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Pharmaceutical strategies to prevent, treat, and recover: Advances and challenges in ischemic stroke and hemorrhagic stroke

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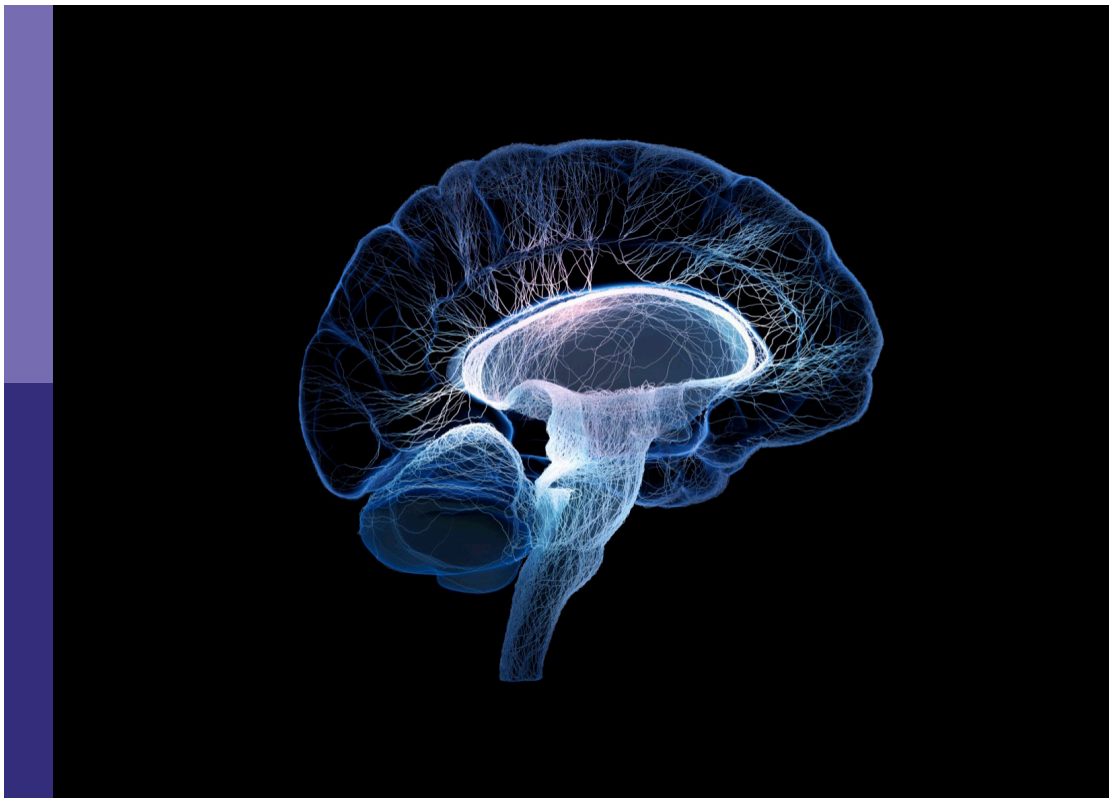

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Pharmaceutical strategies to prevent, treat, and recover: Advances and challenges in ischemic stroke and hemorrhagic stroke

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Editorial: Pharmaceutical strategies to prevent, treat, and recover: advances and challenges in ischemic stroke and hemorrhagic stroke

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stroke, cerebrovascular disease, clinical management, neuropharmacology, neuroprotection

Editorial on the Research Topic
Pharmaceutical strategies to prevent, treat, and recover: advances and challenges in ischemic stroke and hemorrhagic stroke

Stroke stands as one of the primary causes of death and disability globally, particularly with an increasing incidence in developing countries (Campbell et al., 2019). At the present topic, Zhou et al. documented a fatality resulting from cerebral hemorrhage after parathyroid surgery, underscoring the existence of obscure yet highly lethal etiological factors. Complications arising from strokes are prevalent and pose significant threats to life. Zheng M. et al. emphasized the need to consider lumbar cistern blockage as a potential complication following cerebral hemorrhage. However, pharmaceutical therapies for both ischemic and hemorrhagic stroke are still lacking, and the available therapeutic strategies are generally time-sensitive (Jovin et al., 2022). Thus, there is an urgent need to explore the complex regulation network after stroke, which is essential for developing new generations of effective treatment strategies. Besides, translational research and clinical trials are encouraged to improve the prognosis of stroke in the future. This Research Topic includes 17 articles concerning pathological mechanisms, new therapeutic entities, and other attractive aspects of stroke.

Concurrently, stroke research remains at the forefront of technological advancements. Qiu et al. presented a novel method for identifying differentially expressed genes in spatial transcriptomics data, exemplifying the integration of cutting-edge technologies in stroke investigations. The intricate relationship between brain function and structure is elucidated through spatial-omics techniques like spatial transcriptomics, providing researchers with a more precise characterization of biological processes with spatial data. Recognizing the pivotal role of stem cells in brain function recovery (van Velthoven et al., 2013; Bacigaluppi et al., 2016; Tornero et al., 2017), Zhang Q. et al. provided a comprehensive summary of

the progress in stem cell applications within the realm of stroke research, offering valuable insights into their potential therapeutic utility.

The pathological changes following the onset of a stroke are intricate, involving the activation/inactivation of multiple cells and pathways, extending even to the interplay between the brain and gut microbiota (Jeong et al., 2023). These complexities render stroke treatment a formidable task, and various articles on this topic have synthesized novel insights into targets and therapeutic strategies for stroke. Wang J. et al. reviewed approaches such as dietary intervention, fecal microbiota transplantation, probiotics, antibiotics, traditional Chinese medication, and gut-derived stem cell transplantation were explored for their influence on gut microbiota and subsequent regulation of brain recovery post-stroke. Yuan et al. delved into intermittent hypoxic conditions as a potential strategy for ischemic stroke prevention and treatment. Their examination encompassed current evidence, future research directions, and the proposal of intermittent hypoxia as a non-invasive, non-pharmacological, systemic, and multi-targeted intervention to mitigate brain damage.

Contemporary drug research and development are predicated on the identification of specific targets. Two studies included in this topic have uncovered novel druggable targets within this context. Zuo et al. employed CXC3CR1+/GFP mice to elucidated the increased expression of myeloperoxidase (MPO) on microglia after ICH. This MPO-targeted therapeutic interventions consequently facilitated the restoration of motor function. Meanwhile, Zhang C. et al. offer a comprehensive overview of the versatile roles played by 15 specific lncRNAs in the pathological alterations associated with ICH, suggesting valuable insights into potential drug research and development targets.

Traditional Chinese medicine (TCM), often composed of multiple herbs, represents a repository of numerous chemical entities with potential efficacy in stroke treatment. Zheng L. et al. conducted a comprehensive review of studies on treating stroke with *Sanhua decoction*, consisting of *Polygonaceae*, *Magnoliaceae*, *Rutaceae*, and *Apiaceae*, and demonstrated its multiple effects on brain repair. Similarly, Li L. et al. analyzed the potential active pharmaceutical ingredients of another TCM, *Zuogui Pill*, which facilitated neurite outgrowth via mTOR, p53 and Wnt signaling pathways following ischemic stroke. A pressing need persists for high-level evidence regarding herbal medicines, herbal extracts, gut microbiota, or any novel strategy for treating stroke (Zheng L. et al.). This need is pivotal for translating preclinical findings into clinically applicable interventions. Consequently, numerous scholars actively investigate the safety and efficacy of herbal medicines or extracts in stroke treatment through well-designed clinical trials. For instance, a multi-center, open-label, pilot randomized clinical trial conducted by Cui et al. demonstrated the effectiveness of *Ginkgo biloba* extract in protecting patients from cognitive decline 24 weeks after acute ischemic stroke, as assessed through various evaluation methods.

Additionally, specific chemical entities and therapeutic modalities have gained noteworthy attention within stroke research delineated the attributes of TJ-M2010-5, a BBB-permeable drug candidate exhibiting efficacy as a MyD88/NF-κB and ERK pathway inhibitor (Li Z. et al.). Wang X. et al. investigated the systemic administration of dobutamine, revealing its capacity to expedite erythrocyte clearance from the brain to cervical lymph nodes following SAH. Intriguingly, Pichardo-Rojas et al. conducted a comprehensive review highlighting memantine's ability to mitigate NMDA-mediated excitotoxicity, preserve intracellular ATP stores, and up-regulate neuron-specific growth factor expression, with clinical evidence proving its neuroprotective effects in ICH, ischemic stroke, and ischemic stroke-related aphasia. Besides, anesthesias utilized during endovascular thrombectomy (EVT), including ketamine, propofol, sevoflurane, and isoflurane, have been identified as beneficial for post-stroke brain protection (Zhang T. et al.).

Moreover, this topic includes two meta-analyses evaluating the efficacy of inclisiran and mechanical thrombectomy in stroke. Luo et al. analyzed three randomized clinical trials (ORION-9, ORION-10, and ORION-11), concluding that inclisiran did not exhibit significant effects in preventing stroke in atherosclerotic cardiovascular disease patients or those at high risk. Meanwhile, Yang et al. analyzed seven studies (including 1,083 patients) and observed that mechanical thrombectomy with intra-arterial alteplase might enhance functional outcomes without significantly impacting recanalization. These meta-analyses contribute valuable insights to inform clinical decision-making processes.

The assortment of manuscripts within this Research Topic underscores the diverse facets of stroke prevention, treatment, and recovery. These articles contribute novel perspectives to preclinical research and expand the horizons of clinical practice. Notably, the impact of TCM, intestinal microbiota, intermittent hypoxia, and anesthetic medications on stroke prognosis is particularly enlightening. This underscores that, while stroke remains a condition associated with high mortality and disability rates, preventive and therapeutic tools are at our disposal. This realization catalyzes advancing translational research to translate cutting-edge findings in stroke research into clinical applications expeditiously.

We eagerly anticipate further revelations, discussions, and practical applications in emerging areas, such as new drug delivery systems. These developments promise to unveil additional possibilities for stroke prevention, treatment, and recovery, paving the way for continued advancements in stroke-related research and clinical interventions.

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Conflict of interest

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Intermittent hypoxia conditioning as a potential prevention and treatment strategy for ischemic stroke: Current evidence and future directions

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Ischemic stroke (IS) is the leading cause of disability and death worldwide. Owing to the aging population and unhealthy lifestyles, the incidence of cerebrovascular disease is high. Vascular risk factors include hypertension, diabetes, dyslipidemia, and obesity. Therefore, in addition to timely and effective reperfusion therapy for IS, it is crucial to actively control these risk factors to reduce the incidence and recurrence rates of IS. Evidence from human and animal studies suggests that moderate intermittent hypoxia (IH) exposure is a promising therapeutic strategy to ameliorate common vascular risk factors and comorbidities. Given the complex pathophysiological mechanisms underlying IS, effective treatment must focus on reducing injury in the acute phase and promoting repair in the recovery phase. Therefore, this review discusses the preclinical perspectives on IH conditioning as a potential treatment for neurovascular injury and highlights IH pre and postconditioning strategies for IS. Hypoxia conditioning reduces brain injury by increasing resistance to acute ischemic and hypoxic stress, exerting neuroprotective effects, and promoting post-injury repair and regeneration. However, whether IH produces beneficial effects depends not only on the hypoxic regimen but also on inter-subject differences. Therefore, we discuss the factors that may influence the effectiveness of IH treatment, including age, sex, comorbidities, and circadian rhythm, which can be used to help identify the optimal intervention population and treatment protocols for more accurate, individualized clinical translation. In conclusion, IH conditioning as a non-invasive, non-pharmacological, systemic, and multi-targeted intervention can not only reduce brain damage after stroke but can also be applied to the prevention and functional recovery of IS, providing brain protection at different stages of the disease. It represents a promising therapeutic

strategy. For patients with IS and high-risk groups, IH conditioning is expected to develop as an adjunctive clinical treatment option to reduce the incidence, recurrence, disability, and mortality of IS and to reduce disease burden.

KEYWORDS

intermittent hypoxia conditioning, ischemic stroke, vascular risk factors, neurovascular protection, neurovascular restoration, cerebral collaterals, circadian rhythm

Introduction

With an aging population and unhealthy lifestyles, cerebrovascular disease morbidity and mortality rates are gradually increasing, and the disease burden is increasing (Tsao et al., 2022). Ischemic stroke (IS) is the most common cerebrovascular disease and the main cause of death and disability. At present, venous thrombolysis and mechanical thrombectomy are the only two effective methods for the treatment of acute IS (Xiong et al., 2022). Although many neuroprotective drugs have been tested in preclinical trials with animal models to reduce stroke injury, they have not been successfully transferred to the clinical setting (Lyden, 2021). Therefore, it is urgent to identify alternative treatment methods to improve the brain's self-protection capacity. It is also critical to actively control the risk factors for cerebrovascular disease, to reduce its incidence and recurrence rate.

Over the last two decades, the benefits of adaptive regulation have been widely reported (Gidday, 2006; Sommer, 2009). Preconditioning refers to the use of short-term sub-lethal stimuli to improve the tolerance of cells, tissues, and organs to subsequent lethal damage, thus playing an endogenous protective role (Dirnagl et al., 2009). Many preclinical studies have shown that preconditioning schemes, such as hypoxia, ischemia, hypothermia, drugs, and exercise can induce adaptive endogenous protective pathways and exert protective effects against central nervous system diseases, but the exact mechanism underlying these effects remains unclear (Obrenovitch, 2008; Stetler et al., 2014; Stevens et al., 2014; Li et al., 2017; Hafez et al., 2021). Hypoxia conditioning, as a simple, non-invasive, systemic intervention treatment method that does not involve the use of drugs, has more extensive application value than other preconditioning schemes and has great therapeutic potential for a variety of clinical diseases (Navarrete-Opazo and Mitchell, 2014; Mateika et al., 2015; Verges et al., 2015).

Moderate and well-controlled mild intermittent hypoxia (IH) refers to a short period of daily alternating exposure to normobaric hypoxia and normoxia (or hyperoxia) for several weeks (Burtscher et al., 2022). Moderate IH triggers adaptive phenomena that produce beneficial therapeutic effects, improving the body's defense ability against future potential damage in the future (Arkhipenko et al., 2005; Manukhina et al., 2013). Moreover, this type of repetitive moderate IH provides better safety and lasting treatment outcomes compared to acute or persistent hypoxia modes (Zhu et al., 2007; Stowe et al., 2011; Terraneo et al., 2017).

Increasingly, experimental and clinical studies indicate that IH can not only reduce the severity of injury after IS (Stowe et al., 2011; Monson et al., 2014; Selvaraj et al., 2017), but also decrease the risk factors for cerebrovascular disease (Lyamina et al., 2011; Urdampilleta et al., 2012; Serebrovska et al., 2017), suggesting that IH intervention can increase the body's ability to resist injury and promote repair, reduce the risk of developing IS, and exert neurological and cerebrovascular protective effects. Therefore, this paper summarizes the relevant supporting research evidence and proposes the feasibility that IH can be applied to the prevention, treatment, and rehabilitation of IS. This treatment method has great translational prospects, but more basic and clinical studies are still needed to elucidate its mechanism of brain protection and other potentially beneficial effects. Further, the optimal hypoxia therapy protocol requires investigation for safe application to patients and high-risk populations in a clinical settings.

In clinical translational studies of IH, it is noteworthy that IS occurs more often in older adults, more often in males than in females, and most of the affected individuals have comorbidities (Branyan and Sohrabji, 2020; Candelario-Jalil and Paul, 2021). Therefore, it should be noted that the therapeutic effects of IH may vary among individuals. In addition, the opposite circadian rhythms of humans and rodents, which are normally used in experimental studies, may also affect the relative effectiveness of the treatment (Esposito et al., 2020; Lo et al., 2021). However, it is unclear whether the above-mentioned factors affect the therapeutic effect of IH. Taking these factors into account, future experimental and clinical studies can provide more comprehensive and robust evidence for individualized IH treatment. It is hoped that the hypoxia intervention program will be continuously optimized in the future to reduce the incidence of cerebrovascular disease, decrease the disability

and mortality rates associated with IS, improve the functional prognosis of stroke patients, and enhance quality of life.

Intermittent hypoxia: A therapeutic hypoxia protocol

Intermittent hypoxia is a very promising strategy for treating and preventing human diseases that has attracted increasing attention in various fields. Many clinical studies have shown that intermittent exposure to moderate hypoxia can have beneficial effects on both sick and healthy individuals (Zhang et al., 2010; Leone and Lalande, 2017; Liu et al., 2017; Wojan et al., 2021). IH is also safe and effective for the older adults, for whom short periods of alternating exposure to moderate hypoxia and normoxia environments can alter body composition and health status by improving exercise tolerance, metabolism, inflammation, and systemic arterial pressure (Burtscher et al., 2004; Timon et al., 2021, 2022). Studies focusing on central nervous system diseases have shown that hypoxia conditioning can promote health in the aging brain (Burtscher et al., 2021a) and make it more resistant to acute brain injury (Miller et al., 2001; Lin et al., 2003; Stowe et al., 2011, 2012; Wacker et al., 2012a,b), as well as play a protective role against chronic age-related neurodegenerative diseases, such as cognitive impairment (Bayer et al., 2017; Serebrovska et al., 2019c; Wang et al., 2020), Alzheimer's disease (Manukhina et al., 2016), and Parkinson's disease (Burtscher et al., 2021b). IH is expected to become a new therapeutic strategy against aging and neurodegeneration. However, it should be noted that the beneficial effects of hypoxia on the body and its effects as disease treatment depend on factors, such as the concentration, mode, and exposure time of hypoxia.

Hypoxia is a double-edged sword: "dose" is the key

The metabolism of the brain is highly active. Oxygen consumption of the brain accounts for 20% of the amount consumed by the whole body in the resting state. It is very sensitive to changes in oxygen concentration (Magistretti and Allaman, 2015). Therefore, the brain is especially vulnerable to the adverse effects of hypoxia. When an organism first enters into a high-altitude area, acute exposure to low oxygen partial pressure is likely to cause various symptoms collectively known as acute altitude illnesses, among which high-altitude brain edema can be life-threatening (Luks and Hackett, 2022). High-altitude hypoxia environments exert certain effects on the central nervous system of individuals habituated to low altitudes (Wilson et al., 2009), which may lead to cognitive dysfunction (Wang et al., 2022), sleep disorders, and may also induce and aggravate central nervous system diseases (Falla et al., 2021). Hypoxia has also been increasingly recognized as an important factor in the development of neurodegenerative diseases (Burtscher et al., 2021a). During IS, oxygen delivery to the brain is impaired due to vascular occlusion, resulting in neurological deficits. The above shows that maladaptive responses to hypoxia and local severe hypoxia can cause damage to important organs, which is harmful to human health.

In contrast, moderate hypoxia adaptation is beneficial to the body. "Altitude training" and training combined with normobaric hypoxia can enhance physical fitness through hypoxia stimulation, improving hypoxia endurance and sports performance (Flaherty et al., 2016). An epidemiological study found low mortality rates from stroke and coronary heart disease in high-altitude areas of Switzerland (Faeh et al., 2009). High-altitude rodents obtain abundant collateral circulation through gene selection, which can prevent tissue damage after brain, coronary artery, and peripheral artery occlusion (Faber et al., 2021). Many experimental studies in rodents have shown that hypoxic preconditioning can protect important organs (including the heart and brain) from fatal cell damage caused by hypoxia or ischemia (Stowe et al., 2011; Manukhina et al., 2013). Thus, non-lethal moderate hypoxic stimulation as a means of enhancing hypoxic adaptation, which subsequently protects important organs and tissues from similar but more severe damage, is a promising therapeutic strategy.

In summary, hypoxia can induce either physiological adaptation or pathological injury, including death. This is because when oxygen delivery is interrupted or reduced, body cells sense the reduced oxygen supply and respond by initiating endogenous protection through adaptive mechanisms to promote cell survival under hypoxic conditions. However, when the intensity of hypoxia exceeds their maximum tolerance, a cascade of gene expression cascades (like a chain reaction) is initiated, subsequently leading to altered cellular function or even death (Terraneo et al., 2017; Lee et al., 2020; Burtscher et al., 2022). Therefore, whether hypoxia is beneficial or harmful to the body largely depends on the "dose" of hypoxia (Jackman et al., 2014; Navarrete-Opazo and Mitchell, 2014; Serebrovska et al., 2016). Low-dose intermittent exposure to hypoxia (FiO_2 = 9–16%), 3–15 times per day, has been proven to be beneficial in clinical and animal experiments (Navarrete-Opazo and Mitchell, 2014). In addition, it is important to note that differences in hypoxia tolerance between individuals can also affect the outcome of hypoxia (Dempsey and Morgan, 2015).

Different forms of intermittent hypoxia conditioning

Therapeutic IH refers to short-term alternating exposure to normobaric hypoxia and normoxia (or hyperoxia) (Navarrete-Opazo and Mitchell, 2014; Mateika et al., 2015; Serebrovska et al., 2016). As a moderate and non-harmful stressor, it can

promote adaptive responses and exert various beneficial effects on physical health (Gangwar et al., 2020; Behrendt et al., 2022), avoiding the possible harmful effects of continuous hypoxia. Additionally, IH can also improve the defense against potential future damage by initiating endogenous protective mechanisms (Stowe et al., 2011; Manukhina et al., 2013, 2016, 2018; Mallet et al., 2018; Burtscher et al., 2021a; Su et al., 2022). Intermittent hypoxia-hyperoxia (IHH) is a modified IH protocol. The normoxia period is replaced by a moderate hyperoxia period (FiO_2 = 30–40%), resulting in a faster recovery from deoxygenation (Bayer et al., 2017; Glazachev et al., 2017; Dudnik et al., 2018; Serebrovska et al., 2019c, 2022; Afina et al., 2021; Behrendt et al., 2022; Bestavashvili et al., 2022). Compared with IH, IHH offers a more obvious improvement in the clinical parameters and is considered to offer more beneficial effects (Serebrovska et al., 2019a). The underlying mechanism may be related to a more pronounced induction of reactive oxygen species during mild hyperoxia, triggering an intracellular redox signaling cascade that induces the synthesis of intracellular protective proteins with antioxidant and anti-inflammatory effects through the activation of the transcription factors nuclear factor erythroid 2-related factor 2 (Nrf2) and hypoxia-inducible factor (HIF) (Arkhipenko et al., 2005; Serebrovska et al., 2017; Burtscher et al., 2022). Based on the limited animal and clinical studies available, we summarized the potential therapeutic benefits of IH conditioning or IHH conditioning on cerebrovascular protection in **Figure 1**. The protective mechanism of IH and IHH is not completely understood yet, and further research on their molecular details is needed. In addition, IH therapy includes IH exposure and IH training (IHT). The latter consists of a combination of exercise and IH exposure (Morishima et al., 2015; Timon et al., 2021), while the former has a wider range of applications and is more appropriate for groups that, for various reasons, are not suitable for exercise.

Intermittent hypoxia conditioning improves common risk factors of ischemic stroke

Ischemic stroke is often accompanied by hypertension, diabetes, dyslipidemia, and obesity; and these comorbidities further complicate the pathology of IS (Gottesman and Seshadri, 2022). These patients often need a combination of oral medications to control their risk factors, which may bring unavoidable side effects. Therefore, in addition to modifying an unhealthy lifestyle, it is very important to identify effective non-pharmacological interventions for cardiovascular and cerebrovascular diseases and for metabolic risk factors that can be applied as adjunctive therapies for people at high risk for cerebrovascular disease. A growing number of clinical and experimental findings suggest that IH can modulate risk factors associated with cerebrovascular disease (Serebrovskaya et al., 2008; Urdampilleta et al., 2012; Serebrovska et al., 2017, 2019a; Kayser and Verges, 2021), which is a potential therapeutic strategy. This evidence supports the applicability of IH in the preventive treatment of IS.

