stimulates the production of colonic Treg cells by producing IL-10 cytokines and TGF-β, and expressing Foxp3 and cell surface markers CD4 and CD25, thereby reducing infarct size (Sadler et al., 2020). In addition, monocytes/macrophages in the intestinal tract of stroke patients can be activated by intestinal flora. Intrusions of intestinal monocytes into the brain can be detected during the acute phase of stroke. Therefore, monocytes/macrophages also play a role in microbiome mediated stroke prognosis (Singh et al., 2016).

Gut Microbiota-Related Complications Following Ischemic Stroke

Post-stroke infection is an important factor causing worsen outcomes of stroke patients, and more than one-third of patient's condition and treatment are complicated by post-stroke infection complications (Emsley and Hopkins, 2008). Increased susceptibility to infection after ischemic stroke is associated with activation of feedback activity between the CNS and peripheral immune organs (Chamorro et al., 2012). And according to current studies, the increased permeability and dysfunction of the intestinal barrier after stroke can cause bacterial migration and spread of the intestinal microbiome, which may be one of the mechanisms of post-stroke infection (**Figure 1**; Stanley et al., 2016).

After ischemic stroke, the sympathetic nervous system is activated, the intestinal permeability is increased, the intestinal barrier is damaged, and the antibacterial function of the body is reduced. These changes promote the transfer of bacteria to extra-intestinal organs, blood or lymph, participates in local and systemic immunity, and may lead to organ infections and

even sepsis (Hagiwara et al., 2014). The β-adrenergic signaling pathway may play an important role (Wong et al., 2011). When β-adrenergic receptors are blocked, the integrity of the intestinal barrier can be inhibited. For example, feeding stroke mice with propranolol can't completely avoid the occurrence of infection, but can obviously reduce the serious situation of systemic tissue infection after ischemic stroke (Stanley et al., 2016).

In an mice experimental study, germ-free mice were modeled by two methods. The results showed that the intestinal microbiota of mice with post-filament middle cerebral artery occlusion model (fMCAo) had significantly imbalance and the diversity was decreased while those in mice with permanent distal middle cerebral artery occlusion model (cMCAo) had relatively little influence. This suggests that the changes in microbiome are secondary to the stroke and the degree of disturbance is affected by the severity of stroke (Singh et al., 2016).

In turn, gut microbiota disturbance may be one of the important causes of enterogenic infection, sepsis even multiple-organ dysfunction syndromes (MODS) (Lyons et al., 2016; Haak and Wiersinga, 2017). After stroke, the destruction of the integrity of the intestinal barrier provides conditions for bacterial migration. Although in healthy individuals a variety of gut microbiomes are also found in blood and lung tissue (Potgieter et al., 2015; Dickson et al., 2016; Li Q. et al., 2018). However, due to the destruction of the integrity of the intestinal barrier, pathologic translocation of intestinal bacteria in stroke patients increases, leading to an increase in the incidence of post-stroke infection (Tascilar et al., 2010).

Bacterial pneumonia is the most common complication of ischemic stroke patients and one of the early nosocomial infections (Langhorne et al., 2000; Hannawi et al., 2013). Blood or sputum culture samples taken from patients with stroke complicated with pneumonia are often absent of the common pathogens that cause pneumonia, or have much less than those found in patients with common pneumonia (Marik, 2001). In one study, prophylaxis with antibiotics in ischemic stroke patients did not reduce the incidence of pneumonia or death compared with untreated patients (Kalra et al., 2015). In contrast, evidence from several studies suggests that post-stroke pneumonia is associated with the transmission of certain bacteria from the patient's gut microbiota. These bacteria, when translocated, become pathogenic strains (Stanley et al., 2016). This suggests that endogenous factors play a more important role in the onset of post-stroke pneumonia than exogenous infection. Changes in intestinal permeability after ischemic stroke can induce bacterial migration and infection. In addition to direct transmission through the small intestine after, gut bacteria can also travel through the portal vein to the liver, where they can spread indirectly to the lungs after filtering through the blood.

Neuropsychiatric disorders are also common complications of stroke, includes depression, anxiety, mania, dementia, and cognitive impairment (Hackett et al., 2014; Hackett and Pickles, 2014). About one-third of patients experience cognitive impairment within a year of a stroke (Li X. et al., 2017). Gut microbiota dysbiosis can be found in many neurological disorders such as Alzheimer's disease and depression (Cho et al., 2021). The high-abundance Prevotella group expressed more negative emotions and reduced hippocampal functional activation than the group with higher levels of bacteroides (Tillisch et al., 2017). In addition, bacterial metabolites have been linked to cognitive function. Ischemic stroke patients who detect higher levels of TMAO experience more severe cognitive impairment (Zhu et al., 2020). SCFAs producing bacteria (such as Lachnospiraceae and Ruminococcus) were significantly reduced in patients with amnestic cognitive impairment (Liu et al., 2021). Another study found similar results. The abundance of Lachnospiraceae, Clostridiaceae, and Ruminococcus was reduced in the high-risk group compared with the low-risk group (Huang Q. et al., 2021). Gut microbiota may aggravate neuropsychiatric symptoms by common pathogenesis, like neuroinflammatory response. These results suggest that it is feasible to predict the occurrence of cognitive impairment after ischemic stroke by intestinal flora.

Gut Microbiota With Other Types of Stroke

Current studies on the gut-brain axis mainly focus on patients with ischemic stroke. Compared with ischemic stroke, there are fewer clinical and experimental studies on the association between hemorrhagic stroke and intestinal flora. The occurrence probability of intracranial hemorrhage (ICH) and subarachnoid hemorrhage (SAH) are less than that of cerebral infarction, but the mortality and disability rate of ICH and SAH are not low. Although the pathogenesis and clinical manifestations of the two types of stroke patients are different, similar results of microbiome disruption were found between the two stroke patients. Some studies have even shown that the stability disruptions of gut microbiota in patients with hemorrhagic stroke or high NIHSS scores are more severe than those with ischemic stroke and TIA (Zeng et al., 2019; Haak et al., 2021). Like ischemic stroke, some mechanisms of action are also at work in ICH patients. According to a case-control study of hypertension patients in China, intestinal bacterial metabolite TMAO levels are strongly associated with stroke. The association between TMAO and hemorrhagic stroke was significantly higher than that of ischemic stroke (Nie et al., 2018). TMAO has also been shown to be closely associated with the prognosis of ICH patients (Zhai et al., 2021). Inflammation has also been found to play an important role in the brain-gut axis in patients with intracerebral hemorrhage. An animal study has demonstrated that dysregulation of gut flora is associated with dysregulation of pro-inflammatory T cell differentiation in mice after intracerebral hemorrhage, which exacerbates neuroinflammatory responses and causes secondary damage to brain tissue. Neuroinflammation was reduced in ICH mice after intestinal transplantation with fecal gut microbiota from healthy mice (Yu et al., 2021). Intestinal disruption also occurred after ICH. Persistent ileal mucosal injury and increased intestinal permeability were observed in ICH mice. This permeability reached its highest level on the 7th day after intracerebral hemorrhage. Intestinal disruption also occurred after ICH. Persistent ileal mucosal injury and increased intestinal permeability were observed in ICH mice. This permeability

reached its highest level on day 7 of intracerebral hemorrhage. Intestinal bacteria can enter the blood circulation through the broken intestinal mechanical barrier, and lead to systemic inflammation, especially pneumonia (Zhang H. et al., 2021).

As the most common cause of SAH, intracranial aneurysms have a prevalence of about 3% in the population and are associated with 80–85% of non-traumatic SAH (Kassell et al., 1990; Vlak et al., 2011). The current study reveals a partial link between intracranial aneurysms and gut microbiota. Destruction of the gut microbiota by antibiotics can reduce the incidence of intracranial aneurysms in mice (Shikata et al., 2019). And in another study, intracranial aneurysm formation can be induced in normal mice after transplantation of feces from patients with an intracranial aneurysm. Further studies revealed a relationship between aneurysms and the abundance of H. hathewayi in the gut (Li H. et al., 2020). This is a group of anaerobic bacteria that can maintain stable levels of serum taurine, which reduces the risk of aneurysm formation and rupture by inhibiting systemic inflammation. Meanwhile, artificial taurine supplementation also reversed the progression of intracranial aneurysms (Li H. et al., 2020). On the other hand, intestinal microbiota can also play an important role in the rupture of aneurysms. Compare the gut microbiota of patients with ruptured aneurysms (RA) and unruptured aneurysms (URA), researchers found that the genus Campylobacter and C. ureolyticus were significantly increased in the RA group patients (Kawabata et al., 2022). This may be related to the more intense inflammatory response and the remodeling or destruction of blood vessel walls induced by Campylobacter (Nilsson et al., 2018; Kushamae et al., 2020).

Cavernous hemangioma (CA) is also a kind of common vascular disorder will cause cerebral hemorrhage. More abundance Gram-negative bacteria O. splanchnicus and lower levels of gram-positive bacteria F. prausnitzii and B. adolescentis can be found in CA patients, compared with the non-CA patients. However, the most valuable combination of bacteria for diagnosing disease and assessing its severity was not found in this experiment. This indicates that the influence of bacteria on CA is not independent but may play a role together with other factors.

A Promising Biomarker for Predicting Stroke Outcomes

Currently, it has been proved that there is a correlation between intestinal microbiota dysbiosis after stroke and the incidence and progression of stroke. Therefore, it is feasible to predict the disease recovery of patients by indicators related to intestinal microbiome. By comparing samples from stroke patients and control group, several studies got the similar conclusions that stroke patients had a reduction in Firmicutes and Bacteroidetes, while the abundance of Proteobacteria was increased. And the difference of composition ratio in the microbiome was correlated with the severity of the disease. The abundance of TMA-producing bacteria was significantly higher, and the levels of intestinal butyrate-producing bacteria decreased in severe patients compared with mild patients. And the metabolites like TMA or butyrate of these bacterias also had a similar situation (Gu et al., 2021; Haak et al., 2021; Xia et al., 2021).

By examining the difference in intestinal microbiota distribution between acute ischemic stroke patients and healthy participants, a study established a Stroke Dysbiosis Index (SDI), as an independent predictor of severe disease (NIHSS $>$ 8) and poor prognosis (MRS $>$ 2) (Xia et al., 2019). Increased abundance of Enterobacteriaceae and Parabacteroides have a correlation with a higher SDI, while the abundance of fecalibacterium, Clostridiaceae, and Lachnospira decreased. In addition, animal studies have shown that mice that received fecal transplants from patients with a high SDI index experienced severe brain damage, increased levels of IL-17 and T cells, and a significantly higher risk of stroke than mice that received normal fecal transplants.

Treatment and Management Strategies Targeting Gut Microbiota for Ischemic Stroke

Dietary Interventions

Dietary regulation is an important measure to improve the prognosis of stroke (**Figure 2**). At the same time, diet, smoking cessation and blood pressure control are also three important interventions to prevent stroke (Hackam and Spence, 2007). A low-fat diet is recommended to reduce the risk of cerebrovascular disease. Many of the guidelines recommend a diet that reduces saturated fat and cholesterol and increases fruit and vegetables. Specifically, it includes vegetables, grains, poultry, fish and nuts, and cuts out red meat, candy and sugary drinks (Juraschek et al., 2017). While meat from different animals has roughly the same amount of cholesterol, red meat is higher in saturated fat and has about four times as much carnitine as chicken and fish. Carnitine and choline can be converted to TMAO by intestinal bacteria, affecting stroke outcomes. Therefore, stroke patients and high-risk patients should avoid foods such as red meat and egg yolks.

In addition, increasing the consumption of fruits and vegetables can increase fiber intake, which can increase the level of SCFAs production. For example, resistant starches (such as whole grains and legumes) and fructo-oligosaccharides (such as bananas, Onions and asparagus), as metabolic food sources for butyric acid producing bacteria, can increase butyric acid production in the gut (Le Blay et al., 1999).

Energy control is an effective way to promote good health and reshape the intestinal symbiotic microbiome. Some studies suggest that energy restriction to 60–70% of the recommended intake is protective against ischemic stroke (Mitchell et al., 2019). The protective effect of energy control on brain injury after stroke may be realized by promoting glycogen metabolism and adiponectin expression (Ciobanu et al., 2017; Zhang J. et al., 2019). And long-term energy control resulted in significant changes in the composition of intestinal flora in mice experiments, especially the enrichment of bifidobacterial (Huang J. T. et al., 2021).

Probiotics and Prebiotics

Probiotics are a group of living gut microorganisms that are widely believed to be beneficial to the host. Probiotics can affect brain function by altering brain neurochemistry. Current studies

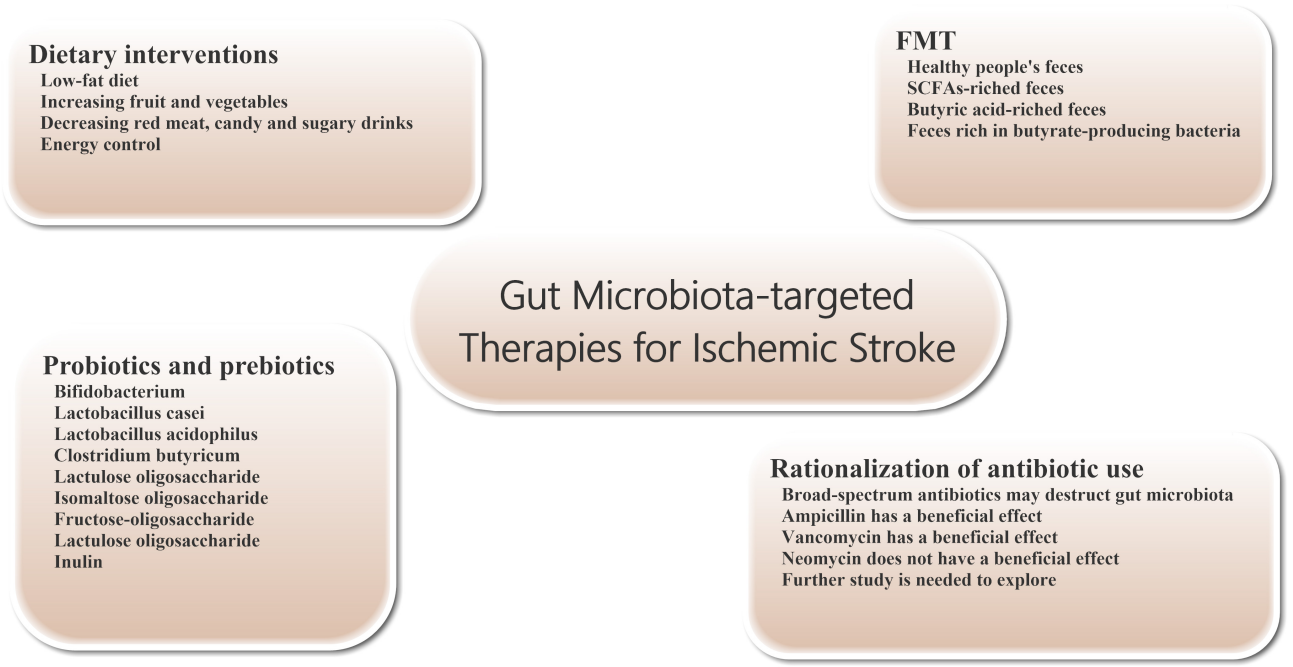

FIGURE 2 | Gut microbiota-targeted treatments and managements for ischemic stroke. Gut microbiota-targeted treatments and managements can be considered for patients with ischemic stroke, including dietary interventions, probiotics and prebiotics supplementation, FMT, and rationalization of antibiotic use. FMT, fecal microbiome transplantation.

have shown that probiotics supplementation can effectively reduce or prevent brain tissue damage in stroke patients. Probiotics may protect tissue from damage by reducing the production of oxygen free radicals and inflammatory cytokines. For example, probiotics can inhibit the production of TNF-α *in vivo*, promote the generation of anti-inflammatory cytokines, and improve the activity of antioxidant enzymes (Luo et al., 2014; Abhari et al., 2016). The severity of brain tissue damage in mice after focal cerebral ischemia was significantly reduced by 2 weeks of daily intake of probiotics such as bifidobacterium, Lactobacillus casei, Lactobacillus bulgaricus and Lactobacillus acidophilus (Akhoundzadeh et al., 2018). Pretreatment with Clostridium butyricum can effectively inhibit apoptosis and enhance antioxidant enzyme activity in rat cerebral ischemia model, thereby improving prognosis (Sun et al., 2016). In addition, regular consumption of lactobacillus probiotics can also alter the expression of brain-derived neurotrophic factor (BDNF) receptors and increase BDNF levels in the brain. There is evidence that elevated levels of BDNF in the brain have a protective effect on ischemic stroke (Bercik et al., 2011; Liang et al., 2015).