Intermittent hypoxia and hypertension

Inadequate production and reduced availability of nitric oxide lead to increased blood pressure (Panza et al., 1993), and long-term chronic hypertension is a common risk factor for stroke. Experimental studies on rodents show that the hypotensive effect of IH in spontaneously hypertensive rats is due to the stimulation of nitric oxide synthesis and storage in blood vessels (Manukhina et al., 2011). In addition, intermittent hypobaric hypoxia can reduce blood pressure in these rats, potentially through the inhibition of the renin-angiotensin system (Chen et al., 2021). In a renal vascular hypertensive rat model, intermittent hypobaric hypoxia also has an anti-hypertensive effect (Li et al., 2019). In human studies, intermittent normobaric hypoxic adaptation for 20 days was reported to reduce blood pressure levels in patients with stage 1 hypertension, accompanied by an increase in nitric oxide synthesis (Lyamina et al., 2011). Another study further confirmed the hypotensive effect of IH conditioning in patients with stage 1 hypertension, confirmed that hypoxic adaptation combined with exercise produced a better and longer-lasting hypotensive effect than oral antihypertensive drugs alone, and found that the hypotensive effect of hypoxia correlated with elevated levels of nitric oxide (NO) and hypoxia-inducible factor-1alpha (HIF-1α) (Muangritdech et al., 2020). Moreover, IH conditioning also provides good anti-hypertensive effects in the presence of comorbid conditions. IHH training in patients with coronary artery disease not only reduced blood pressure, but also improved exercise capacity, reduced angina attacks, enhanced the left ventricular ejection fraction, and reduced blood glucose (Glazachev et al., 2017). In patients with metabolic syndrome, IHH exposure can significantly reduce systolic and diastolic blood pressure (Bestavashvili et al., 2022). Mild IH can reduce blood pressure in patients with obstructive sleep apnea (OSA) complicated with hypertension (Panza et al., 2022). Evidence from studies in animals and humans (**Tables 1**, **2**) suggests that well-controlled mild IH conditioning regimens may be a safe and effective way to prevent and treat hypertension.

Intermittent hypoxia and abnormal glucose and lipid metabolism and obesity

Modern lifestyle frequently entails lack of exercise and high-calorie dietary intake, leading to an increasing number

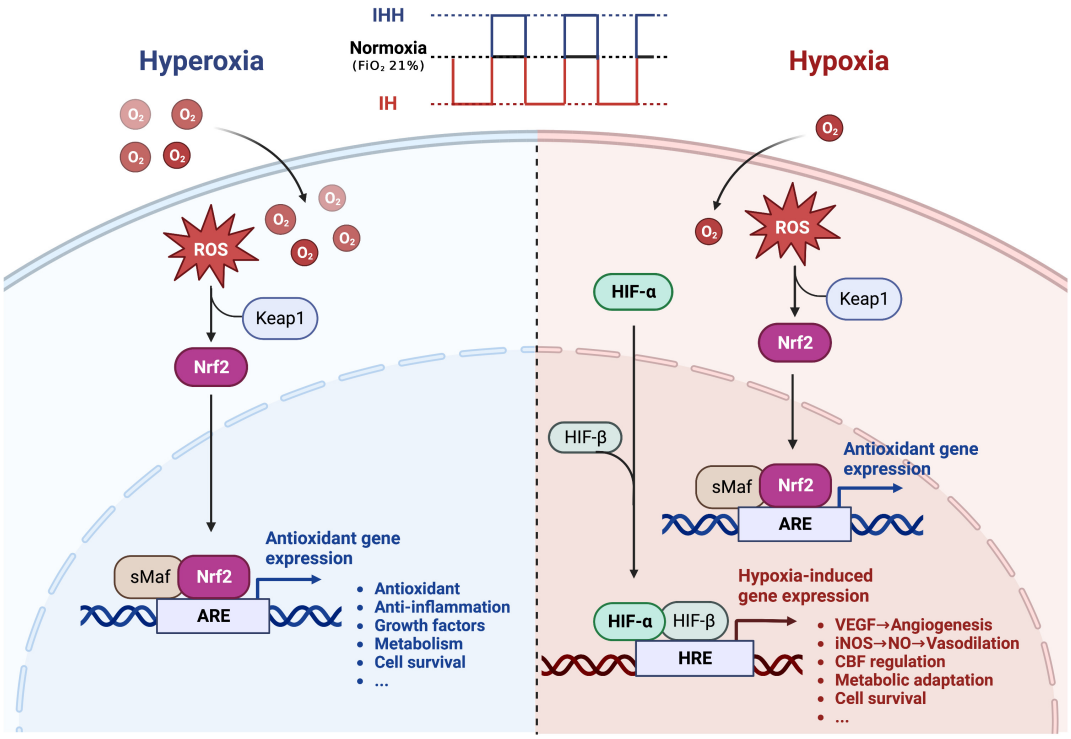

FIGURE 1
The potential therapeutic benefit of intermittent hypoxia conditioning or intermittent hypoxia-hyperoxia conditioning for cerebrovascular protection. During intermittent hypoxia (IH) conditioning or intermittent hypoxia-hyperoxia (IHH) conditioning, moderate amounts of ROS are produced, activating the transcription factor Nrf2, which promotes the synthesis of protective proteins and improves the brain's defense response to severe ischemic-hypoxic injury. In addition, hypoxia activates the transcription factor HIF, which induces numerous genes essential for cell metabolism, proliferation, and survival. Many of these genes play a central role in injury tolerance and promotion of tissue oxygenation, such as vascular endothelial growth factor (VEGF), and inducible NO synthase (iNOS). Replacing normoxia with hyperoxia during the reoxygenation phase can amplify the beneficial effects of intermittent hypoxia. ROS, reactive oxygen species; Nrf2, nuclear factor erythroid 2-related factor 2; ARE, antioxidant response element; HIF, hypoxia-inducible factor; HRE, hypoxia-responsive element. Created with www.biorender.com.

of cases of type 2 diabetes and obesity (Ampofo and Boateng, 2020; Sun et al., 2022), which are, in turn, associated with increased morbidity and mortality. Clinical evidence shows that IH can improve abnormal glucose and lipid metabolism in humans (**Table 1**). A single session of IH can improve cardiopulmonary reflexes and exert a hypoglycemic effect in patients with type 2 diabetes (Duennwald et al., 2013). Moderate IHT lasting for 3 weeks is potentially useful for the management of patients with pre-diabetes and can induce an increase in the expression of HIF-1α mRNA and its target gene, which can be used as an effective non-pharmacological preventive therapy (Lyamina et al., 2011). Another study confirmed that IHT can restore blood insulin levels to normal levels in prediabetic patients, and this was related to the increased expression of PDK-1 mRNA in leukocytes (Serebrovska et al., 2019b). Additionally, in one case, a female patient with obesity and pre-diabetes not only experienced controlled blood glucose but also weight loss through IH treatment (Fuller and Courtney, 2016). For sedentary individuals, 4 weeks of IHT improved insulin sensitivity more than 2 weeks of the same number of IHT sessions, suggesting that a longer IHT schedule may be more beneficial for improving insulin sensitivity (Susta et al., 2020). IHT also lowered blood pressure in this study, but this was not related to the duration of training. In overweight or obese adults, 2 weeks of passive moderate IH improved cardiovascular risk factors by lowering blood glucose, low-density lipoprotein, and cholesterol (Costalat et al., 2018). Three weeks of IHH treatment for patients with metabolic syndrome can improve the blood lipid profile and anti-inflammatory state (Afina et al., 2021). IHH therapy is safe and well-tolerated by patients, can reduce arteriosclerosis, and positively affect liver function by improving the hemodynamics and lipid profile of patients (Bestavashvili et al., 2022). The specific mechanism by which hypoxia improves glucose and lipid metabolism remains unclear. Animal studies have shown that the regulatory mechanism of IH on metabolism may be related to hypoxia-induced epinephrine (Luo et al., 2022), ameliorating insulin resistance *via* the HIF-insulin signaling pathway (Tian et al., 2016), and

TABLE 1 Effects of intermittent hypoxia on vascular risk factors in humans.

References	Participants	Hypoxia protocols	Results
(Bestavashvili et al., 2022)	65 patients with MS: IHH group $n = 32$ (age 56.9 ± 11.7), control group $n = 33$ (age 59.8 ± 10.3)	IHH: 5–8 cycles of 4–7 min hypoxia (11–12%) followed by 2–4 min hyperoxia (30–35%), 5 sessions/week for 3 weeks	SBP↓, DBP↓, improve lipid profile and liver functional state
(Panza et al., 2022)	16 males with OSA and HTN: IH $n = 10$ (age 40.7 ± 9.8), control $n = 6$ (age 46.2 ± 10.3)	IH: 12 cycles of 2 min hypoxia (8%) followed by 2 min normoxia, 5 sessions/week for 3 weeks	SBP↓, DBP↓, accompanied by parasympathetic↑, sympathetic↓
(Muangritdech et al., 2020)	47 HTN patients: IHR $n = 15$, IHT $n = 15$, control $n = 17$	IH: eight cycles of 3 min hypoxia (14%) followed by 3 min normoxia, 2 sessions/week for 6 weeks	SBP↓, Nox and HIF-1α were negatively correlated with SBP
(Glazachev et al., 2017)	46 CAD patients: IHH $n = 27$ (age 52–77); control = 19 (age 43–83)	IHH: 5–7cycles of 4–6 min hypoxia (10–12%) with 3 min hyperoxia (30–35%), 3 sessions/week for 8 weeks	SBP↓, DBP↓
(Lyamina et al., 2011)	37 stage 1 HTN (age ∼32)	IH: 4–10 cycles of 3 min hypoxia (10%) with 3 min normoxia, 1 session/day, for 20 days	SBP↓, DBP↓, normalization of NO production
(Afina et al., 2021)	65 patients with MS: IHH group $n = 32$ (age 44.5–65.5), control group $n = 33$ (age 56.2–66.0)	IHH: 5–8 cycles of 4–7 min hypoxia (11–12%) followed by 2–4 min hyperoxia (30–35%), 5 sessions/week for 3 weeks	Improved lipid profile and anti-inflammatory status
(Gangwar et al., 2020)	40 male healthy volunteers (age 22–25)	On day 0: 1 h hypoxia (13.5%); Days 1–4: hypoxia (12%), 4 h/day for 4 days; on day 5: 1 h hypoxia (13.5%)	Regulate lipid metabolism
(Serebrovska et al., 2019b)	11 prediabetic patients (age 48–70) and seven healthy volunteers (age 44–68)	IH: four cycles of 5 min hypoxia (12%) followed by 5 min normoxia, 3 sessions/week for 3 weeks	Normalizing blood insulin level, correlated with an enhanced mRNA expression of PDK-1 in leukocytes
(Serebrovska et al., 2019a)	55 patients with prediabetes (age 51–74): IHH group $n = 17$, IH group $n = 22$, control $n = 16$	IHH or IH: four cycles of 5 min hypoxia (12%) followed by 3 min hyperoxia (33%) or normoxia, 5 sessions/week for 3 weeks	Reduced blood glucose (fasting and OGTT); decreased total blood cholesterol and LDL
(Costalat et al., 2018)	Six overweight and obese individuals (age 56.2 ± 10)	IH: 70 min of repeated cycles of hypoxia (SpO_2 = 70%) followed by re-oxygenation (SpO_2 = 95%), 5 sessions/week for 2 weeks	A single IH: reduced blood glucose and lactate; 10 sessions IH: decreased LDLc, LDLc/HDLc ratio and SBP
(Serebrovska et al., 2017)	Seven healthy and 11 prediabetic individuals (age 44–70)	IH: four cycles of 5 min hypoxia (12%) followed by 5 min normoxia, 3 sessions/week for 3 weeks	Reduced blood glucose (fasting and OGTT), associated with HIF-1α
(Fuller and Courtney, 2016)	A female patient (age 49) with obesity and pre-diabetes	IH: daily hour-long session of alternating 6 min hypoxia and 3 min normoxia	Weight loss and glycemic control
(Morishima et al., 2015)	21 sedentary men (age 24.3 ± 1.2)	Hypoxic training (15%) for 2 weeks or 4 weeks	Improving insulin sensitivity
(Duennwald et al., 2013)	14 patients with type 2 diabetes	1 h single bout IH: 5 min hypoxia (13%) followed by 6 min normoxia	Reduced blood glucose

DBP, diastolic blood pressure; HTN, hypertension; HIF-1α, hypoxia-inducible factor-1alpha; HDLc, high-density lipoprotein cholesterol; IH, intermittent hypoxia; IHH, intermittent hypoxia-hyperoxia; LDLc, low-density lipoprotein cholesterol; NO, nitric oxide; NOx, nitric oxide metabolites; OGTT, oral glucose tolerance test; PDK-1, pyruvate dehydrogenase kinase 1; SBP, systolic blood pressure.

recovery of mitochondrial activity (Trzepizur et al., 2015; **Table 2**).

Taken together, these results suggest that IH can improve blood pressure, blood glucose, blood lipids, and weight loss; providing a new therapeutic strategy for the treatment and prevention of atherosclerosis and metabolic syndrome. However, more evidence from randomized controlled trials and animal experiments is needed to support these conclusions. Moreover, the above-mentioned clinical and preclinical studies (**Tables 1**, **2**) suggest that IH can improve conditions that are common risk factors for IS, which is not only beneficial to the prevention of cerebrovascular diseases but also helpful to reduce the likelihood of recurrence.

Application of intermittent hypoxia conditioning at different stages of ischemic stroke

The potential therapeutic use of IH in the treatment of cerebrovascular and cardiovascular diseases is the focus of extensive research (Manukhina et al., 2016; Serebrovskaya and Xi, 2016; Mallet et al., 2018). The mechanisms underlying the beneficial effects of hypoxia adaptation have been investigated at multiple biological levels, ranging from systemic physiological responses to genomic regulation and protein modifications (Terraneo et al., 2017; Lee et al., 2020; Burtscher et al., 2022).

TABLE 2 Effects of intermittent hypoxia on vascular risk factors in animals.

References	Subjects	Hypoxia protocols	Results	Mechanisms
(Chen et al., 2021)	SHR and WKY rats	Hypobaric hypoxia (4000 m altitude), 5 h/day for 35 days	ABP↓	Inhibiting RAS activity, downregulating the ACE-Ang II-AT1 axis, upregulating the ACE2-(Ang17)-Mas axis
(Li et al., 2019)	RVH rats	Hypobaric hypoxia (5000 m altitude), 6 h/day for 28 days	ABP↓	Upregulating NOS expression in the nucleus tractus solitarii
(Manukhina et al., 2011)	SHR rats	5–10 min hypoxia (9.5–10%) and 4 min normoxia, 5–8 cycles/day for 20 days	Suppressed the development of hypertension	Prevention of endothelial dysfunction, increased accumulation of NO stores in vascular walls
(Luo et al., 2022)	C57BL/6L mice with HFD	10% oxygen for 1h/day, 4 weeks	Reduce body weight; ameliorate fatty liver	Associated with hypoxia-induced epinephrine
(Tian et al., 2016)	Sprague–Dawley rats	Hypobaric hypoxia (simulate 5000 altitudes) for 6 h/day, 4 weeks	Decreased SAP, serum triglyceride and cholesterol; improved insulin resistance and hepatic steatosis	Ameliorating insulin resistance *via* the HIF-insulin signaling pathway
(Trzepizur et al., 2015)	C57BL/6J mice with HFD	IH: 1 min cycle, hypoxia (5% O_2, 30 s) followed by normoxia (21% O_2, 30 s) for 8 h/day, 2 weeks	Increase insulin and leptin levels; restore endothelial function and mitochondrial activity; limits liver lipid accumulation	Prevented endothelial dysfunction by restoring NO production; improved liver lipid metabolism by restoring mitochondrial activity

ABP, arterial blood pressure; ACE, angiotensin-converting enzyme; Ang, angiotensin; HFD: high-fat diet; HIF, hypoxia-inducible factor; IH, intermittent hypoxia; NO, nitric oxide; NOS, nitric oxide synthase; RAS, renin-angiotensin system; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto.

Preconditioning is a therapeutic strategy that induces endogenous self-protection of vital organs through sub-lethal physiological stimulation (Stevens et al., 2014). Experimental studies in rodents have shown that IH can trigger beneficial effects through preconditioning, improving the brain and heart's defenses against ischemic-hypoxic injury and protecting them from the harmful consequences of ischemia and reperfusion (Stowe et al., 2011; Manukhina et al., 2013). Postconditioning refers to the promotion of recovery from injury by promoting processes such as repair, regeneration, and plasticity, which contribute to an improved prognosis (Pietrogrande et al., 2019; Li et al., 2022). Therefore, the treatment approach of IH preconditioning and postconditioning at different stages of the disease can not only reduce risk of stroke but also enhance neuroprotection to reduce the injury severity and ultimately improve the prognosis of IS (**Figure 2**).

Intermittent hypoxia preconditioning

Experimental studies in rodents show that hypoxia preconditioning before an acute IS can induce endogenous brain protection, increase the brain's resistance to ischemic-hypoxic injury, and produce a neuroprotective effect by activating the hypoxic signal pathway, the anti-inflammatory pathway, antioxidative stress, and autophagy (Liu et al., 2021). How to maximize the protective effect and obtain the greatest benefit depends on the mode of hypoxia. Animal studies have found that either a single episode of IH (Miller et al., 2001) or consecutive days of IH (Lin et al., 2003) reduce the infarct size in a transient focal IS model and have a neuroprotective effect. However, this protective effect diminishes over time. Repeated IH with different concentrations and durations can provide sustained brain protection by regulating epigenetic neurovascular plasticity, inducing a longer tolerance time window (Stowe et al., 2011; Monson et al., 2014; Selvaraj et al., 2017). It is worth noting that IH preconditioning has a protective effect against IS, and hypoxic concentration is the key factor. Studies have confirmed that 10% of IH is neuroprotective, whereas 6% of IH exacerbates tissue damage, and this different outcome is associated with changes in susceptibility to mitochondrial damage (Jackman et al., 2014). A recent study confirmed for the first time that brief, repeated exposure to systemic hypoxia attenuates hypoperfusion-induced cognitive impairment and that this resistance to dementia is heritable, allowing mice offspring to avoid memory loss even in the presence of persistent cerebral hypoperfusion (Belmonte et al., 2022). Due to the unpredictability of IS, few clinical studies have focused on it, with most studies conducted on animal models. Therefore, future research needs to strengthen our understanding of the neuroprotective mechanism of preconditioning in the brain and find the optimal hypoxia mode and applicable population to accelerate clinical translation.

Intermittent hypoxia postconditioning

Hypoxia postconditioning, which is administered during the acute phase of IS, can have a protective effect by improving cerebral energy metabolism (Ren et al., 2020). It also stimulates neural regeneration after IS by promoting the proliferation

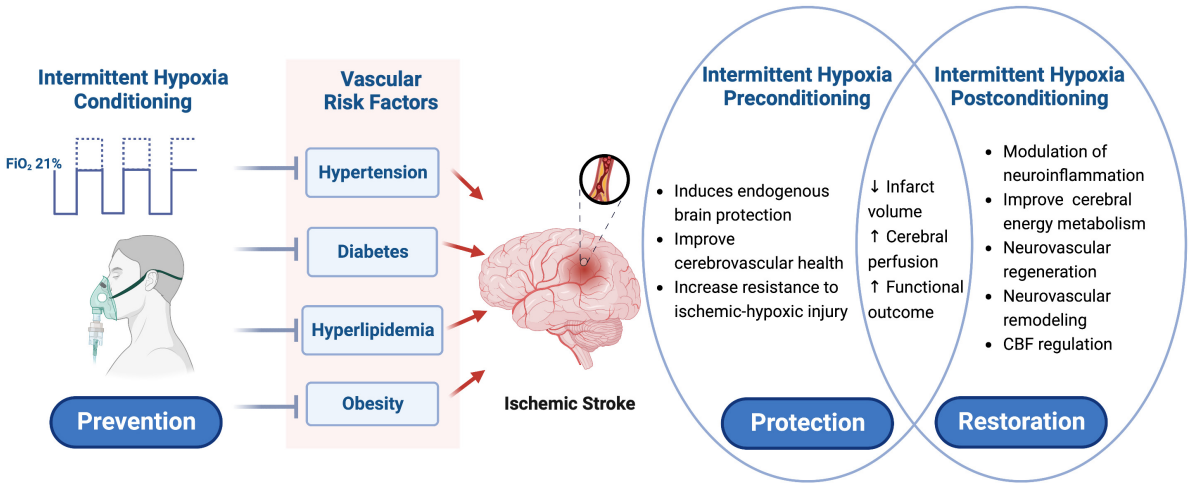

FIGURE 2
Schematic presentation of intermittent hypoxia conditioning as a potential prevention and treatment strategy for ischemic stroke. IH conditioning can improve the conditions considered as risk factors for cerebrovascular diseases, such as high blood pressure, glucose, and lipid content, and high body weight, which is a potential treatment strategy to prevent IS and reduce its incidence and recurrence rate. IH preconditioning induces endogenous cerebral protective mechanisms, improves cerebrovascular health, and enhances the resistance of the brain to ischemic-hypoxic injury. IH postconditioning can modulate neuroinflammation, improve brain energy metabolism, promote neurovascular regeneration and remodeling, and regulate cerebral blood flow. Applying IH conditioning at different stages of IS may ultimately reduce infarct volume, improve cerebral perfusion, and promote neurological recovery. IH, intermittent hypoxia; IS, ischemic stroke. Created with www.biorender.com.

and migration of neural stem cells (Li et al., 2022). Hypoxia postconditioning 48 h after an IS can improve motor function and reduce tissue loss (Pietrogrande et al., 2019). In addition, the adaptation after hypobaric hypoxia in the acute stage can also improve neurological function and reduce the infarct volume after IS, which is related to the fact that hypobaric hypoxia attenuates the inflammatory response by regulating the expression of HIF-α and its target genes (Huang et al., 2014; Qiao et al., 2015). In contrast, some studies have shown that hypoxic postconditioning in the acute phase of IS does not improve neurological function but may increase brain damage instead (Tsai et al., 2008). These differences in results may be related to the dose of hypoxia and the animal model used in each case. A subsequent study, found that hypoxia postconditioning in the chronic stage of IS can improve cognitive dysfunction in mice after stroke by inducing hippocampal neurogenesis and functional synapse formation (Tsai et al., 2013), indicating that hypoxia postconditioning can promote brain repair after injury.

To summarize, based on the current evidence from experimental and clinical studies, IH preconditioning may not only prevent cerebrovascular diseases and reduce their recurrence by controlling risk factors (**Tables 1**, **2**) but also reduce post-stroke brain injury and neurological deficit by activating the adaptive response of the brain (**Table 3**). IH postconditioning may promote neurological recovery (**Table 4**) and the regulation of cerebral blood flow through neurovascular remodeling (Manukhina et al., 2016). Moreover, IH may improve the conditions commonly identified as risk factors for stroke, promote repair and regeneration after injury, and play a role in preventing the recurrence of stroke and reducing the brain tissue injury after recurrence, thus reducing the disability and mortality rates of the disease, and its burden as well. Therefore, IH can be used in the prevention, treatment, and rehabilitation phases of IS and is a very promising adjunctive therapy. However, the present evidence supporting its use for the treatment during the acute recovery phases is limited to a few animal studies and is, therefore, not yet sufficient. Future studies need to further elucidate the neuroprotective and neurorestorative mechanisms of hypoxia conditioning while also considering the effects of age, sex, comorbidities, and the safety and efficacy of its application during the acute and recovery phase, to help accelerate its clinical translation.

Therapeutic effects and mechanism of hypoxia conditioning on ischemic stroke

Ischemic stroke is the most common acute cerebrovascular disease, with complex pathophysiological mechanisms and high heterogeneity. Severe ischemia after vascular occlusion leads to rapid brain injury and cell death, which activates the immune system *in vivo* (Iadecola et al., 2020). Inflammatory signals participate in all stages of the ischemic cascade reaction, leading to early blood-brain barrier (BBB) destruction and infarction progress, but also to later neurovascular repair and

TABLE 3 Beneficial effects of intermittent hypoxia preconditioning in ischemic stroke.