Prebiotics are oligosaccharides with no biological activity, such as lactulose oligosaccharide, isomaltose oligosaccharide, fructose-oligosaccharide, lactulose oligosaccharide and inulin, which can stimulate the growth and reproduction of beneficial bacteria in the intestine without being digested by intestinal metabolism. After entering the intestinal tract (mainly the lower digestive tract or colon), prebiotics can be hydrolyzed and used as nutrients by the beneficial bacteria in the intestinal tract, such as bifidobacterium, and promoting the reproduction and growth of these bacteria. In addition, prebiotics can also affect the production of SCFAs and regulate the production of mucin, thus enhancing the phagocytosis of macrophages (Markowiak and Śliżewska, 2017). In one study, prebiotics effectively reduced the incidence and severity of pneumonia during hospitalization in critically ill patients. Therefore, we believe that the use of prebiotics can play a certain role in alleviating ischemic stroke patients' condition and the onset of infectious complications (Barraud et al., 2013).

Fecal Microbiome Transplantation

The transfer of the entire microbiome from the stool of a healthy donor to the patient's gastrointestinal tract is known as fecal microbiome transplantation (FMT). The technique is already being used to treat patients with severe infections, such as refractory bronchiolitis and pseudomembranous colitis (Eiseman et al., 1958; van Nood et al., 2013). In addition, FMT intervention can also relieve symptoms in patients with Parkinson's disease and reduce autism in children with autism disorder (Aroniadis and Brandt, 2013; Kang et al., 2017). However, because the gut microbiome also has the potential to cause disease, it is important to select suitable healthy people as FMT donors. Transplantation SCFAs-riched feces (particularly butyric acid) can regulate the composition of intestinal microbes, increase lactobacillus species and enhance microbial activity, maintain intestinal wall integrity and reduce intestinal wall permeability, thereby reducing intestinal leakage in patients with ischemic stroke. These positive effects are beneficial to

maintain the integrity of BBB and improve the functional status of brain tissue in ischemic stroke patients (Singh et al., 2016; Chen et al., 2019a,b). For example, transplanting gut microbiota from young mice can improve stroke outcomes in older mice (Spychala et al., 2018). Transplantation of feces rich in butyrate-producing bacteria has also been shown to reduce ischemic stroke injury in diabetic mice (Wang et al., 2021).

Rationalization of Antibiotic Use

In clinical work, about 30% of patients with stroke will have bacterial infection within 1 week of onset (Westendorp et al., 2011), so a significant number of patients receive prophylactic anti-infective therapy with antibiotics, which often include a combination of broad-spectrum antibacterial drugs. However, to date, there is no clear evidence that prophylactic use of antibiotics after stroke benefits patient outcomes. Compared with standard treatment regimen, prophylactic use of antibiotics in stroke patients did not improve the long-term neurological status or mortality and had no significant effects on the incidence of post-stroke complications such as pneumonia. In some patients, intestinal microbiota damage can promote immune suppression, which increases the probability of facultative or mandatory bacterial re-invasion and increases the risk of infection (especially pneumonia) (Kalra et al., 2015; Westendorp et al., 2015). Further studies have shown that extensive destruction of the gut microbiome by untargeted use of broad-spectrum antibiotics after ischemic stroke can worsen stroke outcomes (Benakis et al., 2016; Winek et al., 2016). Studies have shown neuroprotective effects on brain tissue in stroke mice treated with ampicillin or vancomycin. A similar neuroprotective effect was not observed with neomycin (Benakis et al., 2020a,b). These different effects may be related to changes in the composition of intestinal flora. Therefore, further work is needed to explore whether specific antibiotics can have a beneficial effect on the prognosis of patients with ischemic stroke.

CONCLUSION AND PERSPECTIVE

As the most common type of stroke, treatment options for ischemic stroke remain limited despite extensive research. New insights have highlighted the role of gut microbiota in the pathophysiology of ischemic stroke. Ischemic stroke could cause gut microbiota dysbiosis as well as translocation and dissemination of gut microbiota-derived selective strains of bacteria. In turn, changes in gut microbiota affect ischemic stroke-induced brain injury and determine stroke outcomes through multiple mechanisms, including neuroendocrine pathways, bacterial metabolite, and immune response. Gut microbiota dysbiosis may also contribute to some stroke complications such as pneumonia, sepsis, and neuropsychiatric disorders. Some gut microbiota-targeted therapies have shown potential in the treatment and management of ischemic stroke, including dietary interventions, probiotics supplementation, FMT, and rationalization of antibiotic use. Gut microbiota is expected to provide new perspectives for ischemic stroke treatment. However, the efficacy and safety of this treatment strategy for ischemic stroke have not been verified in large scale clinical trials. In addition, it must be recognized that gut microbiota dysbiosis is only one component of the multifactorial brain injury mechanisms of ischemic stroke. Further studies are necessary to broaden our knowledge of the role of gut microbiota in the pathogenesis of ischemic stroke and to facilitate the development of novel therapeutic strategies for ischemic stroke.

AUTHOR CONTRIBUTIONS

ZB wrote the manuscript. ZZ directed the writing of the manuscript, revised the manuscript, and made the figures and the table. GZ and AZ checked the manuscript. AS proposed the idea. FZ supervised the writing of the manuscript. All authors approved the submitted version.

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REVIEW
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Secondary Brain Injury by Oxidative Stress After Cerebral Hemorrhage: Recent Advances

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Intracerebral hemorrhage (ICH) is a clinical syndrome in which blood accumulates in the brain parenchyma because of a nontraumatic rupture of a blood vessel. Because of its high morbidity and mortality rate and the lack of effective therapy, the treatment of ICH has become a hot research topic. Meanwhile, Oxidative stress is one of the main causes of secondary brain injury(SBI) after ICH. Therefore, there is a need for an in-depth study of oxidative stress after ICH. This review will discuss the pathway and effects of oxidative stress after ICH and its relationship with inflammation and autophagy, as well as the current antioxidant therapy for ICH with a view to deriving better therapeutic tools or targets for ICH.

Keywords: cerebral hemorrhage, oxidative stress, research progress, Nrf2, heme oxygenase

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INTRACEREBRAL HEMORRHAGE

Intracerebral hemorrhage (ICH) is the second most common type of stroke in the world, accounting for 10–15% of all strokes (Feigin et al., 2009; Steiner et al., 2014; Kim and Bae, 2017). The early brain damage from ICH is mainly caused by the mass effect from the formation of a hematoma. However, the patient's clinical symptoms are not alleviated or improved when bleeding stops. Therefore, the study of the mechanism of secondary injury occurring after ICH has led to a more extensive and in-depth exploration and discussion among researchers (Babu et al., 2012). Secondary brain damage after ICH mainly comes from processes such as oxidative stress and inflammation (Aronowski and Zhao, 2011). After ICH occurs, blood components quickly migrate from the vessel, including red blood cells, white blood cells, macrophages, and hemoglobin. Additionally, the divalent iron ions produced during hemoglobin cleavage can promote the formation of free radicals, leading to the occurrence of oxidative stress (Xiong et al., 2014). The body's oxidative stress response can then activate nuclear factor-κB (NF-κB), a key regulator of inflammation, inducing the expression of proinflammatory cytokines (Khaper et al., 2010; Hu W. et al., 2015). Proinflammatory cytokines may also participate in promoting the generation of free radicals, leading to a malignant positive feedback loop (Duan et al., 2016).

PATHOPHYSIOLOGICAL MECHANISMS OF ICH

The pathophysiology of ICH is complex and includes the totality of the brain injury, from early hematoma formation and expansion to SBI from oxidative stress, inflammatory response, mitochondrial dysfunction, and cell death. In contrast, oxidative stress is the main source of SBI in ICH.

Mitochondrial Dysfunction and Cell Death

As a structure playing an important role in the redox homeostasis of the whole cell (Georgieva et al., 2017), mitochondria are inextricably linked to the pathophysiological mechanisms associated with SBI in ICH (Chen W. et al., 2020). It has been shown that the incidence of mitochondrial dysfunction in the hematoma area is significantly higher during SBI and that mitochondria with normal functional activity play a significant role in the maintenance of neuronal survival (Kim-Han et al., 2006; Diao et al., 2020; Chen et al., 2021), indicating that mitochondrial dysfunction is closely related to SBI. Cell death after ICH is mainly divided into two categories: apoptosis and necrosis. Numerous preliminary and clinical studies have shown that apoptosis is involved in the physiopathological process of ICH (Chu et al., 2014; Salihu et al., 2016; Zille et al., 2017). After ICH, substances such as inflammatory cytokines that are released in response to oxidative stress activate cysteinases. They injure or kill cells through cysteinase-dependent or independent pathways, allowing apoptosis to occur. The mechanical compression of adjacent tissues by the hematoma and the accumulation of excess glutamate after ICH cause activation of N-methyl-d-aspartate (NMDA) receptors. large influx of Ca^{2+}, intracellular Ca^{2+} overload, and consequent mitochondrial dysfunction occur. Eventually, cellular necrosis occurs due to insufficient ATP production by mitochondria (Zhang et al., 2022).

Oxidative Stress and ROS

Oxidative stress is a state in which oxidant and antioxidant effects in the body are out of balance. Under this state, the body produces excessive free radicals, which cause serious oxidative damage to cells, eventually damaging cell vitality or bringing about cell apoptosis (Sinha et al., 2013). The main component of free radicals in organisms is active oxygen, which includes hydroxyl radicals (·OH), superoxide anions ($O^{\cdot 2-}$), hydrogen peroxide (H_2O_2), and other substances. These are normally found in low concentrations in the body and participate in redox reactions. In low concentrations, part of the active oxygen can also participate in the regulation of the signal transduction pathway, as the role active oxygen plays in insulin signal transduction. In cases with excessively high concentrations, active oxygen may cause lipid oxidation or the oxidation of quality proteins and DNA, and which ultimately, may promote cell apoptosis or death (Steinbrenner and Sies, 2009; Qu et al., 2016). Meanwhile, reactive oxygen species (ROS) is one of the main influencing factors for SBI in ICH (Duan et al., 2016; Qu et al., 2016), and the generation and accumulation of excessive ROS can cause adverse outcomes, such as macromolecular damage, impaired cell signaling, cell death, and tissue damage, which can lead to further SBI (Forrester et al., 2018). In a normal physiological state, ROS would be in a dynamic equilibrium in the body (Turrens and Boveris, 1980), whereas after the occurrence of ICH, a large amount of ROS is generated, and the accumulation of ROS in excess leads to oxidative stress, aggravating SBI (Duan et al., 2016). In addition to this, excess ROS will cause damage to the normal function of mitochondria, producing mitochondrial dysfunction and leading to the generation of additional ROS, which leads to cascading cell damage. This makes the degree of SBI after ICH worse (Qu et al., 2016).

Abbreviations: ARE, Antioxidant response element; BBB, Blood–brain barrier; DFO, Desferrioxamine mesylate; HO, Heme oxygenase; ICH, Intracerebral hemorrhage; MPO, Myeloperoxidase; MSC, Mesenchymal stem cells; ROS, Reactive oxygen species; SBI, Secondary brain injury; SOD, Superoxide dismutase.

OXIDATIVE STRESS AFTER ICH

Oxidative stress plays an essential role in the development of ICH, and there is a causal relationship between the excess increase in free radicals and the damage caused by ICH (Aronowski and Zhao, 2011). After the occurrence of ICH, there is a massive increase in ROS, an imbalance between oxidation and antioxidation occurs. This results in oxidative stress, which leads to damaged brain cells and the destruction of the blood–brain barrier (BBB) (Qu et al., 2016).

Sources of Free Radicals After ICH

There are many sources of free radicals after ICH (**Figure 1**). After bleeding, blood cells dissolve and hemoglobin is released. Simultaneously, the heme in the degradation product of hemoglobin is decomposed into iron, carbon monoxide, and biliverdin under the action of heme oxygenase (Maines, 1997; Wang and Doré, 2007b). The excess iron produced in extracellular space will have harmful effects. The Haber-Weiss reaction will occur under the catalysis of free iron, resulting in enhanced damage to neurons. At the same time, highly reactive toxic hydroxyl radicals are produced in the process. With this, oxidative stress and cell death will occur, resulting in lipid peroxidation, and excitotoxicity will also be enhanced (Regan and Panter, 1996; Goldstein et al., 2003). Moreover, inflammation is another major source of ROS after cerebral hemorrhage. When ICH occurs, blood components will penetrate the injured site. The inflammatory reaction occurs rapidly, leading to the activetion of various inflammatory cells (Wang and Tsirka, 2005a; Wang and Doré, 2007a). Meanwhile, a large number of cytokines, chemokines, and free radicals are released by activated inflammatory cells such as microglia (Aronowski and Hall, 2005; Wang and Tsirka, 2005a; Wang and Doré, 2007a; Gao et al., 2008). Microglia produce two different phenotypes: the M1 phenotype, which is activated by the classical pathway, and the M2 phenotype, which is activated alternately. Microglia of the M1 phenotype can be considered as pro-inflammatory cells. After ICH, microglia of the M1 phenotype secrete large amounts of ROS and pro-inflammatory factors. Microglia of the M2 phenotype are currently considered as protective cells, secreting anti-inflammatory factors and upregulating neuroprotective factors. Study showed that most of the newly recruited microglia at the injury site were M2 phenotype, while M1 phenotype dominated about 1 week after injury. This phenotype shift from M2-dominant to M1-dominant may result from a M2-to-M1 conversion within activated microglia. The imbalance in the number of the two phenotypes produced leads to the accumulation of ROS and a high production of pro-inflammatory factors (Hu W. et al.,

2015). Moreover, the activation of neutrophils also result in the activation of NADPH Oxidase (Nox) and production of ROS (Joice et al., 2009). In addition to the main sources mentioned above, another source of ROS after ICH is mitochondrial dysfunction (Kim-Han et al., 2006; Swanson, 2006). The excess iron produced after ICH will induce mitochondrial dysfunction and produce oxidative damage. Mantle et al. demonstrated that impaired mitochondrial function leads to a significant increase in ROS (Mantle et al., 2001). At the same time, the release of inflammatory mediators and metalloproteinases also mediates oxidative damage (Gong et al., 2000; Alvarez-Sabín et al., 2004). Furthermore, after ICH, glutamate is released into the blood and interacts with N-methyl-D-aspartate receptors, leading to Ca^{2+} overload in the mitochondria (Sharp et al., 2008). Activated α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors may further promote mitochondrial Ca^{2+} overload (Joshi et al., 2015). The loading of mitochondrial Ca^{2+} reduces its transmembrane potential and opens the mitochondrial permeability transition pore (MPTP), which destroys the mitochondria and mitochondrial respiratory chain. This then leads to the release of ROS (Mracsko and Veltkamp, 2014). Besides, the activation of MPTP changes the mitochondria. The internal redox environment promotes the release of active oxygen by positive feedback, forming the "active oxygen-induced active oxygen release" (RIRR) (Zorov et al., 2014).

Effects of Oxidative Stress After ICH

The excessive generation of free radicals is one of the main attributer in the formation of brain damage after ICH. After the occurrence of ICH, a major number of free radicals are produced, while excessive consumption of superoxide dismutase (SOD) occurs at the same time. It result in the occurrence of lipid peroxidation (Chi et al., 2014), which can by oxygen free radicals causing cell damage. Brain is sensible to oxygen free radicals with rich lipid content. Lipids are subject to peroxidation in the presence of large amounts of oxygen free radicals, causing damage to cells and further leading to membrane damage. Damage to the membrane leads to its increased permeability and increased influx of Ca^{2+}(Toda et al., 2009). The activity of membrane proteins such as Na^{+}/Ca^{2+} exchangers is inhibited by cross-linking and polymerisation of membrane lipids due to lipid peroxidation (Eigel et al., 2007), further increasing the concentration of intracellular calcium. The absorbed calcium combines with phosphate to form insoluble calcium phosphate, which affects the process of mitochondrial oxidative phosphorylation and reduces the production of ATP (Li et al., 2014). Because of the increase in Ca^{2+} concentration, phospholipase is activated, and membrane phosphorylation occurs in cell and organelle membranes (Chrissobolis et al., 2011; Gu et al., 2011). Free radicals cause a large amount of damage to neuron. It not only damages the cell membrane, but also may lead to disruption of cellular DNA (Mantle et al., 2001). Free radicals react with the components of DNA molecules, and this can cause damage to the purine and pyrimidine bases and deoxyribose backbone (Dizdaroglu et al., 2002). The DNA damage may cause transcriptional arrest, signal transduction pathway induction, replication errors, and genome instability, leading to neuronal apoptosis (Marnett, 2000; Cooke et al., 2003). Numerous studies have shown that free radicals are closely to pathophysiological process to ICH. Malondialdehyde (MDA) is a product of free radicals, whose concentration level is often used to evaluate the protein oxidation degree of damage. Studies have shown that the level of MDA increases after ICH. The increased levels of MDA can induce the apoptosis of neuron and glial, indicating that an oxidative stress response promotes the occurrence of ICH-induced brain damage (Wagner et al., 2002; Han et al., 2008).