References	Subjects	Model	Hypoxia protocols	Outcomes
(Belmonte et al., 2022)	C57BL/6J mice	Chronic cerebral hypoperfusion	Hypoxia (11%) 1 h, every other day for 8 weeks	Abrogated hypoperfusion-induced memory/plasticity deficits, resistance to dementia is heritable
(Jackman et al., 2014)	C57BL/6J mice	tMCAO	Hypoxia (10 or 6%) 90 s followed by normoxia 90 s, 20 hypoxic episodes per hour	10% CIH is neuroprotective (reduced infarct volume), 6% CIH exacerbates tissue damage
(Stowe et al., 2011)	Swiss-Webster ND4 mice	tMCAO, pMCAO	2 weeks of repetitive hypoxic (8–11%) preconditioning (RHP) or single hypoxic preconditioning (SHP; 4 h, 8% O_2)	RHP protection against stroke persisted for 8 weeks
(Lin et al., 2003)	Wistar rats	tMCAO	Hypoxia (380 mmHg altitude) for 15 h/day, lasted 4 weeks	Reduced infarct volume
(Miller et al., 2001)	C57Bl/6, 129SvEv, Swiss-Webster ND4 mice	tMCAO	48 h before tMCAO, hypoxia (11%) for 2 h	Reduced infarct volume

CIH, chronic intermittent hypoxia; pMCAO, permanent middle cerebral artery occlusion; RHP, repetitive hypoxic preconditioning; tMCAO, transient middle cerebral artery occlusion.

TABLE 4 Beneficial effects of intermittent hypoxia postconditioning in ischemic stroke.

References	Subjects	Model	Hypoxia protocols	Outcomes
(Su et al., 2022)	SD rats	tMCAO	1 week after stroke, IH (13% O_2) for 4 h/day, lasted 4 weeks	Reduce infarct volume and promote motor function recovery
(Li et al., 2022)	C57BL/6 mice	tMCAO	30 min after stroke, IH (8% O_2) for 3 h/day, lasted 13 days	Recovery of neurological function, promote the proliferation and migration of neural stem cells
(Ren et al., 2020)	C57BL/6J mice	dMCAO	15 min after stroke, IH repeated four times	Reduce infarct size
(Pietrogrande et al., 2019)	C57BL/6 mice	Photothrombotic occlusion	48 h after stroke, IH (11% O_2) for 8 h/day, lasted 14 days	Reduce motor deficits and tissue loss
(Qiao et al., 2015)	C57 mice	MCAO	24 h after stroke, IH (simulate 5000 m altitude) for 4 h, lasted 7 days	Accelerate cognitive function recovery
(Huang et al., 2014)	C57BL/6 mice	tMCAO	12 h after stroke, IH (simulate 5000 m altitude) for 4 h	Better neurological performance and smaller infarct size
(Tsai et al., 2013)	SD rats	MCAO	7 days after stroke, IH (12% O_2) for 4 h/day, lasted 7 days	Alleviate long-term memory impairment
(Leconte et al., 2009)	SWISS mice	tMCAO	5 days after stroke, IH (8% O_2) for 1 h/day, 3 times/week, lasted 43 days	Reduced delayed thalamic atrophy

IH, intermittent hypoxia; pMCAO, permanent middle cerebral artery occlusion; tMCAO, transient middle cerebral artery occlusion.

remodeling. Like the complex pathophysiological mechanisms of cerebral ischemia, the protective mechanisms underlying hypoxia conditioning are diverse and intertwined, and have not been fully elucidated. The concept that hypoxia can induce an inflammatory response has been widely recognized from the study of the hypoxia pathway (Eltzschig and Carmeliet, 2011), suggesting that beneficial hypoxic modulation can modulate the body's immunity, not only by reducing early inflammatory damage but also by promoting the repair process after brain injury through anti-inflammatory effects. In addition, it has been shown that hypoxia is involved in neurovascular repair (Walshe and D'Amore, 2008), generation of cerebral collateral vessels (Anan et al., 2009; Zhang et al., 2020), and capillary neovascularization (Milner et al., 2008; Burnier et al., 2016), neurogenesis, and neuroplasticity (Sommer, 2009; Zhu et al., 2010; Tsai et al., 2013; Li et al., 2022) after IS. Therefore, moderate hypoxia conditioning may improve the clinical outcome of IS by regulating these processes to promote endogenous brain repair and regeneration.

Hypoxia and neurovascular protection

The neurovascular unit (NVU) is the structural and functional unit of the central nervous system. It highlights the dynamic interactions between endothelial cells, mural cells (pericytes and smooth muscle cells), basement membrane, astrocytes, microglia, neurons, and extracellular matrix; and the importance of such interactions in the pathophysiology of the CNS (Schaeffer and Iadecola, 2021). The capillary network of the whole brain is composed of endothelial cells and connected by tight junctions, surrounded by the endfeet of pericytes

and astrocytes, constituting an important part of the BBB and maintaining brain homeostasis (Liebner et al., 2018). The close communication between microvascular endothelial cells and surrounding astrocytes plays an important role in maintaining intact neurovascular coupling (Iadecola, 2017). Moreover, the regulation of the microvascular basement matrix membrane, the activation of endothelial cells, and the change of glial cell adhesion directly affect the transmission of information between microvessels and neurons. The organism relies on this complex neurovascular network to achieve the fine regulation of cerebral blood flow and ensure the normal neurological function and steady state of the brain (Zhang et al., 2012). It has been confirmed that IH preconditioning can significantly reduce oxidative stress during ischemia-reperfusion injury and stimulate NO-induced vasodilation (Bertuglia, 2008), thereby maintaining capillary perfusion. In the large vessels, periodic IH increases the blood flow and shear rate of the internal carotid artery, which in turn increases shear-mediated vasodilatation (Iwamoto et al., 2020). It is suggested that hypoxia can improve cerebral blood flow and cerebral perfusion by regulating the diameter of cerebral vessels. In addition, the oxygen uptake capacity of the brain increases during acute periodic hypoxemia to compensate for the reduced oxygen levels in arterial blood (Liu et al., 2017). Repeated IH exposures enhance arterial oxygen delivery and increase O_2 availability (Zhang et al., 2010). These results suggest that hypoxia conditioning can not only affect cerebral vascular reactivity, but also increase cerebral oxygen uptake, oxygen transport, and oxygen use capacity. The studies mentioned above suggest that periodic IH is a promising non-pharmacological treatment strategy for optimizing cerebrovascular health.

Under physiological conditions, hypoxia conditioning can regulate the interactions between various cellular and non-cellular components in the NVU through HIF-dependent or independent pathways (Obrenovitch, 2008; Terraneo et al., 2017; Lee et al., 2020), modulate the organism's response to injury, enhance the resistance of the NVU to ischemic-hypoxic injury, and exert neuroprotective effects (Dirnagl et al., 2003; Stowe et al., 2011, 2012; Wacker et al., 2012a,b; Monson et al., 2014; Selvaraj et al., 2017). HIFs are the major regulators of the hypoxic transcriptional response, and the O_2-sensitive prolyl/aspartate hydroxylase (PHDs/FIH) regulates HIF activity in the transition between normoxia and hypoxia conditions (Kaelin and Ratcliffe, 2008). Under various pathological conditions, hypoxia conditioning serves to improve the clinical outcome of the disease by regulating the cellular environment (Taylor and Colgan, 2017), thereby enhancing mitochondrial metabolism, enhancing antioxidant and anti-inflammatory capacity, and promoting the repair process to reduce the extent of neurological and vascular damage (Walshe and D'Amore, 2008; Tsai et al., 2013; Qiao et al., 2015; Ryou et al., 2017; Manukhina et al., 2018; Pietrogrande et al., 2019; Ren et al., 2020; Li et al., 2022; Luo et al., 2022). Since the underlying cellular and molecular mechanisms are unclear, more relevant research evidence is needed.

Hypoxia and neurovascular regeneration

Cerebral collateral circulation refers to the auxiliary vascular network recruited after arterial occlusion, which can provide partial blood flow compensation for the ischemic area (Liebeskind, 2003). Cerebral collateral vessels refer to the inherent vascular anastomosis among arteries, arterioles, and capillaries (Faber et al., 2014). When cerebral artery stenosis or occlusion leads to ischemia of its downstream brain tissue, good collateral vessel opening can play a role in blood flow compensation. In IS, collateral blood perfusion in the area adjacent to occluded vessels can partially alleviate the ischemic injury caused by insufficient perfusion (Ginsberg, 2018). However, due to the variation in number, diameter, and compensatory capacity of collateral vessels, the degree of protection of collateral circulation against occlusive diseases varies greatly, which directly affects the clinical outcome of patients. Factors affecting collateral vessels include aging (Faber et al., 2011; Ma et al., 2020), genetic background (Lucitti et al., 2016; Faber et al., 2021), and vascular risk factors that can be treated (Biose et al., 2020), including hypertension (Moore et al., 2015), type 2 diabetes (Akamatsu et al., 2015; Nishijima et al., 2016), dyslipidemia, obesity, and metabolic syndrome (Menon et al., 2013). The above-mentioned factors can cause the thinning of collateral vessels and the impairment of the compensation ability of collateral blood flow. The underlying mechanism remains unclear, but may be related to the decrease in eNOS levels and an increase in oxidative stress and inflammation (Faber et al., 2011; Moore et al., 2015; Rzechorzek et al., 2017). Hypoxia conditioning may improve the status of the collateral circulation by improving NO utilization by endothelial cells, antioxidative stress mechanisms, and anti-inflammatory effects (Anan et al., 2009). In addition, mild hypoxia can promote endothelial cell proliferation, increase vascular density, and remodel capillaries and arterioles in mice (Milner et al., 2008; Boroujerdi et al., 2012; Burnier et al., 2016; Halder et al., 2018). Recently, the formation of new collateral vessels induced by arterial occlusion has been confirmed in mouse models for IS and myocardial infarction (Zhang and Faber, 2015; Okyere et al., 2020). Furthermore, it has also been reported that exposure to hypoxia alone can induce the formation of new collateral vessels in the brain and the heart (Zhang et al., 2020; Aghajanian et al., 2021). By gradually acclimating mice to hypoxia and maintaining it for 2–8 weeks, oxygen concentration-dependent new collateral vessel formation and remodeling of intrinsic collateral vessels were observed, and cerebral infarct volume was reduced after subsequent permanent middle cerebral artery occlusion.

The expression of Hif2α, Vegfa, Rabep2, Angpt2, Tie2, and Cxcr4 increased after hypoxia. However, in knockout mice for Rabep2, new collateral vessels could not be formed, and this phenomenon was reversed in the conditional knockouts for Vegfa, Flk1, and Cxcr4 (Zhang et al., 2020). Recently, it has also been found that hypoxia can induce the formation of coronary collaterals in adult mice, and that Vegfa and Rabep2 are required for this (Aghajanian et al., 2021). These results suggest a mechanistic link between embryonic collateral formation and new collateral formation in adult mice. How collateral vessels are formed and how hypoxia promotes collateral vessel neovascularization and remodeling are questions that need to be addressed in the future.

Adult neurogenesis is of great medical significance to cognitive, memory, and motor dysfunction caused by central nervous system diseases (Culig et al., 2022). Experimental evidence shows that intermittent hypobaric hypoxia can promote the proliferation of endogenous neural progenitor cells, leading to an increase in the number of new neurons, and produces antidepressant-like effects. IH can also promote the expression of brain-derived neutrophic factor in the adult hippocampus (Zhu et al., 2010). In a rat model for Alzheimer's disease, IH conditioning protected against neurodegenerative changes and improved cognitive function (Manukhina et al., 2010). The benefits of IH for improving cognitive function have been further confirmed in human studies. Schega et al. (2013) reported for the first time that additional IH conditioning administered before physical exercise can enhance cognitive function and quality of life in the older adults, and demonstrated good tolerance. Wang et al. (2020) reported that moderate IH for eight weeks can reduce arterial blood pressure at rest, enhance cerebral oxygenation and vasodilation in the cerebral cortex during hypoxia, and improve the short-term memory and attention of older adults patients with amnestic mild cognitive impairment. Serebrovska et al. (2019c) showed that IHH conditioning can improve the cognitive function of patients with mild cognitive impairment and reduces the biomarkers for Alzheimer's disease in the peripheral blood while increasing the levels of some inflammatory markers. The upregulation of inflammatory markers may be a potential trigger for cellular adaptation (Serebrovska et al., 2022), but whether these proinflammatory factors mediate the neuroprotective effects is unclear and needs to be further explored.

However, due to the unpredictability of this acute cardio-cerebrovascular disease, the clinical research evidence on hypoxia preconditioning is insufficient. Most rodent studies have been conducted in young healthy mice; but in the clinical setting IS patients are usually middle-aged or older adults, often affected by comorbid hypertension, diabetes, dyslipidemia, and obesity; which complicates the pathology of IS (Candelario-Jalil and Paul, 2021). Whether the same pattern of hypoxia preconditioning can also provide the an equivalent protective effect to aging individuals exhibiting comorbid conditions lacks support from relevant research evidence and needs to be further clarified in future studies. More systematic and comprehensive studies are needed in the future to enhance our understanding of the potential molecular and cellular regulatory mechanisms underlying the beneficial effects of hypoxia, and to provide a strong basis for the application of hypoxia conditioning as a clinical treatment for IS at different stages.

Bridging the gap between preclinical and clinical studies in ischemic stroke: A translational perspective

The current research results provide promising evidence for the application of IH conditioning in the prevention, treatment, and rehabilitation of IS. It should be noted that the inconsistency between the results obtained in rodent models and in the clinical population may affect the translational outcome of IH treatment, and these factors include genetics, age, sex, comorbidity, etc. (Sommer, 2017; Candelario-Jalil and Paul, 2021). In recent years, researchers have noticed that the opposite circadian rhythm between rodents and humans also affects the outcome of stroke treatment (Esposito et al., 2020; Lo et al., 2021). Therefore, it is necessary to optimize the preclinical models of IS to simulate as closely as possible what happens in the actual clinical patients. Although many clinical and experimental studies associated with normobaric IH have been reported, the protocol of hypoxia treatment varies considerably between individual studies. It is not clear which mode of treatment is the best. Therefore, searching for potential clinical biomarkers that can identify hypoxia adaptation and maladaptation may help to provide more accurate and safer IH treatment.

The effect of circadian rhythm

A growing body of research has shown that circadian rhythms interact with multiple aspects of IS pathophysiology, influencing disease susceptibility, degree of damage, repair processes, and response to various treatments, but the underlying mechanism is still unclear (Lo et al., 2021). Recent evidence suggests crosstalk between HIF-1 signaling and the circadian rhythm (Manella et al., 2020; Adamovich et al., 2022). Esposito et al. (2020) reported that three different neuroprotective methods can reduce infarction in rodent models during the day, but not at night, pointing out that circadian rhythm may be one of the factors affecting the clinical

translation of neuroprotection. This study suggests that the response of the body to treatment is different in the active and in the inactive phases, and that the diurnal effect may lead to different outcomes. Moreover, it has also been found in clinical studies that aspirin, a drug used to treat IS, can be orally administered before sleep to reduce platelet reactivity upon waking, which is related to the endogenous circadian rhythm of platelet activation (Bonten et al., 2015). A recent study of circadian gene expression profiles in 12 different mouse organs showed that circadian rhythms exist in the transcription of 43% of protein-coding genes and that most of the best-selling and WHO essential drugs directly target genes that are regulated by circadian rhythms (Zhang et al., 2014). Therefore, it can be speculated that different administration times will have different effects on treatment outcomes and may be beneficial to the treatment of various diseases. Therefore, the effect of the timing of the intervention on the treatment outcome should be considered in preclinical studies of hypoxia conditioning. To our knowledge, no studies on experimental animals or humans have been published in this area, and circadian factors could be included in subsequent studies to provide a basis for the clinical translation of hypoxia conditioning.

Optimizing the preclinical model of ischemic stroke

It is well-known that many differences between clinical and experimental study subjects have resulted in potential treatments identified as promising in rodent IS models that have not shown therapeutic effects in clinical trials. Clinically, stroke is most prevalent in older men and women, and preclinical studies have mostly tested young male animals. Therefore, preclinical studies in animal models of IS affected by aging and comorbidities can more accurately simulate the clinical situation of patients. Aging is a natural phenomenon in which mitochondrial dysfunction, oxidative stress, and inflammation gradually increase with age, resulting in different cellular dysfunctions and a progressive decline in tissue and organ function, which leads to an increased risk of IS and death (Garaschuk et al., 2018). Preclinical studies have also revealed significant differences in the pathophysiology and prognosis of IS between young and old animals, with the latter exhibiting more severe injury and poor recovery. The cellular mechanisms of aging and IS overlap, and beneficial hypoxia conditioning can inhibit mitochondrial dysfunction, oxidative stress, and inflammation, and potentially provide effective treatment for IS among older adults (Burtscher et al., 2021a). In experimental studies, female rodents were found to have milder damage after IS compared to males. This phenomenon disappeared after ovariectomy or beyond reproductive age, suggesting that estrogen and progesterone are potential neuroprotective factors against IS (Alkayed et al., 2000). Uric acid failed to act as a neuroprotective agent in patients with acute IS, but it had a positive effect on functional recovery in female patients (Chamorro et al., 2014). Given the age and sex differences described above, it is necessary to clarify the effect that these factors may have on treatment outcomes in animal models before conducting clinical trials to further evaluate the efficacy and safety of IH treatment. Additionally, common comorbidities such as hypertension, diabetes, hyperlipidemia, obesity, etc. significantly increase the vulnerability of the brain to ischemic injury, eventually leading to worse functional results (Biose et al., 2020; Candelario-Jalil and Paul, 2021). Under the condition of comorbidity and aging, the protective effect of some drugs is weakened or absent. Therefore, whether IH can also induce beneficial effects among older adults affected by multiple comorbidities is still unclear and needs to be further explored.

Conclusion and prospects

Intermittent hypoxia conditioning is a very promising treatment strategy to prevent and treat IS (**Figure 2**). IH can improve the conditions considered to be risk factors for cerebrovascular diseases, such as blood pressure, blood sugar, blood lipid, and weight, and increase the body's resistance to ischemic-hypoxic injury. Therefore, IH may reduce the incidence and recurrence of IS and have a protective effect on the central nervous system, thereby reducing the severity of IS and improving the clinical prognosis of patients. According to the evidence detailed in this review, IH conditioning can reduce damage to the BBB and neuronal cells, and can also induce neovascularization and neurogenesis, contributing to NVU repair and regeneration. Thus, during the recovery phase of IS, IH conditioning promotes neurovascular remodeling, which is beneficial to transition the brain from injury to the repair process. Future research must further clarify the cerebral protective mechanisms of IH conditioning and other beneficial mechanisms, which will help to provide new therapeutic strategies and potential pharmacological targets for the prevention and treatment of cerebrovascular diseases. In addition, issues that need to be solved in future research include the optimal intervention mode, the timing of intervention, the difference in circadian rhythms, sex, age, and comorbidities in response to IH, and the different responses of different brain regions and cell types to IH. At present, IH conditioning for ischemic cerebrovascular disease has been almost exclusively studied in preclinical, animal disease models, and the challenge for the future is how to apply it safely and effectively for the prevention and treatment of IS in clinical patients. Therefore, well-designed controlled clinical trial studies are needed to confirm these findings and determine the optimal target population, time points for intervention, and

mode of hypoxia; and ultimately to provide strong evidence for establishing the most effective individualized hypoxia protocol.

Author contributions

HY investigated and wrote the manuscript. JL reviewed and contributed to the editing.YG participated in the proofreading. XJ and GN contributed to the conception and critically revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

Publisher's note

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Detection of differentially expressed genes in spatial transcriptomics data by spatial analysis of spatial transcriptomics: A novel method based on spatial statistics

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Background: Spatial transcriptomics (STs) simultaneously obtains the location and amount of gene expression within a tissue section. However, current methods like FindMarkers calculated the differentially expressed genes (DEGs) based on the classical statistics, which should abolish the spatial information.

Materials and methods: A new method named spatial analysis of spatial transcriptomics (saSpatial) was developed for both the location and the amount of gene expression. Then saSpatial was applied to detect DEGs in both inter- and intra-cross sections. DEGs detected by saSpatial were compared with those detected by FindMarkers.

Results: Spatial analysis of spatial transcriptomics was founded on the basis of spatial statistics. It was able to detect DEGs in different regions in the normal brain section. As for the brain with ischemic stroke, saSpatial revealed the DEGs for the ischemic core and penumbra. In addition, saSpatial characterized the genetic heterogeneity in the normal and ischemic cortex. Compared to FindMarkers, a larger number of valuable DEGs were found by saSpatial.

Conclusion: Spatial analysis of spatial transcriptomics was able to effectively detect DEGs in STs data. It was a simple and valuable tool that could help potential researchers to find more valuable genes in the future research.

KEYWORDS

spatial transcriptomics, DEGs, spatial statistics, saSpatial, stroke

Introduction

Spatial transcriptomic (ST) is a revolutionary technology that enables us to obtain information on spatial location and amount of gene expression within a tissue section (Stahl et al., 2016). It provides us with a unique opportunity to elucidate the cellular heterogeneity in tissues with a variety of biological conditions such as tumor and ischemia. Thus, enormous progress in the fields of embryology, oncology, and neuroscience has been made in recent years (Maniatis et al., 2019; Baccin et al., 2020).

Detection of differentially expressed genes (DEGs) among the different biological situations is the fundamental for microarray analysis including ST (Dries et al., 2021). Tools such as FindMarkers or FindAllMarkers, originally designed for single-cell RNA sequencing (scRNA-seq), have been applied in ST data (Stahl et al., 2016). FindMarkers is based on the Wilcoxon rank-sum test, a non-parametric test that belongs to classical statistics (Dutta and Datta, 2016). The essential assumption of classical statistics is that each individual must be independent (Griffith, 2005). Gene expression of one spot is very likely to be affected by the nearby ones, which indicates that the individuals of ST data are not independent. It has been demonstrated by recent studies (Svensson et al., 2018; Xia et al., 2019). In this way, the ST data was not suitable for classical statistics. It is necessary to develop a novel method for DEGs detection that simultaneously considers spatial context and gene expression.

Spatial statistics, or called spatial analysis, is a method primarily used in Geography to analyze the effect of spatial location on certain features (Griffith, 2005). For Health science, it is widely used in epidemiology. Unlike classical statistics, there is an essential assumption in spatial statistics: spatial data gathered from nearby areas are dependent on each other. Therefore, recent studies have noted the value of spatial statistics on the analysis of ST data (Xia et al., 2019; Hirz et al., 2022). There are a lot of parameters in spatial statistics and Moran's index (Moran's *I*) is a measure of spatial autocorrelation, which is characterized by a correlation in a signal among nearby locations. Moran's *I* are classified into two categories: global and local. The global Moran's *I* is a measure of the overall clustering of the spatial data, and the local Moran's *I* is developed to evaluate the local spatial autocorrelation analysis (Li and Calder, 2007). Previous studies used the global Moran's *I* to determine if there was global autocorrelation of ST data (Xia et al., 2019; Hirz et al., 2022). How to explore the DEGs *via* spatial statistics remained unclear.

Spatial statistics can be performed using geographical information systems (GIS) (Kistemann et al., 2002). Direct application of GIS for detection of DEGs in ST data was not yet available. Here we described a method named spatial analysis of spatial transcriptomics (saSpatial) to identify the DEGs in both inter- and intra-cross sections.

Materials and methods

Animal preparation

Adult C57BL/6 mice (8 weeks old, 25–35 g), provided by Animal Center of Southern Medical University (Guangzhou, China), were used in the present study. The animals were housed for at least 1 week before surgery under controlled environmental conditions with ambient temperature of 25°C, relative humidity of 65%, and 12/12 h light-dark cycle. The animals were free access to gain food and water. The protocol was reviewed and approved by the Institution Animal Care and Use Committee of The Second Affiliated Hospital of Guangzhou Medical University (NO. B2019-037).