Endogenous Antioxidant Mechanisms in ICH

Antioxidant Mechanism of Heme Oxygenase

HO (heme oxygenase system) is composed of two definite forms: oxidative stress-inducing protein HO-1 and constitutive isoenzyme HO-2.HO-1 is produced under the induction of microglia/macrophages. While HO-2 is usually dominant in the expression of neurons and is the main cause behind the activity of HO in the brain (Muñoz-Sánchez and Chánez-Cárdenas, 2014). As a part of hemoblood degradation, microparticles constitutes a HO system. Under normal circumstances, the HO system regulates hemoglobin levels and protects cells from the harmful effects of intracellular free hemoglobin (Yoshida and Migita, 2000; Kikuchi et al., 2005; Kumar and Bandyopadhyay, 2005). In fact, after the occurrence of ICH, the lysis of red blood cells within the hematoma leads to release of hemoglobin, which is later broken down into heme. The toxic effect of heme causes secondary damage in ICH. Serum proteins and albumin can bind to hemoglobin and prevent toxic effects. However the amount of hemoglobin released from the hematoma is too large, the natural defense mechanism mentioned above cannot completely remove the hemoglobin released from the hematoma (Robinson et al., 2009). After ICH, HO promotes the decomposition of heme to produce carbon monoxide, iron, and other substances (Maines, 1997; Wang and Doré, 2007b), while excess iron is a component of SBI after ICH (Xi et al., 2006; Keep et al., 2012; Jin et al., 2013). However, the effects of these two types of HO after ICH are variable, they will be affected by the type of model and type of cells involved, producing different results (Chen-Roetling et al., 2015). It was revealed that in a collagenase-induced ICH model, although HO-1 induced early brain damage in ICH, neurological function improved over time in WT mice, while it remained unchanged in HO-1-deficient mice. This is because activated microglia/macrophages are a key factor in the rapid clearance of dying cells and elimination of haematomas, as well as an important supplier of neuroprotective molecules. The enhanced HO-1 activity found experimentally may be necessary for the optimal function of ICH-activated microglia/macrophages. This fact may confirm the protective effect of HO-1 induction in the WT group during ICH recovery (Wang and Doré, 2007b). In a therapeutic study of ICH with sulforaphane, HO-1 was upregulated in response to sulforaphane, resulting in a significant reduction in brain damage, and there was a significant correlation between the two (Zhao et al., 2007). At the same time, the

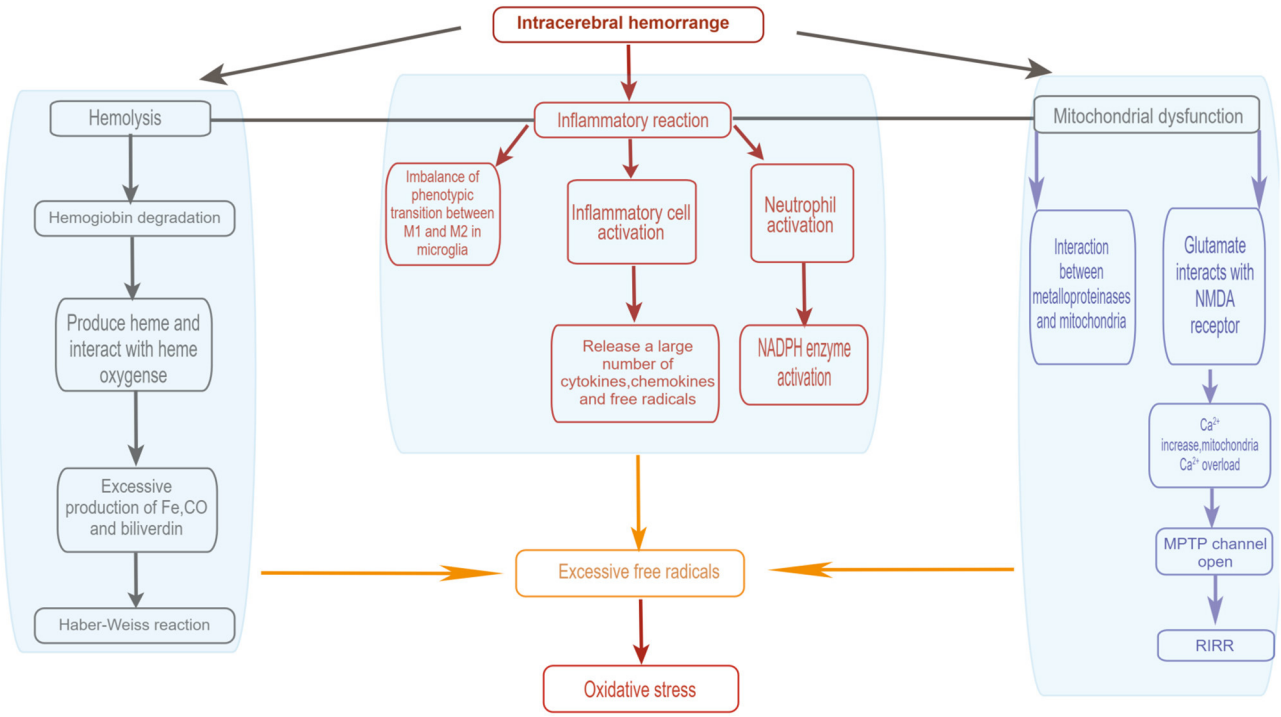

FIGURE 1 | Sources of oxidative' stress after intracerebral hemorrhage. (1) Activated inflammtory cells include leukocytes, macrophages and microglia. (2) After ICH, glutamate is released into the bloodstream, and glutamate interacts with NMDA receptors, leading to an increase in Ca^{2+} concentration and thus Ca^{2+} overload in the mitochondria, which opens the mitochondrial permeability transition pore (MPTP) and disrupts the mitochondrial respiratory chain, resulting in the release of reactive oxygen species. (3) RIRR, ROS-induced ROS release. By Figdraw (www.figdraw.com).

role of HO-2 is more ambiguous. *In vitro* studies showed that knocking out HO-2 can play a role in resisting hemoglobin or heme damage and protecting mouse neurons (Rogers et al., 2003; Regan et al., 2004). However, collagenase injection to ICH mice model, HO-2 knockout, it's had little of any neuronal protection (Wang et al., 2006; Chen-Roetling et al., 2013). Therefore, the antioxidant effect of the HO system after ICH needs to be viewed dialectically. More in-depth research is needed to see if it can play a more beneficial role in the treatment of ICH.

Antioxidant Mechanism of Superoxide Dismutase

Superoxide dismutase (SOD) is an important antioxidant enzyme that plays a role in maintaining O_2^- homeostasis in the antioxidant defense system (Batinic-Haberle et al., 2014). SOD as a system for maintaining homeostasis of O_2^- levels, is composed of three isoforms. Cu/ZnSOD (SOD1) in the cytoplasm, MnSOD (SOD2) in the mitochondria, and Cu/ZnSOD (SOD3) located outside the cell (Fukai and Ushio-Fukai, 2011). Superoxide radicals are first detoxified in the cytoplasm by the action of SOD1 and SOD2, followed by further detoxificatial into water (Valko et al., 2006).

SOD1 deficiency increases superoxide and produces vascular dysfunction in large arteries and microvessels, exacerbates vascular dysfunction produced by angiotensin II, and increases matrix metalloproteinase 9 (MMP-9) expression and activation (Gasche et al., 2001; Didion et al., 2002, 2005). In contrast, SOD1 overexpression will reduce oxidative stress, attenuate the induction and activation of MMP-9, and prevent the development of vascular dysfunction (Morita-Fujimura et al., 2000; Didion et al., 2005). In an ICH model experiment, the work of Yoshinobu et al. verified the inference that overexpression of SOD1 reduce superoxide production and decrease secondary damage in ICH (Wakisaka et al., 2010).

Brain endothelial cells contain a greater number of mitochondria. SOD2 is mainly found in mitochondria. Schroeter et al. design two types of astrocytes and endothelial cells co-culture models. The results showed that the activity of SOD2 in endothelial cells increased after incubation with astrocytes for 48 h. It indicates astrocytes induce SOD2 in brain endothelial cells by either direct contact or exchange of soluble factors. Astrocytes induce BBB properties in brain endothelial cells, and high SOD activity is a prerequisite for normal BBB function (Schroeter et al., 2001). Consequently, SOD2 may have an important share in the protective mechanism of the cerebrovascular system. In the study by Faraci et al. SOD2 reduced brain damage by reducing superoxide production and protecting cells from oxidative stress (Faraci et al., 1985).

In ICH animal experiments, the levels of both SOD1 and SOD2 content in the ipsilateral striatum were reduced and the free radical scavenging system was impaired, leading to the development of neurological impairment in rats, which reflects the development of oxidative damage in the brain after ICH

(Wu et al., 2002). In contrast, in clinical studies, the antioxidant system was impaired after the onset of ICH. The impaired free radical scavenging system after ICH correlates with the decrease in SOD levels (Aygul et al., 2005; Chen et al., 2014). In conclusion, the antioxidant mechanism of SOD has important research significance in SBI after ICH.

Antioxidant Mechanism of Nrf2

Nrf2 belongs to one of the basic regions of the leucine zipper proteins and is the main genomic regulator of the cellular antioxidant defense system. It can participate in the regulation of the antioxidant processes of HO and SOD (Zhao and Aronowski, 2013). Activated Nrf2 is released from Keap1 to increase cell protection and the expression levels of antioxidant target genes so that the cell's defense against oxidative stress is enhanced (van Muiswinkel and Kuiperij, 2005; Kensler et al., 2007). Studies have shown that the activation of Nrf2 plays an important protective role in the occurrence of oxidative stress after an ICH. In mice lacking Nrf2, the damage caused by oxidative stress after ICH is more obvious (Zhao et al., 2007). ROS released by oxidative stress can reduce the damage caused after ICH by activating the Keap1/Nrf2/ARE pathway (Gasche et al., 2001; Wang et al., 2007; Zhao et al., 2007) (**Figure 2**). As the main regulator, the Keap1-Nrf2 pathway can protect cells against endogenous and exogenous stress caused by ROS and electrophiles (Kansanen et al., 2012). Keap1 can bind to Nrf2 and promote its degradation through the ubiquitin proteasome pathway, which has a negative regulatory effect on Nrf2. When Nrf2 is exposed to ROS, it dissociates from Keap1 and transfers to the nucleus. Simultaneously, it activates the antioxidant response element (ARE) that mediates cell survival so as to drive the expression of the target gene of Nrf2 (Kansanen et al., 2013; Qaisiya et al., 2014). Experiments showed that Nrf2 increased significantly at 22 h after the occurrence of ICH, while Keap1 showed a corresponding decrease (Wada et al., 1970). Hence, Keap1 is inhibited and Nrf2 is activated after the occurrence of ICH(Zhao et al., 2007). In addition, results have shown that the degree of brain damage in mice with Nrf2 knockout is more serious, which can indicate a neuroprotective effect of Nrf2 after ICH (Wang et al., 2007; Zhao et al., 2007).

Haptoglobin (Hp) is a glycoprotein that can form a highly stable Hb–Hp complex with free Hb (Wada et al., 1970). Free Hb produced by oxidative stress after ICH may cause strong lipid peroxidation, oxidative DNA damage, and neuronal death (Sadrzadeh et al., 1984; Keep et al., 1998; Wang et al., 2002), so the toxic effect of Hb may be prevented by Hp's neutralization of Hb. It has been shown that after ICH, the levels of Hp in the plasma of animals treated with Nrf2 activators increased significantly, the activation of Nrf2 may be related to an increase of Hp levels (Zhao and Aronowski, 2013). Nrf2 may achieve a protective effect of brain injury after ICH by regulating the level of Hp content in the brain.

At the same time, since excess heme produced after ICH can also have toxic effects, and hemopexin is a protein that can bind to heme with a strong affinity (Nikkil et al., 1991; Tolosano and Altruda, 2002). Reduce of free heme can be achieved by binding hemopexin and heme, thus alleviating the oxidative damage and toxic effects caused by excess heme. There has been evidence that the activation of Nrf2 can enhance the expression of hemopexin (Shen et al., 2006; Kristiansson et al., 2007), which subsequently has a positive effect on clearing out heme (Zhao and Aronowski, 2013).

Other Pathway That Have Antioxidant Effect

Phosphatidylinositol 3-kinase (PI3-K) is an enzyme with serine/threonine protein kinase activity that is involved in immune cell activation signaling and activation. Akt is a widely expressed cytoplasmic serine-threonine kinase that plays an important role in cell survival and apoptosis. The PI3K/Akt pathway is a signaling pathway that is important for neuronal survival regulation (Dudek et al., 1997). Previous studies have shown that PI3K/Akt is a neuroprotective signaling possesses antineuroinflammatory, antioxidative stress, and antiapoptotic properties (Tu et al., 2016; Lv et al., 2017). Therefore, researchers have investigated the role of this pathway in SBI after ICH, showing that activation of the PI3K/Akt pathway by different targets after the occurrence of ICH leads to positive results in terms of the attenuation of neuroinflammation and oxidative stress. It demonstrates the neuroprotective role of the PI3K/Akt pathway in SBI after ICH (Zhao et al., 2019; Chen S. et al., 2020).

ASSOCIATION BETWEEN OXIDATIVE STRESS AND OTHER RESPONSES AFTER ICH

Interaction Between Oxidative Stress and Autophagy After ICH

Autophagy is a degradation process that removes damaged organelles or misfolded proteins. This process can help maintain cell homeostasis and eliminate the damage caused by oxidative stress or protein toxic stress (Jiang et al., 2015). Experimental results have shown that the overload of iron produced under oxidative stress may play a key role in inducing autophagy caused by ICH (He et al., 2008). Previous studies have shown that autophagy act as a negative feedback in the pathological process of ICH (He et al., 2008; Lee et al., 2009; Hu et al., 2011; Wang et al., 2012; Liu et al., 2014). ROS and other free radical substances generated by oxidative stress may induce autophagy. On the other hand, the corresponding stress products can be cleared by autophagy to reduce brain damage (Filomeni et al., 2015; Ismail et al., 2020). The protein p62 binds to ubiquitinated protein in the process of autophagy and transfers it to the autophagosome, which promotes selective protein degradation (Jiang et al., 2015). Phagocytosis regulated by the p62 pathway has a corresponding protective effect on oxidative stress response to brain injury, demonstrating the regulatory effect of autophagy on oxidative stress response after ICH (Rubio et al., 2014). In addition, a series of findings suggest that autophagy can regulate ROS content through the p62 pathway, the chaperone-mediated autophagic pathway, and the mitochondrial pathway. These findings provide a more in-depth theoretical basis for the pathogenesis of cerebral hemorrhage, but due to the diversity of its internal molecular regulatory mechanisms, its specific