Distal MCAO mouse model

The mice were anesthetized using isoflurane (1–2%/oxygen). A cortical stroke model was made by occlusion of the distal middle cerebral artery using a previously reported method (Wen et al., 2019). In brief, a 1 cm skin incision was made between the ear and eye (usually right side). The temporal muscle was removed from the skull. A hole was made by the drill right above the MCA and the artery was coagulated by the electrocoagulation forceps proximal and distal to the bifurcation. The temporal muscle was relocated to cover the burr hole. Suture the wound and place the animal in a nursing box at 32°C to recover from anesthesia and return it to the cage.

Magnetic resonance imaging

Magnetic resonance imaging (MRI) scanning was performed 6 h after stroke onset using a Bruker Biospec 7.0 T system (PharmaScan, Bruker Biospin, Rheinstetten, Germany) with a mouse brain array coil and a transmit only volume coil. The anesthetized animals were secured within the cradle by tooth and ear bars, and a mouse head four-channel phased array surface receiver coil was placed on the head. Body temperature was maintained at 37 ± 0.5°C during the MRI scanning procedure by a closed circuit thermal jacket.

T2 weighted scans using a fast-spin echo sequence: echo time (TE) 33 ms, repetition time (TR) 8,000 ms, field of view (FOV) 30 mm × 30 mm, acquisition matrix 512 × 512, acquiring 0.4 mm thick slices. A four-shot spin-echo planar imaging DWI scan (TE 30.5 ms, TR 8,000 ms, FOV 25 mm × 25 mm, acquisition matrix 96 × 96, three directions x, y, z, *B*-values = 0 and 1,000 s/mm^2, 50 contiguous thick, 0.4 mm thick). The PWI can be performed non-invasively by tagging protons in the arterial blood supply with an inversion pulse by using a T2*-weighted echo-planar sequence: TE 76 ms, TR 11,482 ms, FOV 24 mm × 24 mm, acquisition matrix 128 × 128,

1 excitation, repetition 50 times. All MR images were processed on a commercial workstation (ParaVision Acquisition 6.0.1).

Spatial transcriptomics

Tissue section preparation

The mice were sacrificed under deep anesthesia immediately after MRI scanning. The animals were perfused transcardially with 0.1 M PBS (pH 7.4) only, and brains were surgically removed rapidly and embedded in optimal cutting temperature (OCT) compound (SAKURA). The brains with OCT compound were quick-frozen on dry ice immediately and stored at −80°C until cryosectioning process. The cryosectioning placed in a cryostat (Leica, CM1950) to cryosection the OCT embedded tissue blocks into appropriately sized sections for Visium Spatial slides while keeping the samples frozen. Tissue sections were 10 μm thick each. Tissue sections were placed within the frames of Capture Areas on Visium Spatial slides (10X Genomics).

Fixation, staining, and imaging

Tissue section slides were incubated 1 min at 37°C then were fixed in methanol at −20°C for 30 min. For staining, the slides were incubated in Hematoxylin for 7 min and in Bluing Buffer for 2 min. Then, Eosin was added to the slides and incubated for 1 min. After each staining steps, slides were washed with DNase and RNase free water. Stained tissue sections are imaged by the microscope (Pannoramic MIDI, 3DHISTECH).

Tissue pre-permeabilization

Pre-permeabilization was performed to optimal the suitable permeabilization time. Visium Spatial Tissue Optimization Slides and Reagent Kits (10X Genomics) were used for pre-permeabilization. The tissues were permeabilized in Permeabilization Enzyme for varying amounts of time then the Fluorescent RT Master Mix was added to the tissue sections. For tissue removal, the tissue sections were incubated in Tissue Removal Mix for 60 min at 56°C. The best permeabilization time was selected through the fluorescent microscope (Pannoramic MIDI, 3DHISTECH).

Tissue permeabilization and spatial transcriptomic sequencing

Tissue permeabilization and ST sequencing were performed using Visium Spatial Gene Expression Slides and Reagent Kits. The stained slides were incubated in RT Master Mix for 45 min at 53°C for reserve transcription after permeabilization for appropriate time. Next, Second Strand Mix was added to the tissue sections on the slide and incubated for 15 min at 65°C to initiate second strand synthesis. After transfer of cDNA from the slides, barcoded-cDNA was purified and amplified. The amplified barcoded cDNA was fragmented, A-tailed, ligated with adaptors and index PCR amplified. The final libraries were quantified using the Qubit High Sensitivity DNA assay (Thermo Fisher Scientific, Waltham, MA, USA) and the size distribution of the libraries were determined using a High Sensitivity DNA chip on a Bioanalyzer 2200 (Agilent). All libraries were sequenced by illumina sequencer (Illumina, San Diego, CA, USA) on a 150 bp paired-end run.

Differentially expressed genes detected by spatial transcriptomic analysis

We applied fastp with default parameter filtering the adaptor sequence and removed the low-quality reads to achieve the clean data (Chen et al., 2018). Then the feature-barcode matrices were obtained by aligning reads to the mouse genome using SpaceRanger v1.1.0. In order to minimize the sample batch, we applied the down sample analysis among samples sequenced according to the mapped barcoded reads per spot of each sample and finally achieved the aggregated matrix.

Seurat package (version: 3.2)[1] was used for spot normalization and regression. PCA was constructed based on the scaled data with all high variable genes. Utilizing graph-based cluster method, we acquired the unsupervised the cell cluster result based the PCA top 10 principal and we calculated the marker genes by FindAllMarkers function with Wilcoxon rank sum test algorithm under following criteria: logFC $>$ 0.25; p-value $<$ 0.05; min.pct $>$ 0.1.

Process of spatial analysis of spatial transcriptomics

Construction of spatial map

(a) Read a file with the.bz2 extension in the ST data folder including the contents of gene expression and coordinate of each individual spot (R Script 1).

(b) Export the data to a CSV file with columns of Barcode ID, gene name, standardized expression level (named as 1.csv), and another CSV file with x- and y-coordinate (named 2.csv) (R Script 1).

(c) Modify the coordinates in the 2.csv file. The value of x-coordinate was plus 40,531,000 and the value of y-coordinate is plus 3,460,000.

(d) Construct the spatial map using software Arcmap 10.8 (ESRI 2019. ArcGIS Desktop: Release 10.8. Redlands, CA, USA: Environmental Systems Research Institute). Create a new map document and choose the Add XY Data; import the 2.csv; the coordinate system was using CGCS2000_3_Degree_GK_Zone_40; export the spatial map data to a file with extension.shp (named without.shp in our experiment).

1 https://satijalab.org/seurat/

Data pre-processing

(a) Spilt the 1.csv into files, each of which include one single gene expression. The files are usually named as gene name like A_AQP4.xlsx following the gene of *Aqp4*, a marker of astrocyte cell. It should be added with "A_" before the gene name. It is realized by R Script 2.

(b) Each excel filename is read by Python Script 1 to exclude the potential errors.

(c) Create a new document in Arcmap 10.8 and import all the excel files by Python Script 2. The document is named and saved as T.gdb.

(d) Create another new map document named Joined.gdb. Add T.gdb to without.shp. This adds the gene expression information to spatial map. It was realized by Python Script 3.

Global Moran's index calculation

(a) The global Moran's I statistic is given as:

$$I = \frac{n}{S_0} \frac{\sum_{i=1}^{n} \sum_{j=1}^{n} \omega_{i,j} Z_i Z_j}{\sum_{i=1}^{n} Z_i^2}$$

where Z_i is the deviation of an attribute for feature I from its mean (X_i-$\bar{X}$), $\omega_{i,j}$ is the spatial weight between feature i and j, n is equal to the total number of features, and S_0 is the aggregate of all the spatial weights:

$$S_0 = \sum_{i=1}^{n} \sum_{j=1}^{n} \omega_{i,j}$$

The Z_i-score for the statistic is computed as:

$$Z_i = \frac{I - E[I]}{\sqrt{v[I]}}$$

where:

$$E[I] = \frac{-1}{n-1}$$

$$v[I] = E\left[I^2\right] - E[I]^2$$

It was realized by Python Script 3.

(b) Extract the data of global Moran's I, expected index, variance, z-score and P-value of each gene into one excel file (Python Script 4).

Local Moran's *I* calculation

(a) The local Moran's I statistic is given as:

$$I_i = \frac{X_i - \bar{X}}{S_i^2} \sum_{j=1, j \neq i}^{n} \omega_{i,j} (x_j - \bar{X})$$

Where X_i is an attribute for feature i, $\bar{X}$ is the mean of the corresponding attribute, $\omega_{i,j}$ is the spatial weight between feature i and j, and:

$$S_i = \frac{\sum_{j=1, j \neq i}^{n} \omega_{i,j} (x_j - \bar{X})^2}{n-1}$$

With n equating to the total number of features.

The Z_{I_i}-score for the statistics are given as:

$$Z_{I_i} = \frac{I_i - E[I_i]}{\sqrt{v[I_i]}}$$

where:

$$E[I_i] = -\frac{\sum_{j=1, j \neq i}^{n} \omega_{i,j}}{n-1}$$

$$v[I_i] = E\left[I_i^2\right] - E[I_i]^2$$

(b) The local Moran's I of each spot is realized by Python Script 5 using the files of Step "Construction of spatial map" (d), Step "Data pre-processing" (c and d) (without.shp, T.gdb and Joined.gdb).

(c) The information includes local Moran's I, P-value, Z_{I_i}-score and spatial cluster type (CO type).

Comparison between region of interest and other regions

(a) Using the spatial map constructed in the Step "Construction of spatial map," the ROI is circulated. The spot barcode in the ROI is obtained.

(b) The number of high-to-high (H-H) or low-to-low (L-L) spots in the ROI is extracted and compare with other regions using Chi-Squared Test. It is realized by R Script 3.

(c) Top 20 genes of each comparison are used for bubble plot.

Visualization

(a) For any specific gene identified by the above analysis, add the CSV file to spatial map.

(b) Run Hot Spot analysis by Arcmap 10.8.

(c) Use the Legend, and convert it into graphics.

(d) Export the figure.

Results

Flow of spatial analysis of spatial transcriptomics method

As shown in **Figure 1A**, the spatial maps were constructed based on the spot barcodes derived from the ST data. The gene expression quantities were then connected to the corresponding spots. Global Moran's I was calculated to determine whether it was clustered. If not, the gene was aborted for further analysis. Next, the ROI was circled in the ST maps and the spot barcodes of the ROI were obtained. Then, local Moran's I was used to determine the CO type, which was group into five categories: non-significant, H-H, L-L, high-to-low (H-L), and low-to-high (L-H) spots (**Figure 1B**). The number of H-H and/or L-L spots

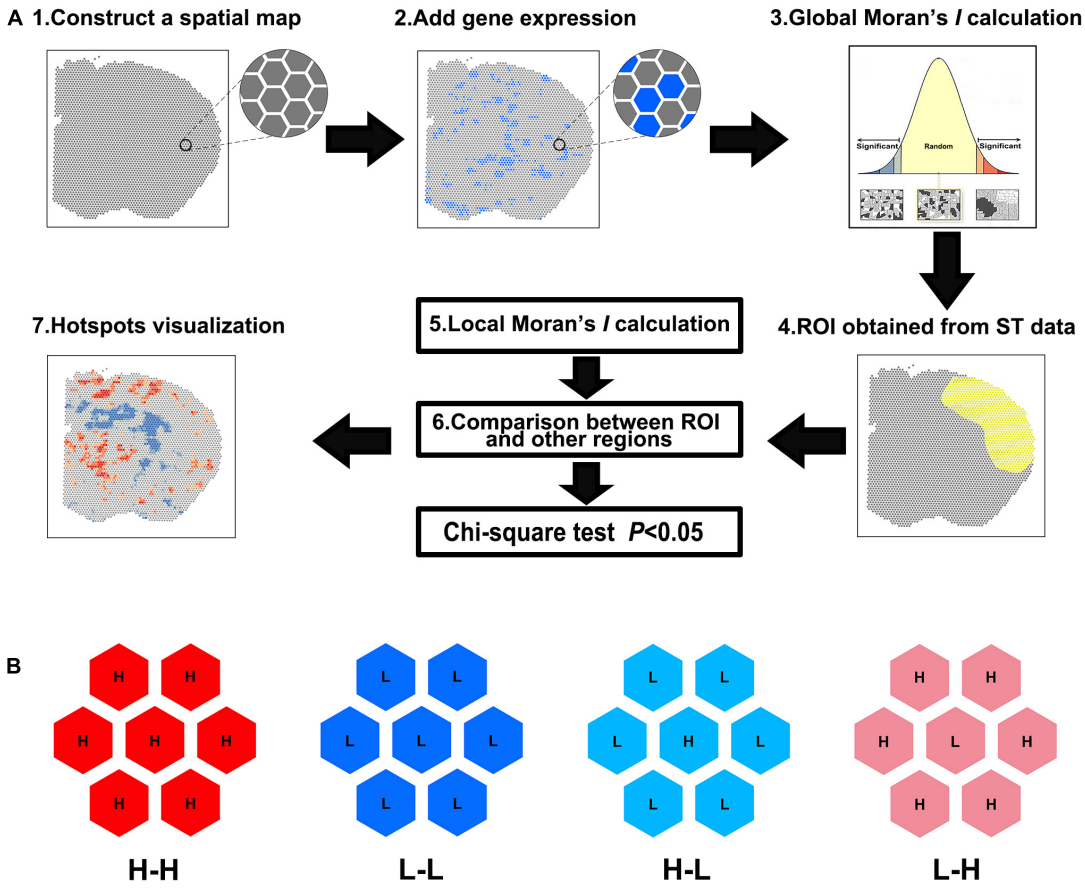

FIGURE 1
Spatial analysis of spatial transcriptomics (saSpatial) flow chart. **(A)** Flow chart of saSpatial. **(B)** Spatial cluster type. ST, spatial transcriptomics; ROI, region of interest; H-H, high-to-high; L-L, low-to-low; H-L, high-to-low; L-H, low-to-high.

were used for the following statistics. Finally, visualization of DEGs was performed using Hot Spot analysis with Arcmap 10.8.

Spatial autocorrelation patterns revealed by global Moran's *I*

Global Moran's *I* measures the spatial autocorrelation based on both the locations and the quantities of features. A value of 0 for Moran's *I* indicated no autocorrelation. In the brain section, no gene showed no autocorrelation. Z-score and *P*-value were used to evaluate the significance of global Moran's *I* (Figure 2). Based on the global Moran's *I*, z-score and *P*-value, the spatial pattern were group into: clustered, random and dispersed (Figure 2). If global Moran's *I* was close to + 1 and z-score was more than 1.65, the pattern was clustered, which means elevated gene expression had similar elevation values close to each other like *Nrgn* (Figure 2). If global Moran's I was close to −1 and z-score was less than −1.65, the pattern was dispersed, which means dissimilar values were next to each other like a checkerboard (Figure 2).

Differentially expressed genes of different regions in normal brain revealed by spatial analysis of spatial transcriptomics

Spatial transcriptomic data of normal brain section was obtained from the 10X Genomics dataset. The brain hemisphere was divided into sensorimotor cortex, basal ganglia, cingulate gyrus, entorhinal cortex, hippocampus, thalamus, and hypothalamus (Figure 3A). The ratio of H-H spots in the sensorimotor cortex was calculated and is shown in Figure 3E. Then we determined the proportion of H-H spots (more than 50% in Figure 3E) in the other brain regions (Figure 3F). For instance, *Arpp19* mostly enriched in the sensorimotor cortex while it also was found in the cingulate gyrus and entorhinal cortex, which was verified in ST map (Figures 3B–D,F). DEGs

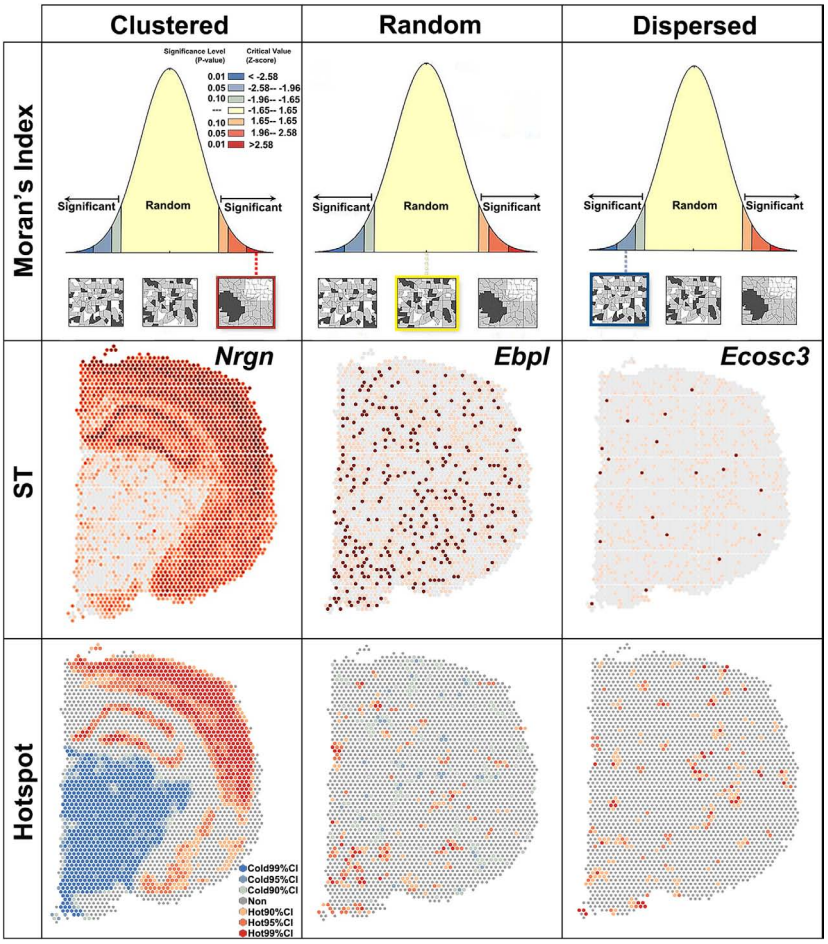

FIGURE 2
Global Moran's index revealing three spatial autocorrelation patterns. Clustered, $z > 1.65$ and $P < 0.1$; random, $-1.65 < z < 1.65$ and $P > 0.1$; dispersed, $z < -1.65$ and $P < 0.1$. ST, spatial transcriptomic; CI, confidence interval.

in other brain regions were also identified and typical genes are shown in Figure 3G. All these results indicated that saSpatial was able to detect DEGs of ROI within a section.

Up-regulated differentially expressed genes of ischemic penumbra and core

Spatial analysis of spatial transcriptomics was then applied to determine the DEGs in the brain section from ischemic stroke. Ischemic stroke was induced in a mouse model by occlusion of the distal middle cerebral artery. MRI scanning was performed 6 h after stroke onset to determine the ischemic penumbra (mismatch of perfusion- and diffusion-weighted imaging) and core. Penumbra has been defined as brain tissue at a risk of infarction (Albers et al., 2018). The brains were then harvested for ST sequencing. Ischemic core and penumbra and the corresponding area in the normal brain section were marked with reference to HE and MRI images (Figure 4B).

Considering numerous gene expressions were similar in the ischemic penumbra and core, which was also proved by the following data (Figure 4A), there were three comparisons of the number of H-H spots: between the ischemic penumbra or core and the corresponding area in the normal brain sections; between the ischemic penumbra or core and the normal region in the ischemic stroke brain section; and between ischemic core and penumbra (Figure 4A). Bubble plots of the top 20 DEGs for each comparison identified by saSpatial are shown in Figure 4A. For instance, *Gm42418* was highly expressed in the ischemic hemisphere while there was no significant difference between ischemic ore and penumbra. In contrast, *Cdk5r2* was significantly increased in the ischemic core but not in the penumbra (Figure 4C). Hence, *Cdk5r2* but not *Gm42418* was a marker gene for ischemic core. As for ischemic penumbra, saSpatial identified the up-regulated DEGs like *Hspa1a*, which was like a thin loop around the core (Figure 4C).

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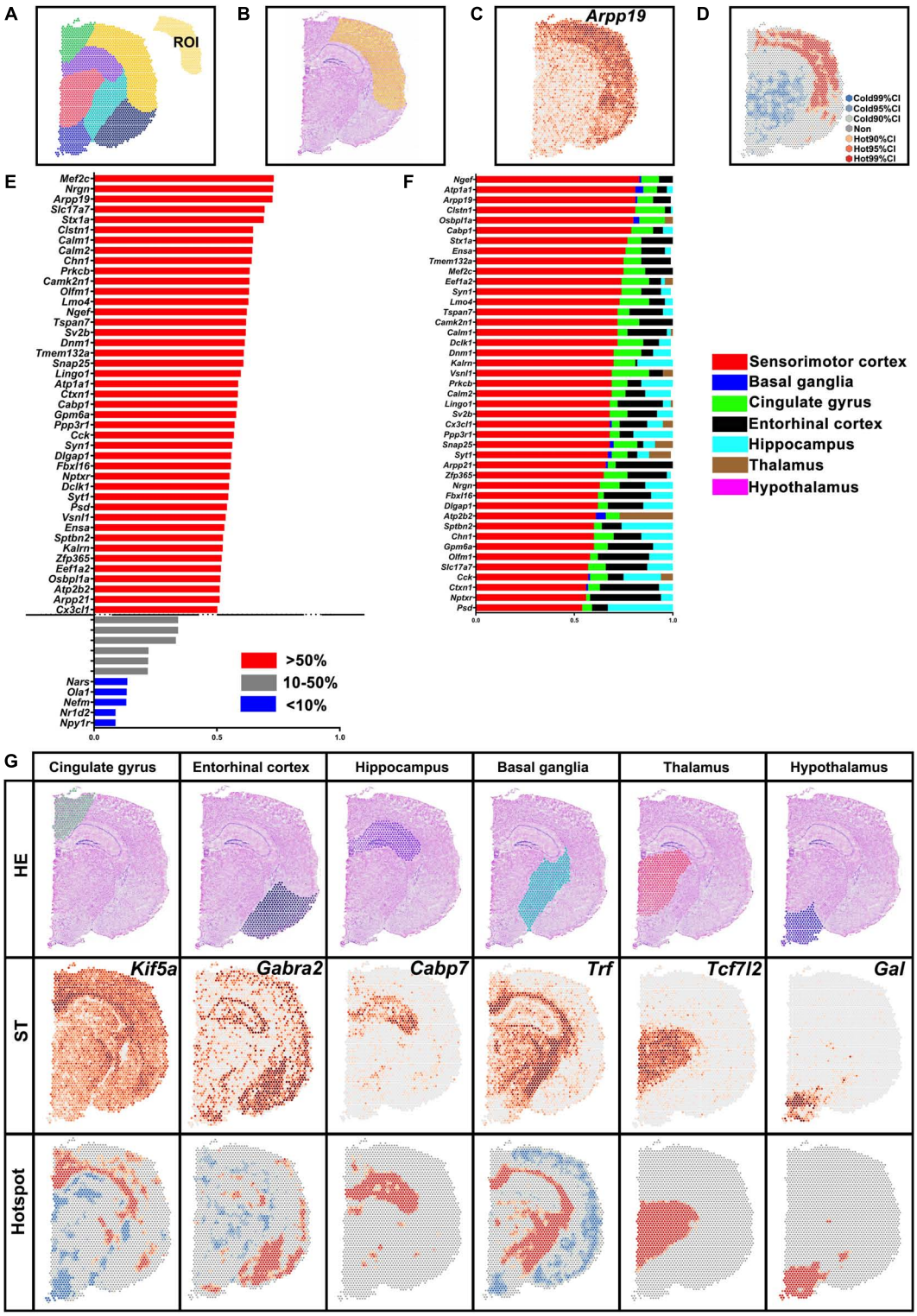

FIGURE 3

Differentially expressed genes (DEGs) in the different brain regions. **(A)** Brain region. ROI indicates sensorimotor cortex. **(B)** Yellow area indicates sensorimotor cortex in HE images. **(C)** *Arpp19* of ST map. **(D)** Hotspot of *Arpp19*. **(E)** DEGs in the sensorimotor cortex. Ratio = The number of H-H spots in sensorimotor cortex/The total number of spots in the section. **(F)** Septicity of DEGs in the sensorimotor cortex. Ratio = The number of H-H spots in each region/The total number of H-H spots. **(G)** Marker genes of each brain region. ST, spatial transcriptomic.

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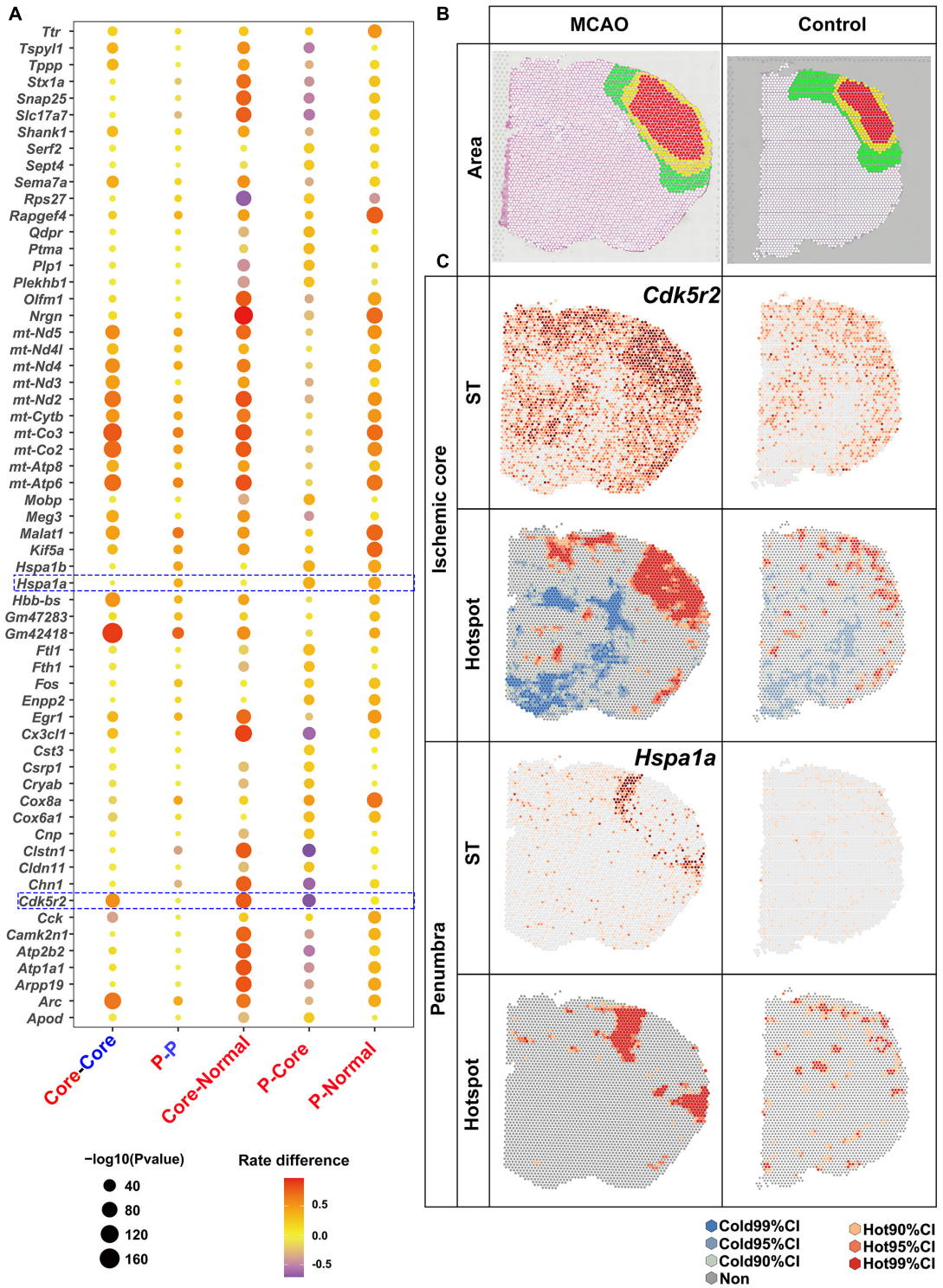

FIGURE 4
Upregulated DEGs revealed by saSpatial. **(A)** Bubble plots of top20 DEGs. *P*-values are indicated by circle size; scale adjacent to the plot. The rate difference between stroke and NC group is indicated by color. NC, normal control. P, penumbra. **(B)** Ischemic brain area. Ischemic core (red), penumbra (yellow), and normal area (green) in the sensorimotor cortex. **(C)** Typical DEGs of ischemic core and penumbra. *N* = 3. CI, confidence interval.

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Down-regulated differentially expressed genes of ischemic penumbra and core

Substantial attention was paid to the up-regulated expression, while expression of numerous genes were halted by ischemia. In contrast to the up-regulated DEGs as described above, the number of L-L spots was used for detection of down-regulated DEGs. Bubble plots of the top 20 DEGs for each comparison are shown in Figure 5A. There were a lot of genes down-regulated in ischemic area while most of them showed no significant differences between ischemic core and penumbra

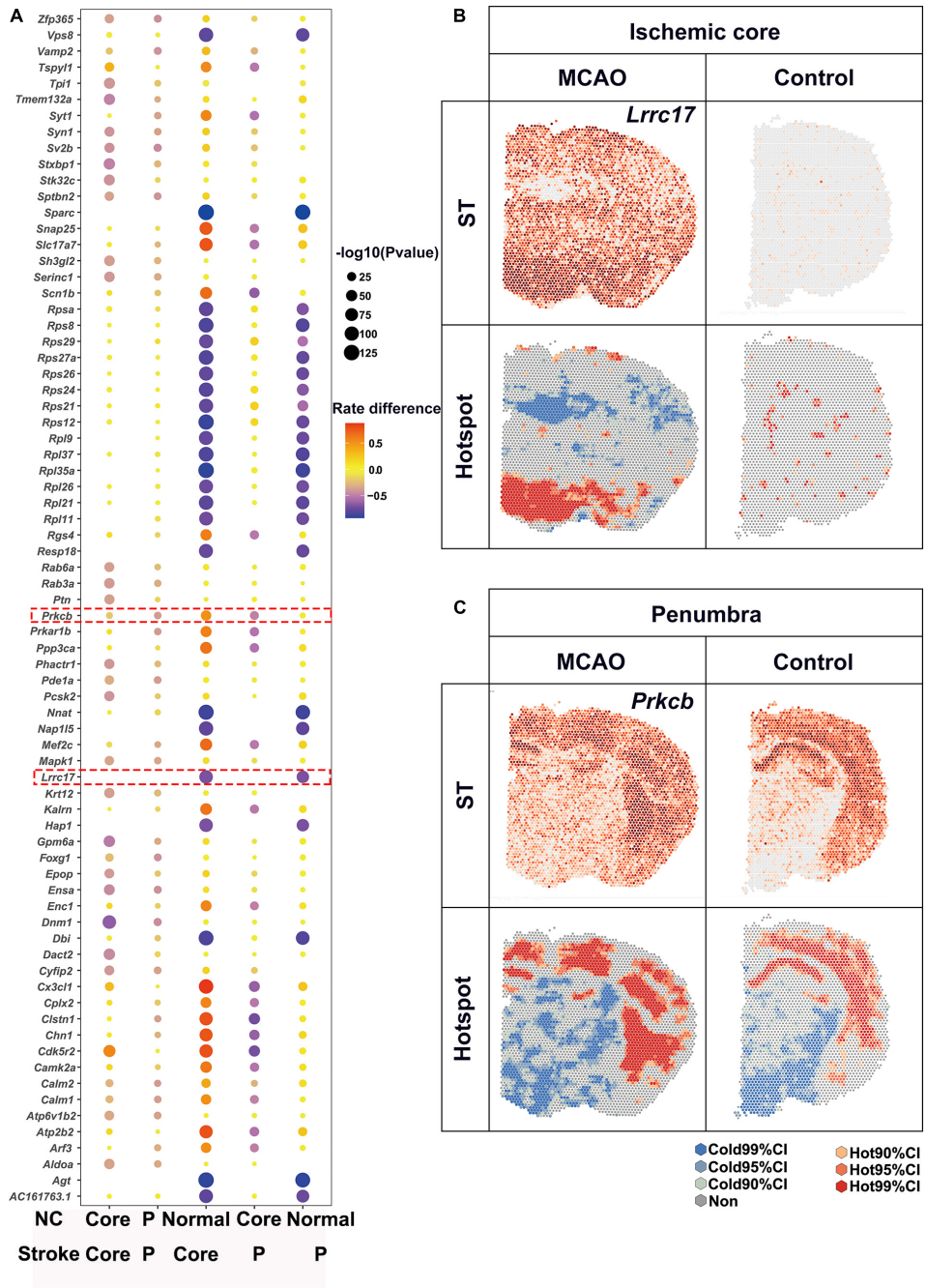

FIGURE 5
Downregulated DEGs revealed by saSpatial. **(A)** Bubble plots of top20 DEGs. *P*-values are indicated by circle size; scale adjacent to the plot. The rate difference are indicated by color. NC, normal control. P, penumbra. Typical DEGs of **(B)** ischemic core and **(C)** penumbra. *N* = 3. ST, spatial transcriptomic; CI, confidence interval.

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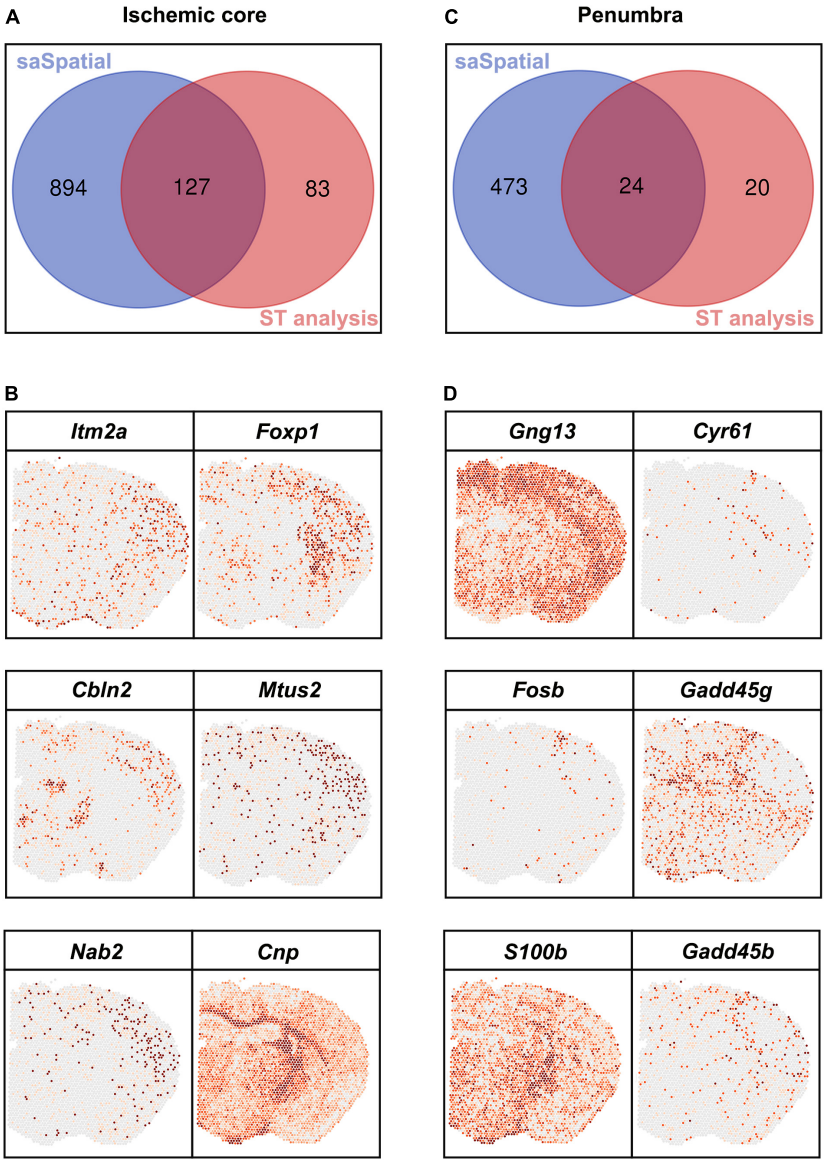

FIGURE 6
Comparison between saSpatial and FindMarkers. **(A)** Venn map of ischemic core. **(B)** DEGs only found by saSpatial in the ischemic core. **(C)** Venn map of penumbra. **(D)** DEGs only found by saSpatial in the penumbra. *N* = 3. CI, confidence interval.

such as *Vps8* and *Sparc* (Figure 4A). For the ischemic core, *Lrrc77* was a marker gene (Figure 5B). As for the ischemic penumbra, down-regulated DEGs like *Prkcb* were identified (Figure 5C).

Comparation of spatial analysis of spatial transcriptomics with FindMarkers

Differentially expressed genes detected by saSpatial were compared with that detected by FindMarkers (Figure 6). For the ischemic core, FindMarkers found only 210 DEGs while there were 1,021 DEGs identified by saSpatial (Figure 6A and Supplementary Table 1). The majority of DEGs detected by FindMarkers could be also found by saSpatial (Figure 6A) while numerous genes like *Itm2a*, *Foxp1*, *Cbln2*, *Mtus2*, *Nab2*, and *Cnp* were not detected by FindMarkers (Figure 6B). For the ischemic penumbra, FindMarkers can only find 44 DEGs and the majority of DEGs were missed (Figure 6C). saSpatial found that there were 497 potentially valuable DEGs in the ischemic penumbra (Figure 6C) such as *Gng13*, *Cyr61*, *Fosb*, *Gadd45g*, *S100b*, and *Gadd45b*, which were confirmed by ST maps (Figure 6D).

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Differentially expressed genes of different cortical layers

The sensorimotor cortex is composed of six layers. DEGs in each layer were revealed by saSpatial and the top 3 DEGs in each layer are shown in Figure 7A. Layer V is the internal pyramidal layer and contains large pyramidal neurons, the axons of which form the corticospinal tract. saSpatial showed that *Ighm* was a marker gene in Layer V (Figure 7B).

Differentially expressed genes of different cortical layers in the ischemic core

Different cortical layers responded differently to ischemic insult. Six layers of ischemic core and that in the normal brain section were marked with reference to the HE images (Figure 8A). The fraction of DEGs in each layer is shown in Figure 8B. The ST and Hotspot maps are shown in Figure 8C.

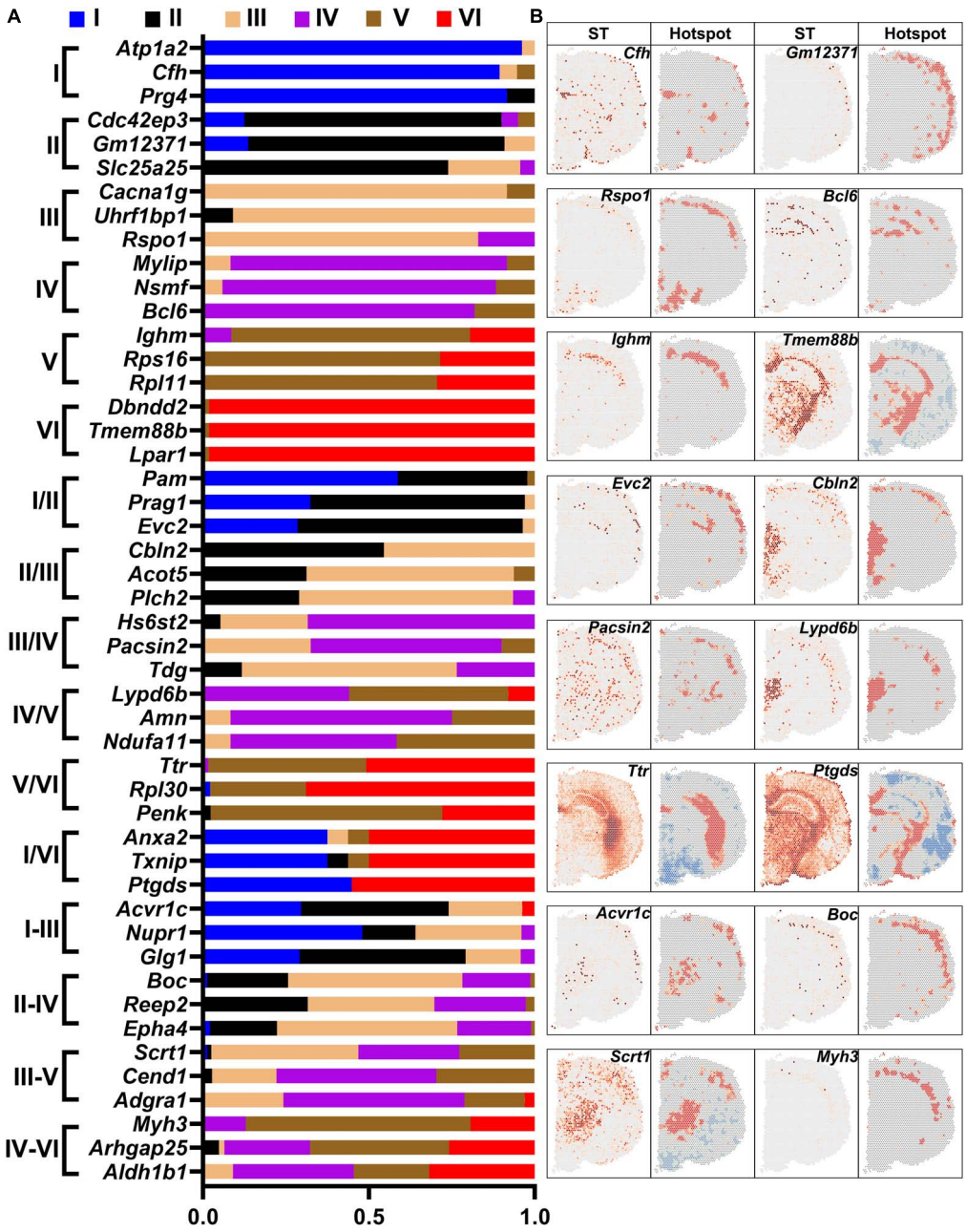

FIGURE 7
Differentially expressed genes (DEGs) in the cortex layers. **(A)** Top 3 DEGs in the layers. **(B)** Typical DEGs. ST, spatial transcriptomic.

For example, *Cbln4*, which was not found in the normal brain, was up-regulated in the ischemic core and enriched mainly in the Layer III. *Scn4b* was up-regulated in the Layer V while it was down-regulated in other layers (Figures 8B,C).

Discussion

In the present study, we developed a simple, reliable and reproduceable method, named saSpatial, to detect the

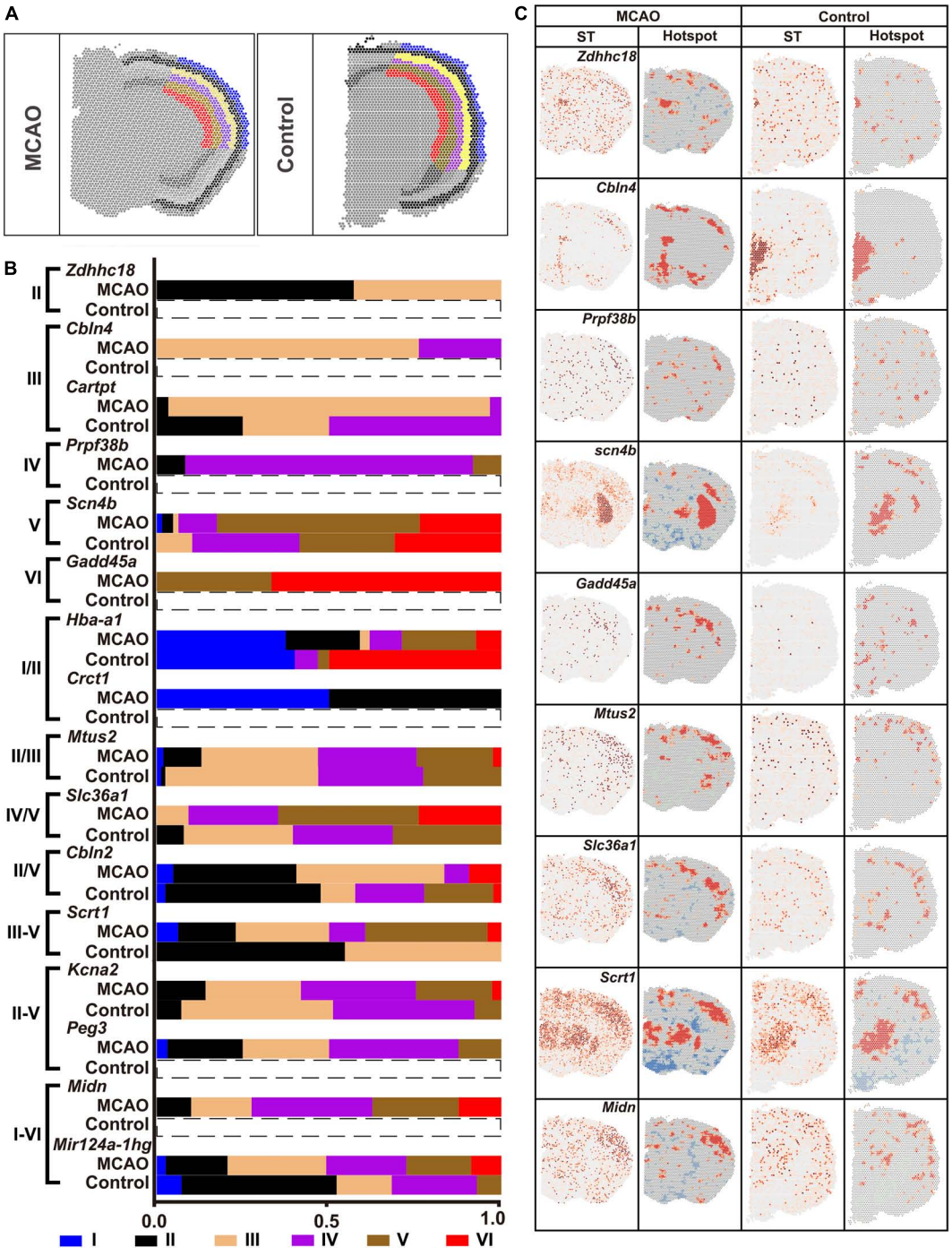

FIGURE 8
Heterogenicity of DEGs within the ischemic cortex. **(A)** Six layers of cortex in the ischemic cortex. **(B)** Typical DEGs. **(C)** ST map and Hotspot of typical DEGs. ST, spatial transcriptomic.

DEGs in ST data. DEGs from different biological situations within the same section or across the different sections can be clearly detected by saSpatial. Heterogenicity of a particular region can also be characterized by saSpatial. In comparison to the traditional method, saSpatial was able to detect more valuable DEGs.

Classical statistics-based methods such as FindMarkers detected the DEGs only referred to the value of gene expression (Dries et al., 2021). It neglects a basic fact that gene expression of cells is effected by the other cells especially the nearby ones. Several other methods such as SpatialDE have been developed to explore the variable genes from the spatial context while they failed to detect the DEGs of any specific ROI (Svensson et al., 2018; Xu and McCord, 2021; Zhu et al., 2021). Based on the spatial statistics, the CO types of any individual spot included information about both the effect of spatial location on gene expression and gene expression quantity. The chi-squared test was used to compare the number of H-H or L-L spots between the ROI and the other regions. This simple approach provided a tool for analyzing the ST data to detect the DEGs and characterize their heterogeneity within the ROI. To the best of our knowledge, it was the first tool to detect the DEGs in ST data with regard to location and value of gene expression simultaneously. We then used this tool to investigate the expression and distribution of genes in the normal and ischemic stroke brain sections.

Firstly, we used saSpatial to detect the DEGs in different regions of the normal brain section. Many previous studies have used ST to characterize the genetic heterogeneity in brain section. Usually, they used snRNA data to find the gene expressions in certain regions and projected the findings onto ST data like the multimodal intersection analysis (MIA) method (Baccin et al., 2020; Moncada et al., 2020). In contrast, saSpatial detected DEGs directly and quickly. In the present study, saSpatial found various DEGs in the different brain regions, such as *Arpp19* for sensorimotor cortex and *Cabp7* for hippocampus.