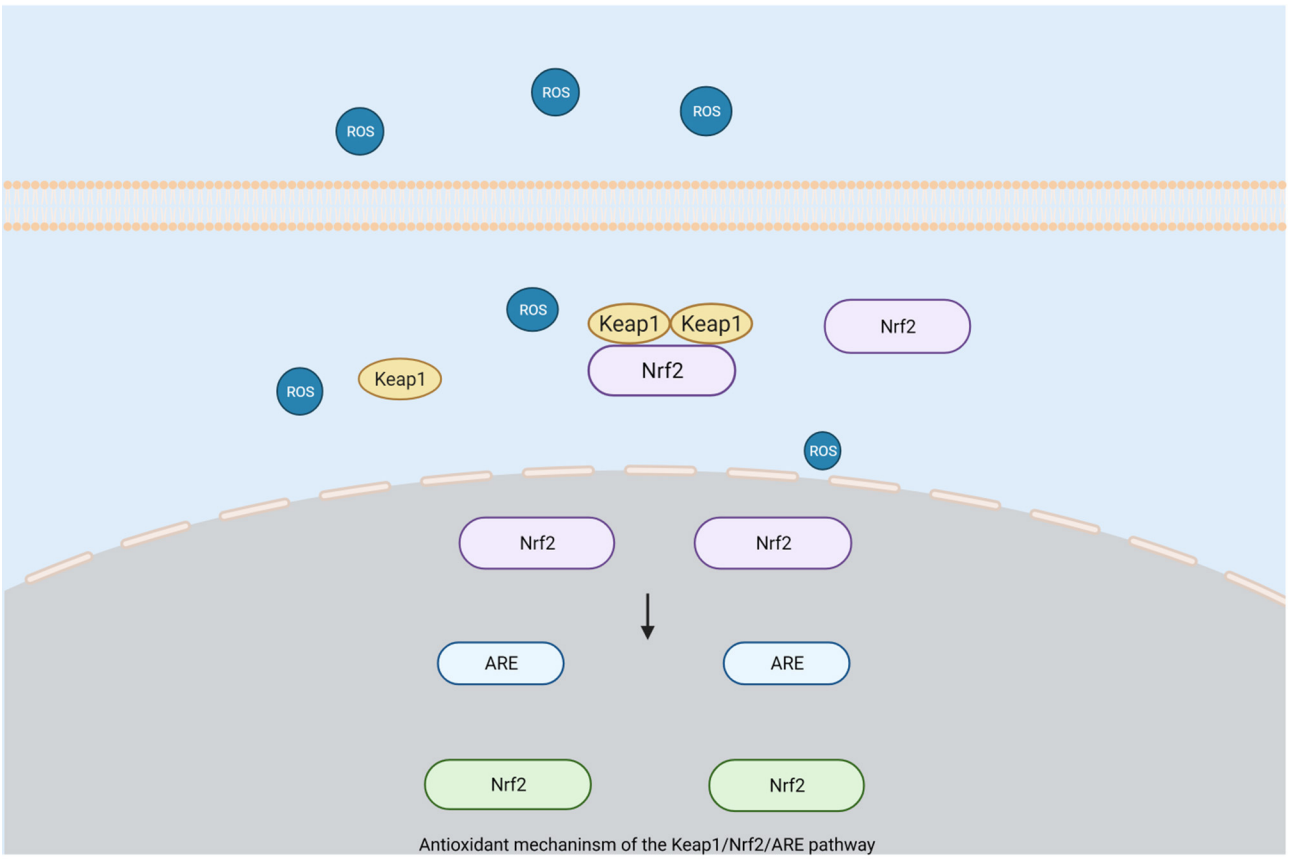

FIGURE 2 | Antioxidant mechanism of the Keap1/Nrf2/ARE pathway. When Nrf2 is exposed to reactive oxygen species, it will dissociate from Keap1 and translocate to the nucleus, while activating antioxidant response elements (ARE) that mediate cell survival to drive the expression of Nrf2 target genes for protective purposes. (Created with BioRender.com).

results need to be further investigated and discussed (Tan et al., 2015). Another study established a cellular module associated with ICH and found that this module is enriched in protein ubiquitination pathways that regulate neuroinflammation and autophagy and enhance the oxidative effects associated with the Nrf2 pathway. It also modulates microglia function and improves the antioxidant capacity of the body after ICH, which reinterprets the relationship between autophagy and oxidative stress after ICH from another perspective (Durocher et al., 2019).

Interaction Between Oxidative Stress and Inflammatory Response After ICH

A growing body of experimental research has shown that inflammation plays an increasingly important role in the pathophysiology of SBI after ICH. There is a close interaction between inflammatory reaction and post-ICH oxidative stress. The oxidative stress reaction that occurs after ICH can trigger an inflammatory reaction, which subsequently can cause corresponding brain damage through the oxidative stress pathway (Mracsko and Veltkamp, 2014). For example, pro-inflammatory cytokines, such as tumor necrosis factor and interleukin 1, is expressed under the induction of free radical substances. Free radical substances produced by oxidative stress play an important role in the induction and occurrence of inflammatory reactions (Khaper et al., 2010; Hu X. et al., 2015), while pro-inflammatory cytokines can also produce free radicals, which promote the occurrence and progress of oxidative stress (Khaper et al., 2010).

The inflammatory response after ICH leads to the activation or infiltration of inflammatory cells and the mass production of inflammatory factors and chemokines. Excessive inflammatory factors activate the NF-κB signaling pathway. It inhibits the Nrf2 pathway, which leads to oxidative stress (Aronowski and Zhao, 2011; Chaudhary et al., 2013; Zhou et al., 2014; Saha et al., 2020). At the same time, after ICH, activated inflammatory cells not only produce a large number of inflammatory factors but also promote the production of ROS and other free radical substances (Matsuo et al., 1995; Yang et al., 2015). In addition, in the inflammatory response, microglia with unbalanced phenotype transfer can cause a large amount of ROS and inflammatory factors to be released during the process of phenotypic conversion (Hu W. et al., 2015). *In vitro* experiments have also shown that microglia can induce the production of ROS (Cui et al., 2015; Yang et al., 2015), in an animal simulation experiment of ICH,

when microglia were suppressed, the production of ROS and brain damage were both reduced. It demonstrates the important effect of microglia on the oxidative stress response after ICH (Wang and Tsirka, 2005b). Moreover, while Nrf2 reduces oxidative damage through the Keap1/Nrf2/ARE pathway, it also protects against inflammatory responses through the Nrf2/ARE pathway. There is a common pathway between these two (Chen et al., 2006; Thimmulappa et al., 2006). This may be achieved by inhibiting NF-κB (Thimmulappa et al., 2006), an important regulator of many pro-inflammatory genes, to achieve anti-inflammatory protection, and NF-κB regulators also play an important role in protection from oxidative stress. There is a clear and significant relationship between oxidative stress and inflammation after ICH, which provides a great reference point for further study of the injury mechanism after ICH.

CLINICAL IMPACT OF OXIDATIVE STRESS IN AND TREATMENT OF ICH

Clinical Impact of Oxidative Stress in ICH

There are a variety of markers that can reflect the level of oxidation after ICH. Substances such as malondialdehyde(MDA), SOD and glutathione-mercapto peroxidase (GSH-Px) are commonly used to monitor the course of the oxidative stress response after ICH. Researchers have studied the impact of OS in plasma and cerebrospinal fluid (CSF) of patients with ICH on their clinical outcomes. The results showed that elevated MDA in CSF and total antioxidant status in plasma were associated with harmful outcomes, while higher plasma SOD and GSH-Px were associated with favorable outcomes in ICH (Masomi-Bornwasser et al., 2021). This reflects the predictive power of oxidative markers for ICH outcomes. Also, data suggest that leukocyte 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels are higher in ICH patients than in healthy subjects (Chen et al., 2011). Since guanine is the most easily oxidized of the five nucleobases, and 8-OHdG is its oxidation product. Therefore, in response to this finding, some investigators recently analyzed serum oxidized guanine levels in patients with ICH and obtained its correlation with mortality, demonstrating that 8-OHdG could be a new independent predictor of ICH outcome (Lorente et al., 2020). Myeloperoxidase (MPO) is one of the common markers of oxidative stress, and thus the changes in MPO after ICH were clinically studied. The results showed that elevated serum MPO concentrations in ICH patients were associated with increased oxidative stress and correlated with ICH prognosis, suggesting that serum MPO levels may be one of the useful biomarkers for determining the prognosis of ICH (Zheng G. R. et al., 2018). In summary, it can be concluded that oxidative stress and its markers are of great significance in the clinical study of ICH.

Antioxidant Therapy After ICH

Since oxidative stress damage after ICH is caused by excessive ROS and other free radical substances, antioxidant treatment after ICH can be divided into the following directions (**Table 1**):

Mitigating Brain Damage From Oxidative Stress by Preventing Excess Production of Free Radicals, Such as ROS, Immediately After ICH

Since the lysis of red blood cells after ICH produces hemoglobin, heme, and free radical substances, haematoma can be regarded as the source of excessive free radical production. Therefore, if blood clots is removed through surgical interventions, it should reduce oxidative damage. Minimally invasive surgery (MIS) for the evacuation of clots may demonstrate the feasibility of this idea, and related animal studies have found that the neurological function of experimental animals is improved through MIS treatment or when combined with other therapies. This provides good protection, as oxidative damage and cell apoptosis are correspondingly reduced (Wu et al., 2011; Liu et al., 2016; Wang et al., 2019). In the same way, as desferrioxamine mesylate (DFO) is an iron chelator, DFO can bind to excess iron produced after ICH, thereby reducing free radical production and oxidative damage. The therapeutic efficacy of DFO for ICH has been demonstrated in several preclinical studies (Okauchi et al., 2010; Hatakeyama et al., 2013; Xie et al., 2014). The tolerability, safety, and maximum tolerated dose of DFO in patients with ICH were determined in a phase I clinical trial (Selim et al., 2011). Preliminary results from a phase II trial showed that DFO reduced perihematomal edema (Yeatts et al., 2013). Meanwhile, some studies have found a positive prognostic effect of DFO use in patients after ICH, but more trials are needed (Zhao et al., 2015). The work of Zhu et al. (2021) suggests that the combined use of DFO and other scavengers may be a routine and more effective approach to treat oxidative damage after ICH. However, coverage of the CNS after ICH may be inadequate due to limitations possibly due to systemic toxicity associated with intravenous DFO, and no therapeutic effect was shown in the iDEF trial evaluating intravenous DFO after ICH (Yeatts et al., 2013; Selim et al., 2019). Therefore, the effect of DFO on ICH needs to be further investigated in depth. Additionally, a selenium nanocomposite material has been developed to reduce oxidative damage after ICH by preventing the cellular accumulation of ROS. The positive effect on oxidative damage shows its great potential for the clinical treatment of ICH and brain damage related to oxidative stress (Yang et al., 2021).