Then, saSpatial was used to detect the DEGs in the ischemic brain. Ischemic stroke was one of the leading causes of mortality and morbidity (Hankey, 2014). Ischemic insult resulted in significant changes of gene expressions (Xu et al., 2017). In the present study, saSpatial found various DEGs in the ischemic brain area. Ischemic brain area was divided into ischemic core and penumbra, of which DEGs was hard to distinguish. saSpatial can successfully distinguish the DEGs in the ischemic core and penumbra including the up- and down-regulated ones. Furthermore, more DEGs were found by saSpatial than that by FindMarkers.

Finally, saSpatial characterized heterogenicity of the gene expressions in the normal and ischemic cortex. Cortex genetic heterogenicity has been proved by many other studies (Stahl et al., 2016). Cortex was made up of six layers, each of which was a limited area like Layer I. How to identify the heterogenicity within a small region in ST data remained challenge. We used saSpatial and found that *Cfh* dominated in the Layer I and *Ighm* was the marker gene of Layer V. As for the ischemic cortex, *Cbln4* was up-regulated in the ischemic core and enriched mainly in the Layer III while *Scn4b* was up-regulated in the Layer V and down-regulated in other layers.

Limitations

Spatial analysis of spatial transcriptomics was based on the ST data. Hence, the resolution of saSpatial relied on the raw ST data. A spot in ST data represented genes of an area including about 10 cells. Thus, saSpatial cannot reveal the cellular types. Secondly, CO types of spots mainly consider the effect of adjacent spots while some genes were affected by remote ones. Finally, the DEGs identified by saSpatial needs to be verified by the additional biological experiment.

Conclusion

In conclusion, saSpatial was constructed based on local Moran's *I* and successfully detected the DEGs in ST data. It would help the potential researchers to find more valuable DEGs in the future study.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. ST data was available in the GEO site: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE199066 and https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE199067. The code was provided in the **Supplementary material**.

Author contributions

ZQ and SL collected the data and prepared the first draft. ML helped perform the animal model and MRI scanning. SZ and ZW analyzed as part of the data. YJ designed the whole study and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2022.1086168/full#supplementary-material

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Effects and mechanism of myeloperoxidase on microglia in the early stage of intracerebral hemorrhage

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Objectives: (1) To clarify the dynamic relationship between the expression of myeloperoxidase (MPO) and microglial activation of intracerebral hemorrhage (ICH), (2) to explore the effect of inhibition of MPO on microglial activation, and (3) to observe the improvement in the neurobehavior of mice with inhibition of MPO.

Methods: C57 BL/6 mice and CX3CR1 + /GFP mice were used to establish a phosphate-buffered saline (PBS) group, an ICH group, and a 4-aminobenzoic acid hydrazide (ABAH) group. Longa score, open field locomotion, hind-limb clasping test, immunohistochemistry, immunofluorescence, blood routine detection, and flow cytometry were used.

Results: The neurobehavior of the mice was significantly impaired following ICH ($P < 0.01$); the expression of MPO was significantly increased following ICH, and reached a peak value at 6 h post-injury ($P < 0.001$). Moreover, the microglial activation increased significantly following ICH, and reached a peak level at 24 h post-injury ($P < 0.01$). Following inhibition of MPO, the activation of microglia in the ICH group decreased significantly ($P < 0.001$). Moreover, the neurobehavior of the ICH group was significantly improved with MPO inhibition ($P < 0.05$).

Conclusion: MPO may be an upstream molecule activated by microglia and following inhibition of MPO can improve secondary injury resulting from ICH.

KEYWORDS

myeloperoxidase, intracerebral hemorrhage, microglia, activation, inhibition, neurobehavior

Introduction

Intracerebral hemorrhage (ICH) accounts for 10–15% of all strokes and is characterized by high lethality and disability. ICH damage to the brain consists of either primary hematoma compression effects or secondary hematoma breakdown products, both of which cause damage to the brain parenchyma. The main factor leading to poor prognosis of ICH is secondary brain injury. The mechanisms that cause secondary injury to ICH are mainly inflammatory response, local active oxygen free radical release, and apoptosis around hematomas (Xi et al., 1999; Cherubini et al., 2000; Wang et al., 2007). ICH results in neuronal cell death and the release of factors such as damage-associated molecular patterns (DAMPs) that induce localized inflammation in the injured brain region. And such focal brain inflammation aggravates secondary brain injury by exacerbating blood-brain barrier breakdown, microvascular failure, brain edema, oxidative stress, and by directly inducing neuronal cell death (Shi et al., 2019). Therefore, the inflammatory response is currently considered to be the key factor causing secondary brain injury following ICH (Wang et al., 2007). Inflammatory responses include microglial activation, leucocyte infiltration, enzyme activation, and release of numerous mediators of injury, such as hemoglobin, iron, reactive oxygen and nitrogen species (Garcia et al., 1994).

Following ICH, microglial cells, as important neuroinflammatory effector cells, are the first non-neuronal cells to generate an immune response to acute brain injury (Walentynowicz et al., 2018). *In vitro*, activated microglia are polarized, showing two polarizing phenotypes, "classically activated" proinflammatory (M1) or "alternatively activated" anti-inflammatory (M2) cells (Xiong et al., 2016). At present, it is believed that when ICH occurs, microglia are activated and the phenotype undergoes short-term dynamic changes (Wang et al., 2013; Lee et al., 2016; Liu et al., 2021). The expression of the M1 microglia in the early stage of ICH is much higher than that of M2 microglia, showing a global pro-inflammatory state (Lan et al., 2017). M1 macrophages are neurotoxic, and M2 macrophages promote a regenerative growth response in adult sensory axons (Kigerl et al., 2009). M1-type microglia are produced and release a series of inflammatory mediators and biologically active factors, such as TNF-α, IL-1β, IL-6, IL-8, etc., lead to inflammation following ICH, and cause further secondary damage. Therefore, microglia plays a key role in the initial inflammatory response following ICH. Supportive treatment is currently the main therapy for ICH (Thabet et al., 2017). A recent study has shown that use of pinocembrin (molecular formula: $C_{15}H_{12}O_4$) to inhibit M1 microglia can protect brain tissue following ICH (Lan et al., 2017). However, the specific mechanism of microglial activation and polarization in ICH is still unclear.

Myeloperoxidase (MPO) is a leukocyte enzyme secreted by activated neutrophils, monocytes, and macrophages, and it possesses peroxidase activity (Arnhold and Flemmig, 2010). Experiments have shown that, in addition to being abundant in neutrophils, MPO is also expressed in other myeloid cells, such as microglia (Gray et al., 2008). In a mouse model of ICH, bleeding causes upregulation of inflammatory factors in the brain, a large number of blood myeloid cells are recruited into the brain, and MPO is synthesized and released to evacuate hematomas. On the other hand, the inflammatory network regulated by MPO may trigger subsequent injury. Therefore, MPO is considered to be a biomarker for the diagnosis and prognosis of ICH (Wang et al., 2007). Studies have shown that the classic MPO inhibitor, 4-aminobenzoic acid hydrazide (ABAH), can inhibit MPO activity, increase the proliferation of stroke neurons, and improve neurogenesis (Forghani et al., 2015; Kim et al., 2016). Stefanova et al. (2012) found that inhibition of MPO in PLP-a-SYN mice can inhibit microglial cell activation and, at the same time as MPO peaks at 6 h, M1 microglial cells are also activated. From the results of previous studies, it is not difficult to speculate that there may be some connection between MPO and microglia in ICH. Therefore, we hypothesized that the key target cells regulated by MPO in ICH are microglial cells. The activating effect of MPO on microglial cells was verified by experiments, and the effects and mechanism of MPO on microglial cells in the early stage of ICH were investigated. This could provide an endogenous treatment strategy for clinically difficult ICH cases.

Materials and methods

Experimental animals

The C57BL/6 mice and CX3CR1 + /GFP mice used in this experiment (had the same genetic background as C57BL/6 mice, including immune cells of myeloid origin, including microglia in the central nervous system; expression of the green fluorescent protein (GFP) gene was composed and provided by the Medical Laboratory Animal Center of Lanzhou University) were kept in a quiet environment, freely fed and watered, keep the living environment of mice for 12 h each day and night. Of these, 37 C57BL/6 mice were male, 8 weeks old, weighing 20 ± 2 g; 21 CX3CR1 + /GFP mice were all male, 8 weeks old, weighing 20 ± 2 g. All animal experiments were approved by the Medical Laboratory Animal Ethics Committee of Lanzhou University Second Hospital. All animals were euthanized in accordance with ethical animal laboratory practice.

Establishment of intracerebral hemorrhage model

All experimental mice were placed in the experimental environment for at least 2 weeks prior to experimentation

in order to adapt the mice to the environment, allowing the mice free access to food and water. The animals were anesthetized by intraperitoneal injection with a 1% sodium pentobarbital solution (50 mg/kg), and then venous blood from the mice was added to an EP tube soaked with heparin using the tail-capped blood collection method. The temperature of the mice was maintained at 37 °C using a thermostatic blanket. The individual mice were placed in a stereotactic frame, the skin of the head was cut, and the scalp was wiped with a hydrogen peroxide solution so that the skull was fully exposed, and a skull hole (approximately 1 mm in diameter) was drilled on the right side of the sagittal line. A 26-gauge needle was inserted into the striatum on the right side (coordinates: $X = -2.0$ mm; $Y = -0.5$ mm; $Z = -3.5$ mm), and a micro-infusion pump was used, the ICH mice were perfused with 10 μL of autologous blood, and the control mice were perfused with ice-cold phosphate-buffered saline (PBS; pH 7.4). The needle was removed, the drilled hole was filled with bone wax, and the skin incision was sutured with a No. 4 suture.

Behavioral experiments

Longa score: A score of 2–3 indicates that the model was successfully created.

0 points: normal, no neurological deficits.
1 point: left forelimb cannot fully extend, mild neurological deficit.
2 points: when walking, mice looped to the left, with moderate neurological deficits.
3 points: when walking, mice collapsed to the left, with severe neurological deficits.
4 points: mice cannot walk on their own, losing consciousness.

Open field locomotion: We used opaque plastic to make a cube box of dimensions 50 cm × 50 cm × 50 cm. The bottom of the box and the inner walls were white. The mouse was placed at a specific corner facing the central area and timing was commenced. The mouse's activities within 15 min were automatically recorded. The open field in the video was divided into a central area and a surrounding area by SMART v3.0. The central area accounted for 25% of the total area, and the mouse's movements were tracked simultaneously. We observed the exercise distance, exercise speed, rest time, and the time the mouse spent in the central area in order to evaluate the exercise ability of the mouse.

Hind-limb clasping test: The tail suspension of the mouse was lifted, the movements of the hind limbs of the mouse within 15 s were recorded, and the hind-limb clasping and muscle strength were observed.

Immunohistochemical and immunofluorescence labeling

We first divided the mice into an ICH group and a PBS group and observed iba1 antibody cells and MPO staining at 6, 12, and 24 h. Six C57 mice were used in each group. We then used the CX3CR1 + /GFP mice for MPO immunofluorescence with six mice per group. We used 40 μm-thick brain slices for immunohistochemistry. For iba1 staining, we used the ABC method. The antibodies were Rabbit anti iba1 (Solarbio Life Sciences, Beijing, China, 1:600), Goat anti Rabbit (Solarbio, 1:300), and streptavidin–horseradish peroxidase conjugate (STR-HRP). For MPO fluorescence staining, we used Rabbit anti MPO (Solarbio, 1:600) and Goat anti Rabbit (Solarbio, 1:300). Following each antibody staining, the brain slices were washed three times with PBST (PBS + Triton). Images of the immunohistochemically labeled sections and the fluorescently immunolabeled sections were obtained using an Olympus CKX41 fluorescence inverted microscope (Olympus, Tokyo, Japan). The images were captured in the FITC and TRITC channels using Cell-P imaging software (Olympus). When the two images were merged, the cells appeared yellow.

Blood routine detection

Before perfusion and taking of the brain slices, 1 ml of blood was collected using the orbital sinus blood collection method in an EP tube soaked with heparin. The EP tube was placed in a routine blood testing machine, and the results were recorded automatically.

Flow cytometry

The brain tissue was placed in pre-chilled flow cytometry buffer [1% bovine serum albumin (BSA), 1 mM EDTA, pH 7.4, 0.1 M PBS, 50 U/ml DNAseI], the striatal area was gently shaken three times, and the tissue was broken up and passed twice through a 70 μm nylon filter in order to generate a single cell suspension, and then the cells were centrifuged at 4 °C, 1,200 rev/min, the supernatant was discarded, flow buffer was added, and the cells washed with 1 × flow cytobuffer, at 4 °C, 1,200 rev/min. After discarding the supernatant and transferring the suspension to a flow tube, flow cytometry was performed. The FITC channel was used for screening. The results were analyzed using FlowJo software.[1]

1 https://www.flowjo.com

Statistical analysis

All data were expressed as the mean ± standard error on the mean (SEM). Comparison between two groups was performed using the unpaired Student's *t*-test. Comparison between multiple groups was performed using one-way analysis of variance (ANOVA) following the *post-hoc* test. $P < 0.05$ was taken to be statistically significant. Prism 8 statistical graphing software[2] was employed, and FlowJo software was used for flow cytometry analysis.

Results

Inhibition of myeloperoxidase activity promotes recovery of motor function following intracerebral hemorrhage

In order to explore whether the activity of MPO affects the recovery of motor function following ICH, we injected ABAH into the striatum (CPu) of mice in order to inhibit the activity of MPO (**Figure 1A**). We used the Longa score, the open field test, and the hind-limb clasping test to comprehensively evaluate the motor behavior ability of the control, ICH, and ABAH intervention groups, respectively, (ABAH-1/ABAH-2). The Longa score showed that, compared with the PBS group, the neurological deficits in the ICH group were more obvious, and the neurological behavior of the ABAH group was improved compared with that of the ICH group (**Figure 1B**). In order to confirm that ABAH intervention improved neurological function following ICH, we used the open field test to evaluate the motor ability of experimental animals (**Figures 1C,D**). The PBS, ICH, and ABAH-2 groups were tested at 24 h, and the ABAH-1 group was tested at 6 h, because the ABAH-1 group was given the first dose of ABAH before the model was established, and the ABAH-2 group was given its dose after the behavior of the ABAH-1 group was tested. The results showed that, compared with the PBS group, the exercise time of the ICH group was significantly reduced, while the difference between the ABAH-1 group and the ICH group was not statistically significant, but the exercise state of the ABAH-2 group was significantly improved (**Figure 1C**). In the hind-limb clasping experiment, we found that, compared with the PBS group, the ICH and ABAH-1 groups had weaker hind-limb clasping ability and more obvious symptoms of paralysis. The hind-limb clasping ability of the ABAH-2 group was significantly improved (**Figure 1E**).

In summary, we found that use of the MPO activity inhibitor ABAH significantly improved motor ability following ICH.

2 https://www.graphpad.com

Intracerebral hemorrhage can induce changes in the number and proportion of inflammatory cells in peripheral blood

Finally, we studied the changes in the number and proportion of neutrophils and monocytes in the peripheral blood of mice following ICH in order to evaluate the level of inflammation-related cells activated following ICH. Cytological examination of the peripheral blood of mice in each group demonstrated that neutrophils and macrophages increased in proportion and quantity following ICH (**Figures 2A–D**).

Inhibition of myeloperoxidase activity reduced microglial proliferation following intracerebral hemorrhage

In order to explore whether MPO activity affected changes in microglia following ICH, we used flow cytometry to count the microglia in the brains of mice 24 h after modeling. At the same time, in order to compare the levels of microglia in the normal physiological state, we specifically introduced a normal group as a control. The results showed that the number of microglia in the ICH group increased significantly, while those in the ABAH group decreased significantly (**Figures 3A–C**).

Microglia and myeloperoxidase were activated following intracerebral hemorrhage

Next, we used immunohistochemical and immunofluorescence techniques to detect microglia and MPO activity following ICH. In order to better understand the activation characteristics of microglia and MPO in the pathophysiology of ICH, we selected three time points (6, 12, and 24 h post-injury) in order to locate and quantify the expression of iba-1 and MPO in the mouse control group and the ICH group. The results showed that microglia were significantly activated in the ICH group compared with the PBS group, and the most significant activation was found at 24 h (**Figures 4A–C**). In the immunofluorescence detection of MPO, we found that the activity of MPO significantly increased following ICH, but the activation peak of MPO appeared at 6 h post-injury (**Figures 4D–F**). To further verify the relationship between microglia and MPO activation following ICH, we used a special CX3CR1 + /GFP gene-edited mouse, which could specifically display microglia via GFP fluorescence. Using immunofluorescence, we found that the expression of GFP and MPO in the ICH group was significantly increased compared with the other three groups, while the expression of

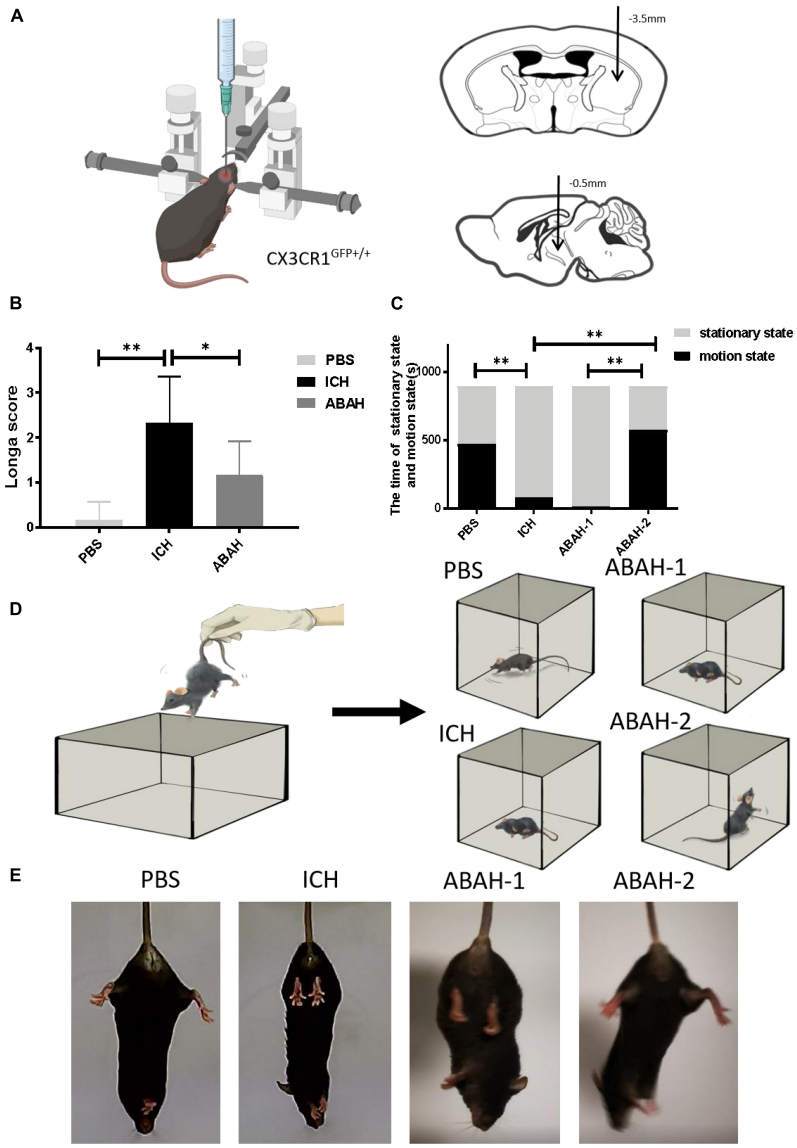

FIGURE 1
Inhibition of myeloperoxidase (MPO) activity promotes recovery of motor function following intracerebral hemorrhaging (ICH). **(A)** Establishment of intracerebral hemorrhage model; **(B,C)** Longa scores of PBS, ICH, and ABAH groups (*$P < 0.05$, **$P < 0.01$, $n = 6$ in each group); open field locomotion in the PBS, ICH, and first and second ABAH treatment groups. The times of movement and rest of the mice in the open field were recorded. The total time was 900 s (*$P < 0.05$, **$P < 0.01$, $n = 6$ in each group); **(D)** open field mode diagram; **(E)** hind-limb clasping test in the PBS, ICH, and first and second ABAH treatment groups. The degrees of hind-limb opening and closing in mice were observed.

GFP and MPO in the ABAH group was significantly reduced compared with the ICH group (**Figures 5A–C**). The results of the immunofluorescence determinations also showed that the expressions of MPO and GFP were spatially overlapped.

Discussion

ICH is a serious central nervous system disease, accounting for approximately 10–15% of all stroke cases, and its mortality rate is as high as 30–67%. Therefore, ICH is one of the greatest challenges for current neurosurgical diagnosis and treatments (Li et al., 2020). Microglia are a special class of mononuclear phagocytes, which play a very important role in the development of the central nervous system and the occurrence, development, and prognosis of central nervous system diseases (Uff et al., 2022). Microglia, neutrophils, and macrophages in the blood are the main executors of inflammatory response to ICH, and inflammatory response is one of the main causes of poor prognosis for ICH patients (Liu et al., 2022).

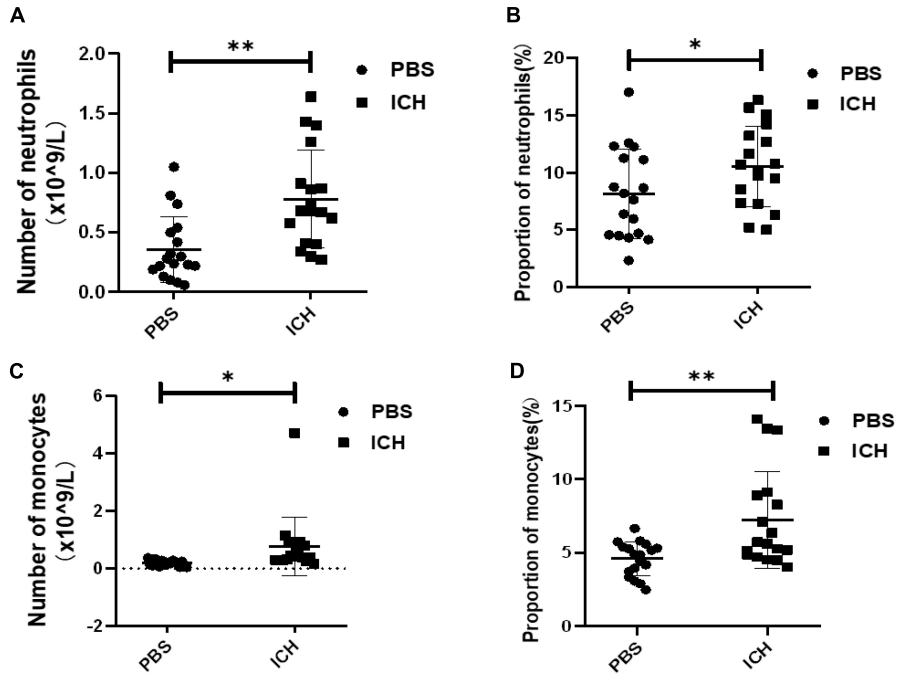

FIGURE 2
ICH can induce changes in the number and proportion of inflammatory cells in peripheral blood. **(A)** Quantity of neutrophils in peripheral blood (**$P < 0.01$, $n = 18$ in each group); **(B)** neutrophils/peripheral blood ratio (*$P < 0.05$, $n = 18$ in each group); **(C)** quantity of peripheral blood mononuclear cells (*$P < 0.05$, $n = 18$ in each group); **(D)** ratio of mononuclear cells to peripheral blood (**$P < 0.01$, $n = 18$ in each group).

Existing studies of therapeutic strategies involving microglia for ICH divide the latter into two main aspects: inhibiting the proinflammatory activity of microglia/macrophages and improving the regulatory properties of myeloid cells that display potential repair and anti-inflammatory properties. The former study subjects include Minocycline, complement 5a receptor antagonist (C5aRA), recombinant C1q/TNF-related protein 9 (rCTRP9), and the leukotriene B4 (LTB4) receptor antagonist, Bortezomib (a proteasome inhibitor), Fingolimod, etc. The study subjects of the latter aspect include Sphingosine-1-phosphate receptor (S1PR) agonists, statins, cannabinoid receptor-2 (CBR2) agonist, peroxisome proliferator-activated receptor γ (PPAR γ) activators, mammalian target of rapamycin (mTOR) inhibitors, Sinomenine, etc. Some of these medications and methods still lack the experimental verification necessary for their safe use in humans, but preclinical data support the use of inactivating agents or inhibitors of proinflammatory microglia/macrophages while enhancing the regulatory phenotype as part of a therapeutic approach to improving the prognosis of ICH (Wang, 2010; Shao et al., 2019; Bai et al., 2020).

Therefore, clarifying the molecular mechanisms related to the activation of microglia, neutrophils, and macrophages is the key to developing new therapies for ICH in the future. According to previous studies, MPO is an important regulatory molecule in the development of ICH, which can participate in the development and prognosis of stroke in various ways. Therefore, MPO is expected to become an important intervention target for ICH in the future (Wang et al., 2022). In summary, we have reason to believe that MPO can participate in the occurrence and development of ICH by changing the activation state of microglia. However, the relationship between MPO and microglial activation has not been experimentally verified in ICH, so this hypothesis was investigated in our study.

First, after the MPO inhibitor ABAH was given to ICH mice *in vivo*, the effects of MPO on ICH motor behavior were verified by behavioral experiments such as Longa score, open field test, and hind-limb clasping test. The experimental results showed that ICH could cause severe neurological dysfunction compared with the control group, which was highly consistent with the clinical manifestations of ICH patients. However, it was surprising that the MPO inhibitor ABAH improved motor behavior effectively following ICH. This preliminary experiment proved that the intervention of MPO activity is effective for the recovery of motor function following ICH, and it also verified our previous hypothesis that MPO can be used as a therapeutic target for ICH. In order to further explore the changes in MPO and microglia during the development of ICH, we analyzed these changes in the time dimension. The results showed that microglia were activated following ICH and peaked at 24 h. The expression of MPO also increased following ICH, peaking at 6 h following ICH. Because the peak of MPO expression appeared

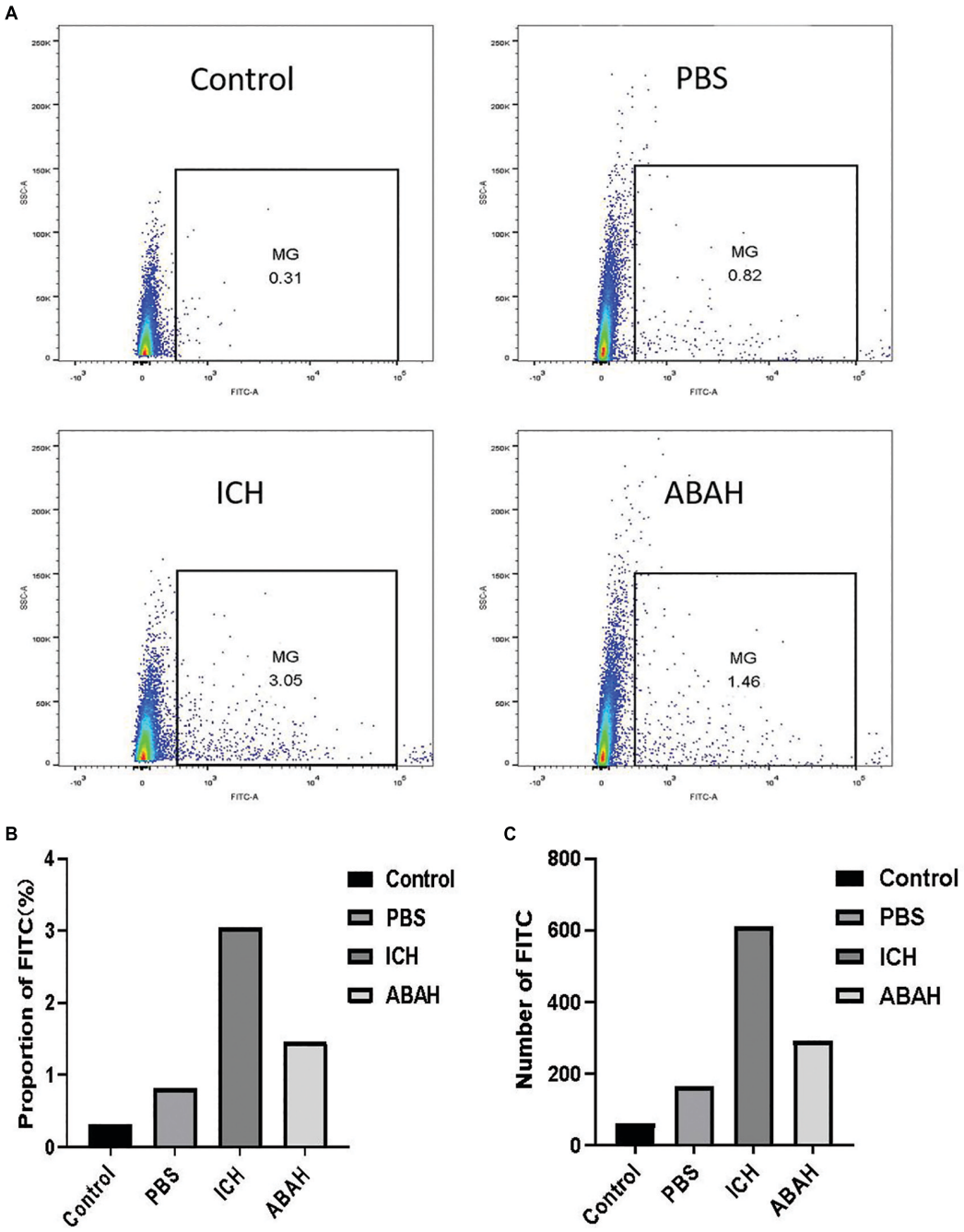

FIGURE 3
Inhibition of MPO activity reduces microglia proliferation following ICH. **(A)** Flow cytometry FITC channel, to detect the content of microglia in the control, PBS, ICH, and ABAH groups (set the total number of living cells to 20,000, n = 1 in each group); **(B)** proportion of FITC in the control, PBS, ICH, and ABAH groups; **(C)** number of FITC in the control, PBS, ICH, and ABAH groups.

earlier than the peak of microglial activation, we believe that the high degree of expression of MPO is one of the causes of microglial activation following ICH. To further verify the relationship between microglia and MPO activation following ICH, we used a special CX3CR1 + /GFP gene-edited mouse, which could specifically display microglia *via* GFP fluorescence. Using the immunofluorescence detection of gene-edited mouse tissues, we found that the expression levels of GFP and MPO in the ICH group were significantly higher than those in the other three groups, while the expression levels of GFP and MPO in the ABAH group were significantly lower than those in the ICH group. At the same time, due to the consistency of GFP

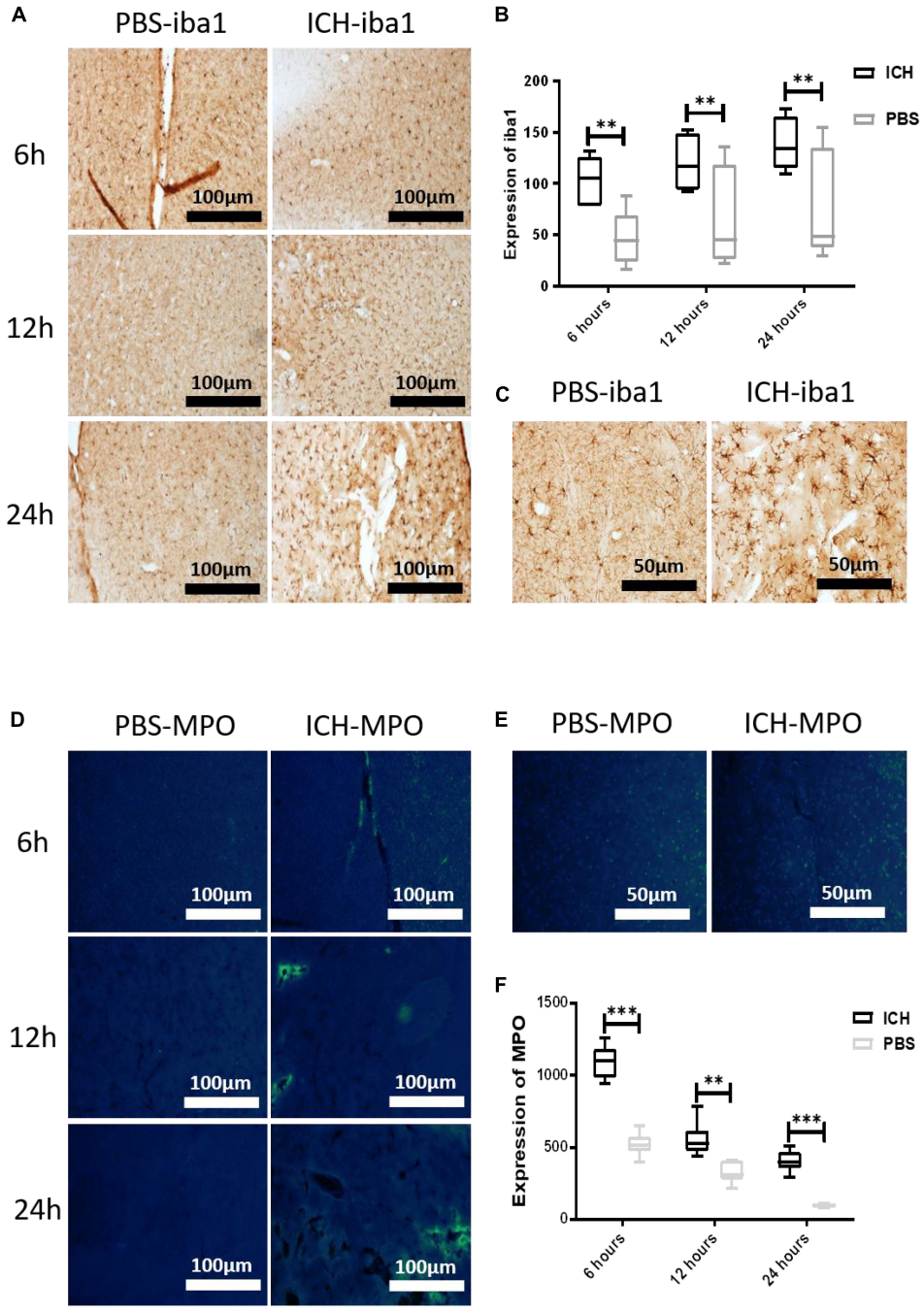

FIGURE 4
Microglia and MPO were activated following ICH. **(A)** Immunohistochemistry of the PBS and ICH groups at 6, 12, and 24 h post-injury. The labeled molecule was iba1 (10×, 20×); **(B)** statistics of iba1 positive expression (**$P < 0.01$, $n = 6$ in each group); **(C)** immunohistochemistry of iba1 in the PBS and ICH groups at 24 h post-injury (10×, 40×); **(D)** immunofluorescence of MPO in the PBS and ICH groups at 6, 12, and 24 h post-injury (10×, 20×); **(E)** immunohistochemistry of MPO in the PBS and ICH groups at 6 h post-injury (10×, 40×); **(F)** statistics of MPO molecular expression (**$P < 0.01$, ***$P < 0.001$, $n = 6$ in each group).

and MPO in terms of spatial expression, these experimental results further proved that MPO is closely related to the pathophysiological development of microglia following ICH. Therefore, in order to verify whether MPO can affect the activation of microglia following ICH, we used flow cytometry to count the number of microglia in normal mice, ICH mice, and mice treated with MPO inhibitor following ICH. The results showed that, compared with the normal physiological

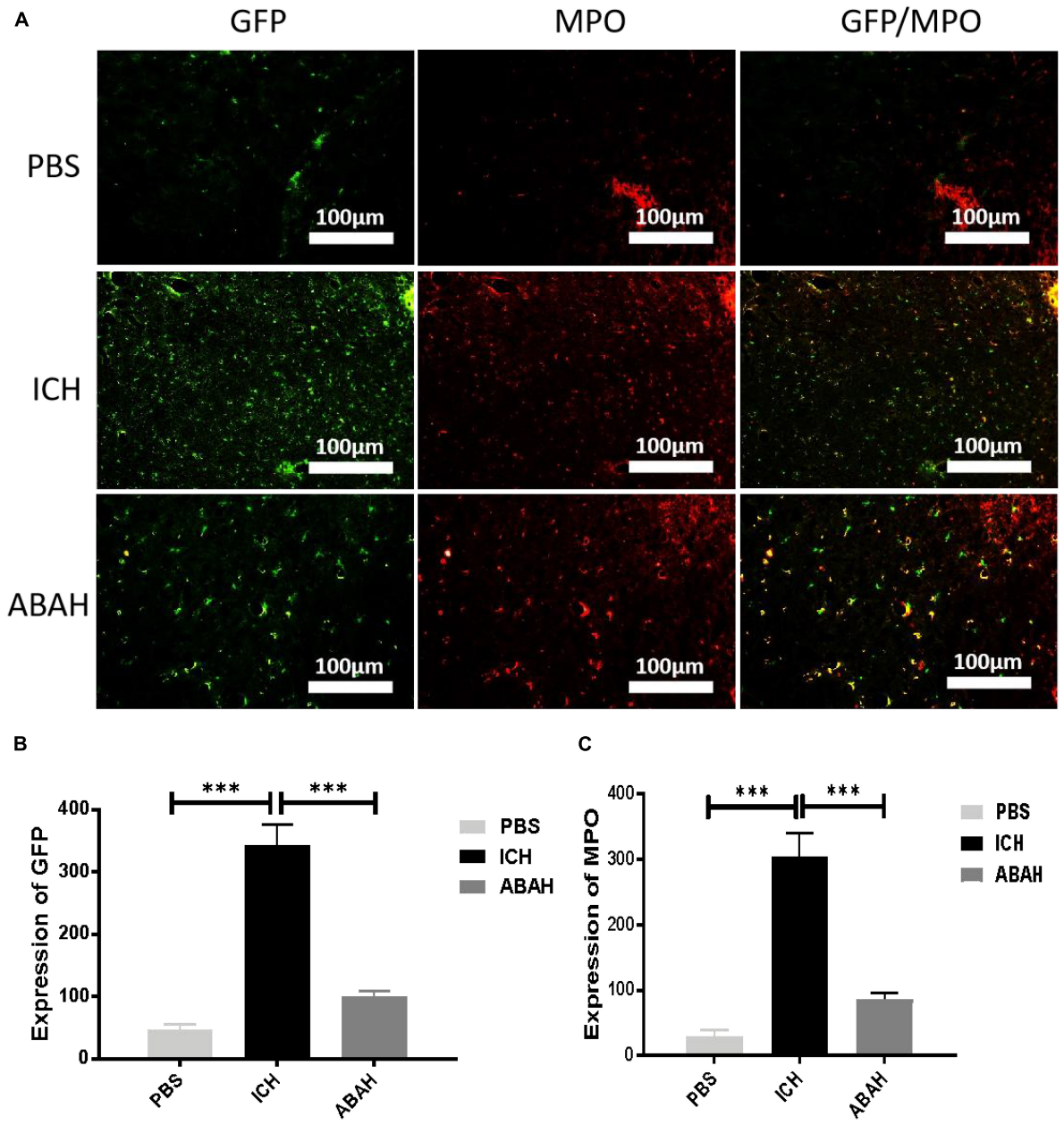

FIGURE 5
Inhibition of MPO activity reduces microglial proliferation following ICH. **(A)** Immunofluorescence double staining was performed in the PBS, ICH, and ABAH groups at 24 h post-injury. The labeled molecules were microglia and MPO; **(B)** microglia expression (***$P < 0.001$, $n = 6$ in each group); **(C)** MPO expression (***$P < 0.001$, $n = 6$ in each group).

state, microglial cells proliferated significantly following ICH, but the inhibition of MPO activity could significantly inhibit this change. Therefore, based on the above experimental data, we proved that ICH induced proliferation of microglia, and this proliferation was closely related to MPO activity. At the same time, due to the destruction of the blood-brain barrier, neutrophils and macrophages in the blood entered the central nervous system following ICH and participated in the inflammatory response. Therefore, we performed peripheral blood cytology tests on the ICH mice, and the results showed that neutrophils and macrophages in the peripheral blood of ICH mice increased in both proportion and quantity.

Our study, however, had certain limitations. We did not validate the specific type of activated microglia in our experiments, and we will continue to supplement our data with this information in subsequent experiments. In addition,

we did not verify the regulatory role of MPO activity in microglia proliferation using *in vitro* experiments, nor did we discuss the signal transduction mechanism in the activation process. We plan to further clarify the specific activation mechanism between MPO and microglia by means of molecular and cellular experiments, clarify the interaction between MPO and microglia, and find other possible targets for additional MPO inhibitors.

In summary, our studies have shown that ICH can cause a large degree of proliferation of microglia in the brain and increase the number of neutrophils and macrophages in the circulation. The increased MPO activity following ICH is closely related to the proliferation of microglia, and so is expected to become an important therapeutic target for ICH in the future.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

This animal study was reviewed and approved by the Medical Laboratory Animal Ethics Committee of Lanzhou University Second Hospital.

Author contributions

WZ, YW, JS, and YZ contributed to the conception and design of the study. WZ and YW performed data analysis and drafted the manuscript. YW and YZ participated in editing the manuscript. JS has made contributions to the implementation of some topics. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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TJ-M2010-5, a novel CNS drug candidate, attenuates acute cerebral ischemia-reperfusion injury through the MyD88/NF-κB and ERK pathway

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Background: Cerebral ischemia-reperfusion injury (CIRI) inevitably occurs after vascular recanalization treatment for ischemic stroke. The accompanying inflammatory cascades have a major impact on outcome and regeneration after ischemic stroke. Evidences have demonstrated that TLR/MyD88/NF-κB signaling contributes to CIRI. This study aimed to investigate the druggability of MyD88 in the central nervous system (CNS) and the neuroprotective and anti-neuroinflammatory effects of the MyD88 inhibitor TJ-M2010-5 on CIRI.

Methods: A middle cerebral artery occlusion (MCAO) model was used to simulate CIRI in mice. BV-2 cells were stimulated with oxygen glucose deprivation/reoxygenation (OGD/R) or lipopolysaccharide, and SH-SY5Y cells were induced by OGD/R *in vitro*. Neurological deficit scores and cerebral infarction volumes were evaluated. Immunofluorescence staining was performed to measure neuronal damage and apoptosis in the brain. The anti-neuroinflammatory effect of TJ-M2010-5 was evaluated by analyzing the expression of inflammatory cytokines, activation of microglia, and infiltration of peripheral myeloid cells. The expression of proteins of the MyD88/NF-κB and ERK pathway was detected by Simple Western. The concentrations of TJ-M2010-5 in the blood and brain were analyzed by liquid chromatography-mass spectrometry.

Results: The cerebral infarction volume decreased in mice treated with TJ-M2010-5, with the most prominent decrease being approximately 80% of the original infarction volume. Neuronal loss and apoptosis were reduced following TJ-M2010-5 treatment. TJ-M2010-5 inhibited the infiltration of peripheral myeloid cells and the activation of microglia. TJ-M2010-5 also downregulated the expression of inflammatory cytokines and inhibited the

MyD88/NF-κB and ERK pathway. Furthermore, TJ-M2010-5 showed good blood-brain barrier permeability and no neurotoxicity.

Conclusion: TJ-M2010-5 has an excellent therapeutic effect on CIRI as a novel CNS drug candidate by inhibiting excessive neuroinflammatory responses.

KEYWORDS

TJ-M2010-5, drug, cerebral ischemia-reperfusion injury, neuroinflammation, Myd88 inhibitor

Introduction

Stroke is an acute cerebrovascular disease in which focal neurological loss suddenly occurs in the relevant parts of the brain due to infarction or hemorrhage (Hankey, 2017). The most common type of stroke is ischemic stroke, accounting for 70%–80%. The cornerstone of effective ischemic stroke care continues to be timely reperfusion treatment, either intravenous recombinant tissue plasminogen activator (rtPA) and/or mechanical thrombectomy (Prabhakaran et al., 2015). Although clinical use of intravenous rtPA and/or mechanical thrombectomy result in high reperfusion rates of acute cerebral infarction, the benefits of reperfusion therapy are incomplete in about half of the patients treated (Chamorro et al., 2021; van Horn et al., 2021). When reperfusion occurs, a seemingly paradoxical increased injury can occur, such as hemorrhagic transformation, which limits the use of rtPA (Liu et al., 2017). Evidence has shown that acute immune-inflammatory reactions related to reperfusion can lead to secondary brain injury and expand the scope of brain injury (Iadecola and Anrather, 2011; He et al., 2021; Przykaza, 2021). Cerebral ischemia-reperfusion injury (CIRI) inevitably occurs after cerebral infarction. CIRI makes the original ischemic necrosis area more than double, and a specific drug is lacking in clinical practice. Studies have shown that myeloid differentiation factor 88 (MyD88) plays a vital role in CIRI (Wang et al., 2011; Ma et al., 2020; Zhong et al., 2020; Qin et al., 2022).

Following ischemic stroke, damaged neurons release damage associated molecular patterns (DAMPs), such as high mobility group box 1 (HMGB1) (Singh et al., 2016). DAMPs spread when reperfusion and were sensed by toll-like receptor (TLR), leading to a series of inflammatory cascade (Akira and Takeda, 2004; Hanisch et al., 2008). MyD88 is an adaptor molecule linking TLR or interleukin (IL) receptors signaling to the downstream activation of nuclear factor-κB (NF-κB) (Janssens and Beyaert, 2002). TLRs identify DAMPs and activate MyD88/NF-κB and ERK signaling, resulting in the expression of pro-inflammatory factors (Li et al., 2018; Chen et al., 2020). Furthermore, inflammatory factors enhance immune cell activation and regulate the cell death of inflammatory tissues (Takeuchi and Akira, 2010), which increases DAMPs, leading to the inflammatory cascade and expanding the scope of damage (Silvis et al., 2020). Blocking the TLR/MyD88/NF-κB signaling can downregulate inflammatory responses and alleviate CIRI (Gao et al., 2009; Bohacek et al., 2012; Wang et al., 2016).

By analyzing the structural domain of MyD88 and using computer-aided systems such as drug design and virtual screening, the small-molecule aminothiazole derivative MyD88 inhibitor, TJ-M2010 series (WIPO Patent Application Number: PCT/CN 2012/070811) has been innovatively developed, which can specifically bind to the Toll/Interleukin-1 receptor (TIR) domain of MyD88 and prevent homodimerization of MyD88. TJ-M2010-5 (TJ-5), one of TJ-M2010 series, has the best water solubility and bioavailability. The chemical structure of TJ-5 (Figure 1A) and its interaction with the MyD88 TIR domain have been described in a previous study (Xie et al., 2016). Specifically, TJ-5 interacts with amino-acid residues of αE, βD, βC, αA, DD loop, and EE loop of MyD88 with a nonbond interaction and the energies (docking score) is -883.298 kJ/mol (Xie et al., 2016). Our previous studies have shown that TJ-5 can inhibit the activation of peripheral innate immune cells such as macrophages and dendritic cells in hepatic, myocardial, and renal ischemia-reperfusion (I/R) animal models (Zhang et al., 2016; Miao et al., 2020; Zhou et al., 2022). However, the druggability of MyD88 is still unknown, especially in central nervous system (CNS) diseases. Due to the requirement of CNS drugs to cross the blood-brain barrier (BBB) and the frangibility of neurons to ischemia and hypoxia, as well as the particularity of the central nervous immune system (Banks, 2016), the effect and mechanism of MyD88 inhibition against CIRI are not clear. Here, we focused on the anti-neuroinflammatory effect of TJ-5. The neuroprotective potential of TJ-5 as a CNS drug candidate for CIRI treatment was evaluated.