Neutralizing Excessive Free Radicals Produced by Oxidative Stress to Reduce Brain Injury

Neutralizing excess ROS and restoring the normal functions of endogenous antioxidant enzymes and scavengers is an effective way to eliminate excess free radicals. Many free radical scavengers have been evaluated in clinical trials. As a free radical scavenger, Nxy-059 showed good safety and tolerability in its ICH efficacy study, but unfortunately efficacy itself was not shown to be significant (Lyden et al., 2007). In the most recent study on ROS scavengers, the combination of N-acetylcysteine and selenium significantly slowed the progression of perihematomal edema in patients with ICH and reduced the time to reach the target Richmond Agitation Sedation Scale (RASS) and the length of stay in the ICU, although there was no significant change in neurological outcomes (Kim et al., 2021). As a free radical scavenger, edaravone has been used in Japan in patients with

TABLE 1 | Antioxidant therapy for cerebral hemorrhage.

Treatment	Mechanism of action	Research progress	Whether it has been tested in clinical trials and/or animal models	Reference
MIS surgical treatment	Reduces the excessive production of free radicals such as ROS in the body by expelling blood, thus reducing oxidative damage	In animal studies, neurological function was better protected in animals using MIS alone or in combination with other therapies	Has been tested in clinical trials	Wu et al., 2011; Liu et al., 2016
Desferrioxamine mesylate (DFO) treatment	Binds to excess iron produced after brain hemorrhage, thereby reducing free radical production and mitigating oxidative damage	Preliminary results from phase II trial show that DFO reduces perihematoma edema	Has been tested in clinical trials	Selim et al., 2011
Selenium nanocomposite therapy	Blocking excessive production of intracellular reactive oxygen species, thus reducing oxidative damage after cerebral hemorrhage	Good efficacy for oxidative damage after cerebral hemorrhage was achieved in animal experiments	Has been tested in animal models	Yeatts et al., 2013
Nxy-059 treatment	Reduce brain damage by scavenging excess free radicals such as ROS already produced by oxidative stress through neutralization	Did not show significant efficacy	Has been tested in clinical trials	Yang et al., 2021
Edaravone treatment	Reduce brain damage by scavenging excess free radicals such as ROS already produced by oxidative stress through neutralization	Recent clinical studies have shown that edaravone does not cause clinically significant QT prolongation as defined by the ICH E14 guidelines for the treatment of patients with cerebral hemorrhage, providing assurance of its safety	Has been tested in clinical trials	Nakamura et al., 2008
Combined treatment with N-acetylcysteine and selenium	Reduce brain damage by scavenging excess free radicals such as ROS already produced by oxidative stress through neutralization	Recent clinical studies have shown that this treatment modality slows the progression of perihematomal edema PHE in ICH patients and reduces the time to achieve the target RASS (Richmond Agitation Sedation Scale) and the length of stay in the ICU	Has been tested in clinical trials	Lyden et al., 2007
Nrf2 activator carotenoid therapy	Activation of the Nrf2 pathway to enhance antioxidant effects, thereby reducing brain damage from oxidative stress	Radiothione activates Nrf2 in ICH-affected brain tissue and reduces ICH-induced neutrophil counts, oxidative damage, and behavioral defects in animal studies	Has been tested in animal models	Wang et al., 2007; Zhao et al., 2007
Novel Nrf2 activator RS9 therapy	Activation of the Nrf2 pathway to enhance antioxidant effects, thereby reducing brain damage from oxidative stress	The results of the ICH mouse experiment suggest that the activation of Nrf2 by RS9 exerts a neuroprotective effect mediated by the attenuation of oxidative stress	Has been tested in animal models	Shimizu et al., 2021

acute cerebral obstruction. Studies on its use in the therapy of ICH have been highly successful, and it has demonstrated significant neuroprotective effects in animal studies of ICH. In clinical trials, edaravone has also been shown to have better efficacy and safety for patients (Edaravone Acute Infarction Study Group, 2003; Nakamura et al., 2008; Shang et al., 2015; Shimizu et al., 2021).

Activation of the Nrf2 Pathway to Enhance Antioxidant Effects Aimed at Reducing Brain Damage From Oxidative Stress

Nrf2 is a key transcription factor for antioxidant response element (ARE) regulatory genes, which play an important regulatory role in cell survival. In oxidative stress after ICH, Nrf2 induces and up-regulates cytoprotective and antioxidant genes, attenuates tissue damage, and exhibits significant protective effects (Wang et al., 2007; Zhao et al., 2007). Radiothione is a natural isothiocyanate that induces the expression of several Nrf2-responsive genes. It is able to activate Nrf2 pathways and protect neurons from oxidative stress damage after ICH, manifesting a marked neurofunctional protective effect (Wang et al., 2007; Zhao et al., 2007). In a different study, Masomi-Bornwasser J and colleagues found that RS9, a novel Nrf2 activator, upregulated the expression of antioxidant enzymes such as SOD1 and HO-1 by activating the Akt-Nrf2 pathway. It also showed a protective effect on BBB and neuronal cells in the SBI of ICH (Sugiyama et al., 2018). It can be inferred that RS9 may be one of the effective therapeutic candidates for

the treatment of SBI after ICH. The above experiments reflect that activation of the Nrf2 pathway is an effective antioxidant modality that significantly attenuates SBI produced by oxidative stress after ICH. This provides a new direction of thought for the treatment of ICH.

Other Antioxidant-Related Treatments

Hypothermia treatment is a known effective means of neuroprotection. According to Song's study, subhypothermia treatment in a rat model of ICH significantly achieved neuroprotective effects by inhibiting ICH-induced neuronal autophagy and apoptosis, reduced neutrophil infiltration and oxidative DNA damage (Song et al., 2018). Furthermore, clinical studies have shown that subhypothermia treatment for 8–10 days in patients with ICH can significantly reduce perihemorrhagic edema and decrease mortality (Staykov et al., 2013).

Mesenchymal stem cells (MSCs) are pluripotent cells with anti-inflammatory, antiapoptotic, and immunomodulatory properties (Nakano and Fujimiya, 2021). Numerous studies have shown that MSCs can reduce neurological deficits resulting from ICH (Zheng H. et al., 2018). The exosomes secreted by MSCs are considered the main mechanism of MSC therapy (Han et al., 2018). The release of exosomes has been shown to inhibit neuroinflammation and brain hemorrhage injury (Li et al., 2021), mainly through antiapoptotic, oxidative stress, and anti-inflammatory effects (Cai et al., 2020; Duan et al., 2020). Furthermore, Wang et al. evidenced the protective effect of umbilical cord MSC-derived exosomes on injured lateral hippocampal neurons after ICH (Wang et al., 2022). The long-term 5-year safety and possible beneficial effects of autologous MSCs transplantation have been clinically substantiated (Zheng H. et al., 2018). This is encouraging for the potential of MSC exosome therapy for application in the treatment of secondary injury after ICH.

CONCLUSION

Oxidative stress is the main source of secondary injury after ICH. It is closely related to other processes, such as inflammation and autophagy. Therefore, the mechanism between oxidative stress and secondary injury after ICH should be further explored. After more in-depth research, it is hopeful that there will be a clearer trajectory for the clinical treatment of oxidative damage after ICH, in addition to contributing to the research and development of related drugs. More extensive literature on the topic may also inspire us to develop new clinical treatments for ICH.

AUTHOR CONTRIBUTIONS

LS and LM collected information and drafted and revised the manuscript. SC contributed to collecting information and editing the manuscript. LM directed the work and finalized the manuscript. All authors agreed to be accountable for the content of the work. All authors contributed to the article and approved the submitted version.

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