Materials and methods

Animals and groups

Male C57BL/6 mice (Beijing Vital River Laboratory Animal Technology Co. Ltd., Beijing, China) weighing 22–28 g and aged 8–10 weeks were used. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tongji Hospital (Wuhan, China). All procedures were performed in accordance with specific pathogen-free standards. Mice were

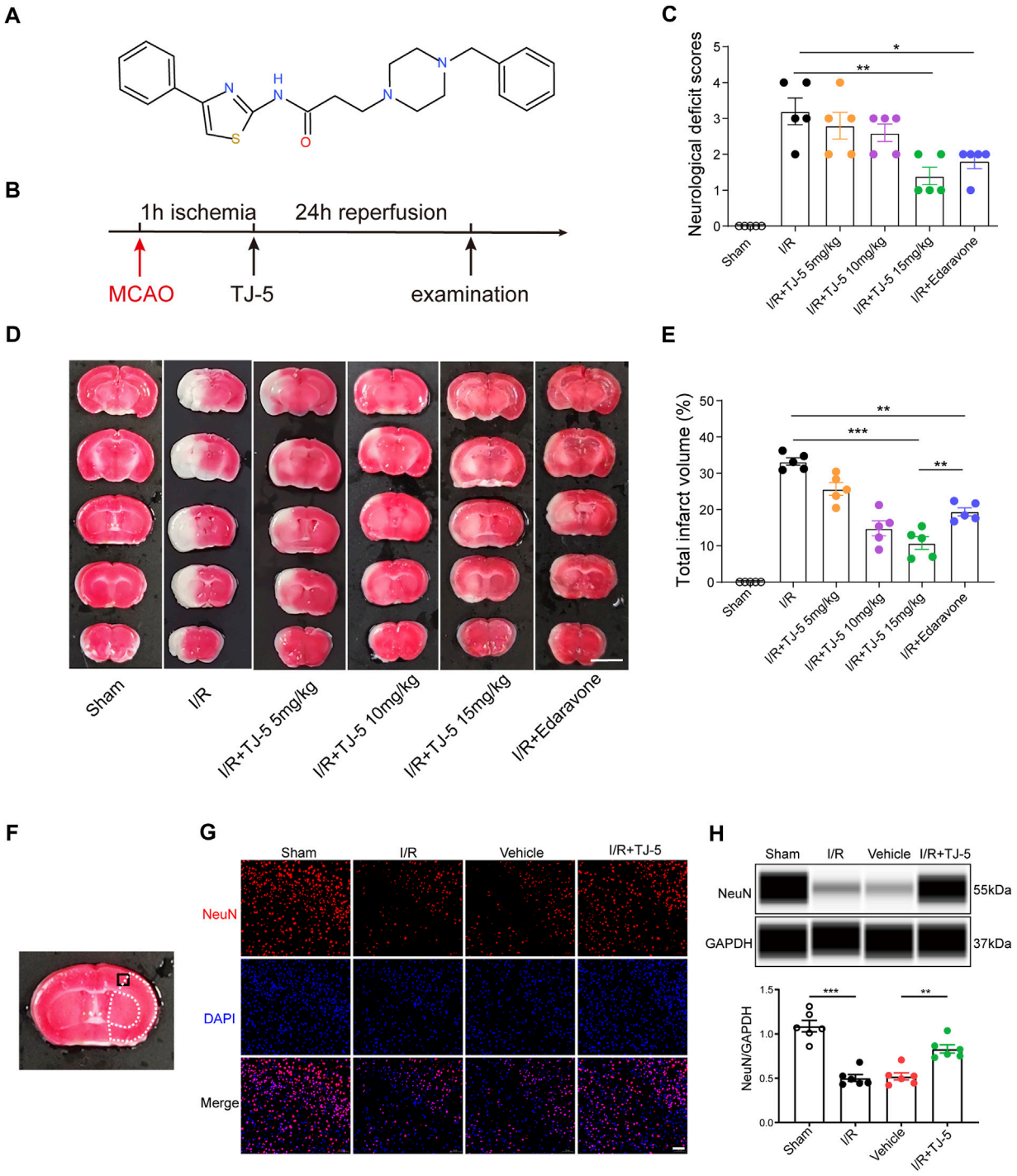

FIGURE 1
Neuroprotective effect of TJ-5 treatment in CIRI mice. **(A)** The molecular structure of TJ-5. **(B)** Experimental timeline of TJ-5 treatment in CIRI mice. **(C–E)** Representative TTC-stained slices at 24 h after reperfusion (scale bar = 5 mm) and statistical analysis of neurological deficit score and infarct volume. Values are mean ± SEM and analyzed by two-way ANOVA. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **(F)** Black boxed area illustrates cortical region represented in the NeuN, TUNEL and Iba-1 images. White dashed line areas illustrate ischemic core (medial) and peri lesion (lateral) regions. **(G)** Representative NeuN immunofluorescence staining image (scale bar = 50 μm). **(H)** NeuN protein levels in the brain were analyzed. All the experiments were repeated three times. Values are mean ± SEM. (**$p < 0.01$, ***$p < 0.001$).

randomly divided into the following seven groups: sham, I/R, vehicle (I/R + saline), I/R + TJ-5 (5 mg/kg), I/R + TJ-5 (10 mg/kg), I/R + TJ-5 (15 mg/kg), and I/R + edaravone (3 mg/kg).

Cerebral ischemia-reperfusion injury model

The mice were anesthetized with 1% pentobarbital sodium solution *via* intraperitoneal injection, and the body temperature was maintained at 37.0°C–37.5°C. The middle cerebral artery occlusion (MCAO) model was established according to the Longa method, as described previously (Longa et al., 1989). In brief, the right carotid artery was carefully separated and exposed. A silicon-coated embolic suture (Doccol, United States) was inserted slowly from the external carotid artery (ECA) into the internal carotid artery (ICA) until it reached the middle cerebral artery (MCA). After 1 h of ischemia, the embolic suture was withdrawn. The surgical procedure in the sham group was the same as that in the I/R group, but the MCA was not obstructed.

Neurological deficiency score

After 24 h of reperfusion, each group of mice was scored blindly, according to the scoring system of the Longa method (Longa et al., 1989). The score was 0 for no obvious neurological deficit, one for inability to fully extend the left forelimb, two for turning to the left, three for leaning to the left while walking, and four for the inability to walk spontaneously and impaired consciousness.

TTC staining

The mice were sacrificed 24 h after I/R. Mouse brains were harvested for the measurement of cerebral infarct volume. The brains were frozen at −80°C for 5 min and cut into five 2-mm thick slices. The slices were then placed in a small dish, 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution (Sigma, United States) was added, and 4% paraformaldehyde was used for fixation. Images were captured using a digital camera. ImageJ software was used to measure the volume of the brain infarction.

Cell culture and treatment

BV-2 microglial and SH-SY5Y cells were cultured as previously described (Zhao et al., 2020). A lipopolysaccharide (LPS)-stimulated BV-2 cell model was established to mimic severe neuroinflammation induced by multiple inflammatory mediators after reperfusion (Li et al., 2021). BV-2 cells were pretreated with different concentrations of TJ-5 for 2 h before LPS (1 μg/ml) stimulation (#L2880, Sigma, United States). Twenty-4 hours later, BV-2 cells and the culture supernatants were harvested for subsequent experiments. In addition, ST2825, a recognized MyD88 inhibitor, was used as positive control group.

The oxygen glucose deprivation/reoxygenation (OGD/R)-induced SH-SY5Y cell model was established to mimic CIRI as previously described (Dong et al., 2021). Briefly, SH-SY5Y cells were cultured in glucose-free Dulbecco's Modified Eagle Medium, and the flasks were placed inside an incubator (1% O_2, 94% N_2, 5% CO_2) for 4 h. OGD cells were then incubated in standard culture conditions with or without TJ-5 for 24 h of reperfusion. OGD 4 h/R 24 h SH-SY5Y cells were harvested, and the Annexin V/PI Apoptosis Kit (Multi- Science Biotech, China) was used to detect apoptosis by flow cytometry.

CCK-8 assay

BV-2 and SH-SY5Y cells were seeded in a 96-well plate at a density of 1×10^4 cells/well. Twenty-4 hours later, different concentrations of TJ-5 (0, 1, 5, 10, 20, and 30 μM) were added and co-cultured with cells for 24 h. Cell Counting Kit-8 (CCK8, Sigma, Shanghai, China) was used to measure cell viability. Experiments were conducted according to the manufacturer's instructions. After incubation at 37°C for 2 h, absorbance was measured at 450 nm using a microplate reader (Synergy 2, BioTek Instruments, United States).

Immunofluorescence staining

As mentioned previously, immunofluorescence staining was performed on paraffin-embedded brain slices (Ye et al., 2020). After standard histological procedures, the slices were treated with the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) reaction mixture to detect apoptosis according to the manufacturer's protocol (Roche, Germany). Furthermore, the slices were used for immunofluorescence with rabbit anti-NeuN antibody (1:300, CST) and rabbit anti-Iba-1 antibody (1:500, CST). After incubating overnight at 4°C, goat anti-rabbit IgG (1:1000) secondary antibodies were applied, and the brain slices were incubated for 2 h at room temperature. Finally, the slices were washed and labeled with 4′,6-diamidino-2-phenylindole for 10 min at room temperature. Images were captured using a fluorescence microscope.

For BV-2 cells immunofluorescence staining, the cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.5% Triton X-100 for 10 min. After blocking with goat serum, the cells were sequentially incubated with the primary antibody, secondary antibody, and Hoechst in sequence. Images

were captured using a confocal microscope. The fluorescence intensity was analyzed using ImageJ software.

Quantitative real-time PCR

Total mRNA was extracted from the brain cortex using TRIzol reagent and reverse-transcribed into cDNA using the PrimeScript™ RT reagent kit (Takara, Japan), following the manufacturer's instructions. RT-qPCR was performed using SYBR Green Real-time PCR Master Mix (Takara, Japan) with the Step One System (Life Technologies). The results were expressed as fold change from the untreated control and analyzed using the $2^{-\Delta\Delta Ct}$ method. The primers were as follows (5′–3′): MyD88 forward: TTTATCTGCTACTGCCCC AACG, reverse: GCGGCGACACCTTTTCTCA; TLR4 forward: ATGCTGCAACTGATGTTCCTTC, reverse: GATGTTAGA CCTTTCTTCCTCCC; GAPDH forward: TGTTCCTACCCC CAATGTGTCC, reverse: GGAGTTGCTGTTGAAGTCGCAG; TNF-α forward: ATGGCCTCCCTCTCATCAGT, reverse: TGG TTTGCTACGACGTGGG; IL-6 forward: AGTGGCTAAGGA CCAAGAC, reverse: ATAACGCACTAGGTTTGCCGA; iNOS forward: ATTCACAGCTCATCCGGTACG, reverse: GGATCT TGACCATCAGCTTGC; IL-1β forward: GCACTACAGGCT CCGAGATGAA, reverse: GTCGTTGCTTGGTTCTCCTTGT.

Isolation of immune cells

After the mice were anesthetized, 0.9% saline was used for transcardial perfusion. The right brain hemispheres were homogenized in a 6-well plate using 2 ml Hank's balanced salt solution (HBSS, Solarbio) per well. Collagenase IV (1 mg/ml) was added to remove myelin. The brain homogenate was filtered through a 70 μm cell strainer and centrifuged at 300 *g* for 5 min at 4°C. Then, 2 ml of 30% Percoll (Sigma) were added to resuspend the brain cell precipitate, and the resuspended cells were slowly added to a 15 ml centrifuge tube containing 3 ml of 70% Percoll. The intermediate layer cells were analyzed by flow cytometry after density-gradient centrifugation.

Flow cytometry

Approximately 5×10^5 cells were suspended in 200 μL HBSS, and anti-mouse CD16/CD32 (5 ng/μL) was used to block Fc receptor binding. The cells were stained with allophycocyanin-conjugated CD45 antibody (1 ng/μL), FITC-conjugated CD11b antibody (1 ng/μL), and phycoerythrin-conjugated Ly6G antibody (1 ng/μL) and incubated in the dark at 4°C for 30 min. Finally, the cells were washed with HBSS buffer, resuspended in 200 μL of HBSS, and analyzed using a flow cytometer (BD FACSCalibur).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) kits were used to detect the levels of TNF-α, IL-1β, and IL-6 in the brain. Brain tissue was homogenized in 1 ml of phosphate-buffered saline using a tissue homogenizer. Then, the homogenates were centrifuged at 14,000 *g* for 10 min at 4 °C. The supernatant was immediately transferred for measurement following the manufacturer's instructions. BV-2 cells were incubated with different treatments and the supernatants were collected and measured.

Capillary electrophoresis immunoassay (Simple Western)

Here, Simple Western, a novel immunoassay to detect proteins in the brain, was used, as previously described (Kannan et al., 2018; Nanki et al., 2018). Briefly, the prepared proteins were diluted to a concentration of 0.5 mg/ml using a sample preparation kit (Protein Simple, United States). Then, according to the manufacturer's instructions, the prepared reagents were added to the detection plate sequentially for processing in an automated capillary electrophoresis system (Simple Western system). The primary antibodies recognized HMGB1, TLR4, MyD88, NeuN, P-ERK, P-IκBα, IκBα, NF-κB p65 (CST, 1:50), Histone H3 (ABclonal, 1:50), and GAPDH (ABclonal, 1:1000). Compass for SW software v4.0.0 (Protein Simple, United States) was used to quantitatively analyze the signal intensity (area) of the protein.

Detection of drug concentration by liquid chromatography-mass spectrometry

The mice were intravenously injected with 15 mg/kg TJ-5 at the time of reperfusion 1 h after MCAO or Sham. The mice were then anesthetized at 5 min, 10 min, 30 min, 1 h, and 2 h, or 6 h post-dosing. Blood was collected to prepare serum samples, and the ipsilateral ischemic hemisphere (IL brain), contralateral non-ischemic hemisphere (CL brain), heart, and liver samples were obtained after transcardial perfusion with 0.9% saline. Liquid chromatography-mass spectrometry (LC-MS) was performed to determine the concentration of TJ-5 in the samples. The time-concentration curve, area under the curve (AUC), and pharmacokinetic (PK) parameters of TJ-5 were analyzed using the PKslover 2.0 PK software (Zhang et al., 2010). BBB permeability was evaluated using the brain-to-serum partition coefficient (K_p), which was calculated as AUC_{brain}/AUC_{serum}.

Statistical analysis

All experimental data were statistically analyzed using the professional analysis software GraphPad 8.0. The data obtained

are expressed as mean ± standard error of the mean values (SEM). Different groups were compared with a *t*-test or two-way ANOVA, as appropriate. $p < 0.05$ was considered statistically significant.

Results

TJ-5 improves neurological function and reduces the infarct volume and neuronal loss in cerebral ischemia-reperfusion injury mice

To investigate the therapeutic effects of TJ-5 in cerebral I/R-induced acute injury, TJ-5 or edaravone was injected intravenously in the intervention groups at 1 h of ischemia, and CIRI mice were evaluated after 24 h of reperfusion (Figure 1B). The results showed that the neurological deficit score of the TJ-5 15 mg/kg group was significantly lower than that of the I/R group (Figure 1C). The infarct volume was evaluated using TTC staining. We found that TJ-5 significantly reduced the infarct volume, especially in the cortex, with an increase in dosage (Figure 1D). The percentage of infarct volume was 34.24% in the I/R group, while that in the most effective TJ-5 15 mg/kg group was only 8.47%, a reduction of approximately 80%. Furthermore, compared with 18.59% in the edaravone group, TJ-5 at 15 mg/kg achieved a better effect (Figure 1E). Intravenous TJ-5 at 4 h reperfusion still effectively reduced infarct volume (Supplemental Figure S1). We used TJ-5 at 15 mg/kg in the I/R + TJ-5 group in subsequent trials because of its superior effect. Immunofluorescence staining of neuron-specific nuclear protein (NeuN) in the brain showed that the number of neurons in the injured hemisphere in the I/R + TJ-5 group significantly increased compared to that in the vehicle group (Figure 1G). TJ-5 attenuated this decrease in NeuN protein levels (Figure 1H). Taken together, these data indicate that TJ-5 has powerful neuroprotective effects and reduces neuronal loss caused by CIRI.

TJ-5 inhibits apoptosis and alleviates the excessive inflammatory response in CIRI mice

To investigate the protective effects of TJ-5 on CIRI-induced inflammatory responses, TUNEL fluorescence staining was used to detect apoptosis. The scope of apoptosis in the whole brain and the apoptotic cells in the ischemic penumbra of the cortex were significantly reduced by treatment with TJ-5 (Figures 2A, B). The mRNA expression and protein levels of TNF-α, IL-1β, and IL-6 in the brain were detected by RT-qPCR and ELISA, respectively. The results showed that I/R injury significantly increased the expression and production of inflammatory factors in the brain tissue, whereas TJ-5 reduced the expression of TNF-α, IL-1β, and IL-6 (Figures 2C, D). These results suggested that TJ-5 reduced the extent of damage by inhibiting the neuroinflammatory response and apoptosis.

TJ-5 inhibits activation of microglia and infiltration of peripheral myeloid cells in CIRI mice

We further explored how TJ-5 inhibited excessive inflammatory responses at the cellular level. Microglia in the brain were labeled with Iba-1, and the results indicated that the number of Iba-1 positive cells in the I/R group increased, while that in the I/R + TJ-5 group significantly decreased (Figure 3A). Flow cytometry results showed that the proportions of $CD11b^{+}CD45^{hi}Ly6G^{+}$ neutrophils (PMNs) and $CD11b^{+}CD45^{hi}Ly6G^{-}$ mononuclear macrophages (Mo/MΦ) increased, and the proportion of $CD11b^{+}CD45^{int}$ inactive microglia decreased in CIRI mice. TJ-5 attenuated this proportional change and inhibited the activation and infiltration of inflammatory cells (Figure 3B). Injecting TJ-5 at the time of reperfusion reduced the number of brain-infiltrating $CD11b^{+}CD45^{hi}$ myeloid cells and increased the proportion of inactive microglia (Figures 3C, D). Thus, TJ-5 inhibited both the infiltration of myeloid cells and activation of microglia to alleviate neuroinflammation in CIRI mice.

TJ-5 inhibits neuroinflammation *via* the MyD88/NF-κB and ERK signaling pathway in CIRI mice

MyD88/NF-κB and ERK signaling plays a vital role in neuroinflammation induced by CIRI (Qin et al., 2022), and the effect of TJ-5 on this pathway in CIRI mice needs to be identified. The results showed that TJ-5 downregulated the expression of HMGB1, TLR4, and MyD88 and inhibited the phosphorylation of ERK in the ischemic hemisphere (Figures 4A–G). Phosphorylation of inhibitor complex alpha (IκBα) and nuclear translocation of NF-κB p65, which lead to the excessive expression of pro-inflammatory factors, were examined. The results indicated that phosphorylation of IκBα and nuclear translocation of NF-κB p65 was reduced by TJ-5 treatment in the ischemic hemisphere (Figures 4H–K). Therefore, TJ-5 inhibits the MyD88/NF-κB and ERK signaling pathway to alleviate neuroinflammation.

Blood-brain barrier permeability and pharmacokinetics of TJ-5

To investigate the blood-brain barrier permeability of TJ-5, the concentration of TJ-5 in the IL and CL brains was measured and compared with that in the serum, liver, and heart (Figure 5A). The concentrations of TJ-5 in each tissue are

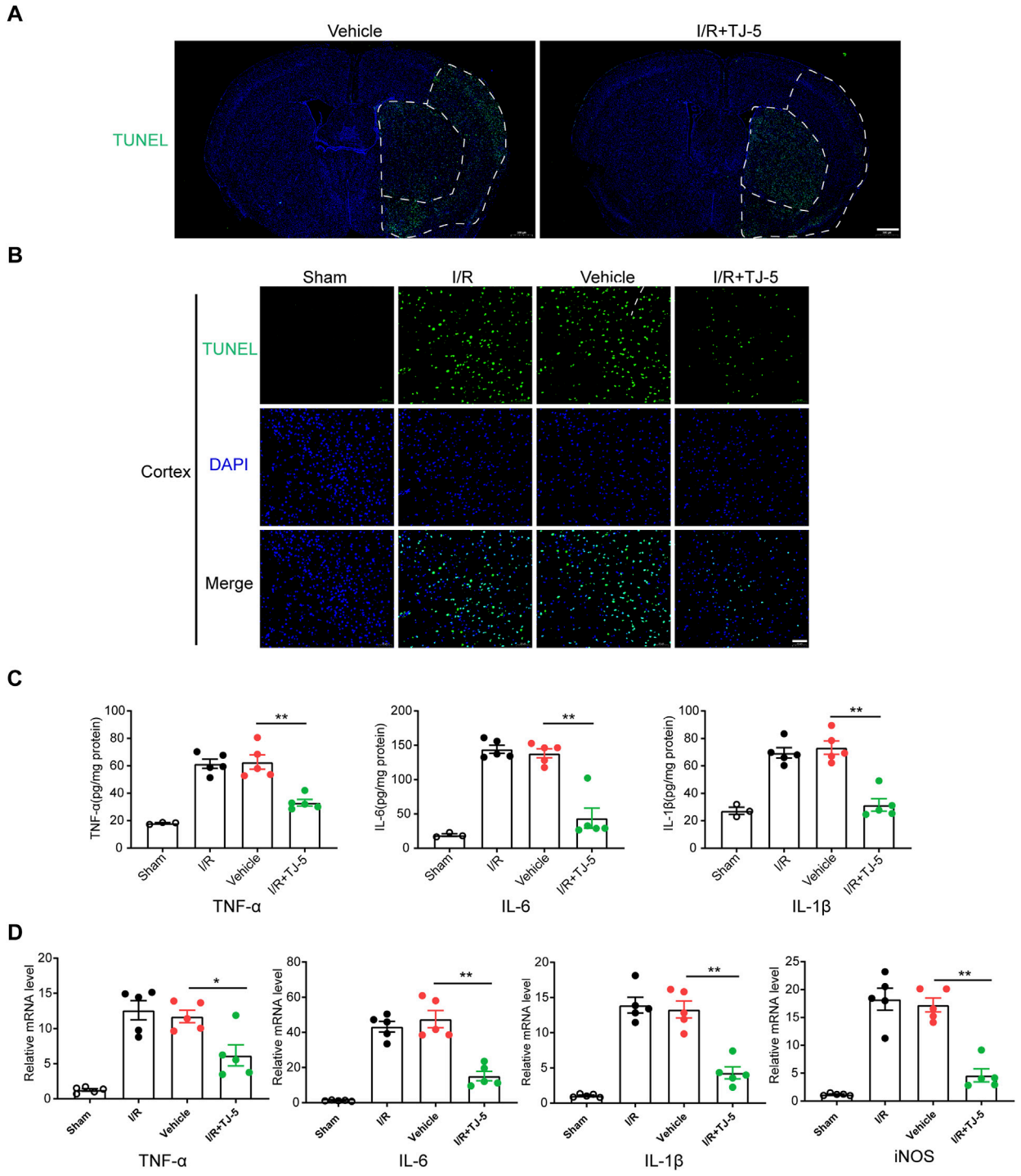

FIGURE 2
Effect of TJ-5 treatment on the neuroinflammatory response and apoptosis in CIRI mice. Mice were injected intravenously with TJ-5 (15 mg/kg) after 1 h ischemia. **(A)** Representative TUNEL staining image of whole brain slices (scale bar = 500 μm), white dashed line areas illustrate ischemic core (medial) and peri lesion (lateral) regions; the range of apoptosis in the brain was reduced with TJ-5. **(B)** Representative TUNEL staining image of cortex; the number of apoptotic cells were reduced with TJ-5 treatment (scale bar = 50 μm). **(C)** TNF-α, IL-6, IL-1β, and iNOS mRNA expression levels in the brain were downregulated with TJ-5 treatment. **(D)** The content of TNF-α, IL-6, and IL-1β in the brain were reduced with TJ-5 treatment. All the experiments were repeated three times. Values are mean ± SEM. (*$p < 0.05$, **$p < 0.01$).

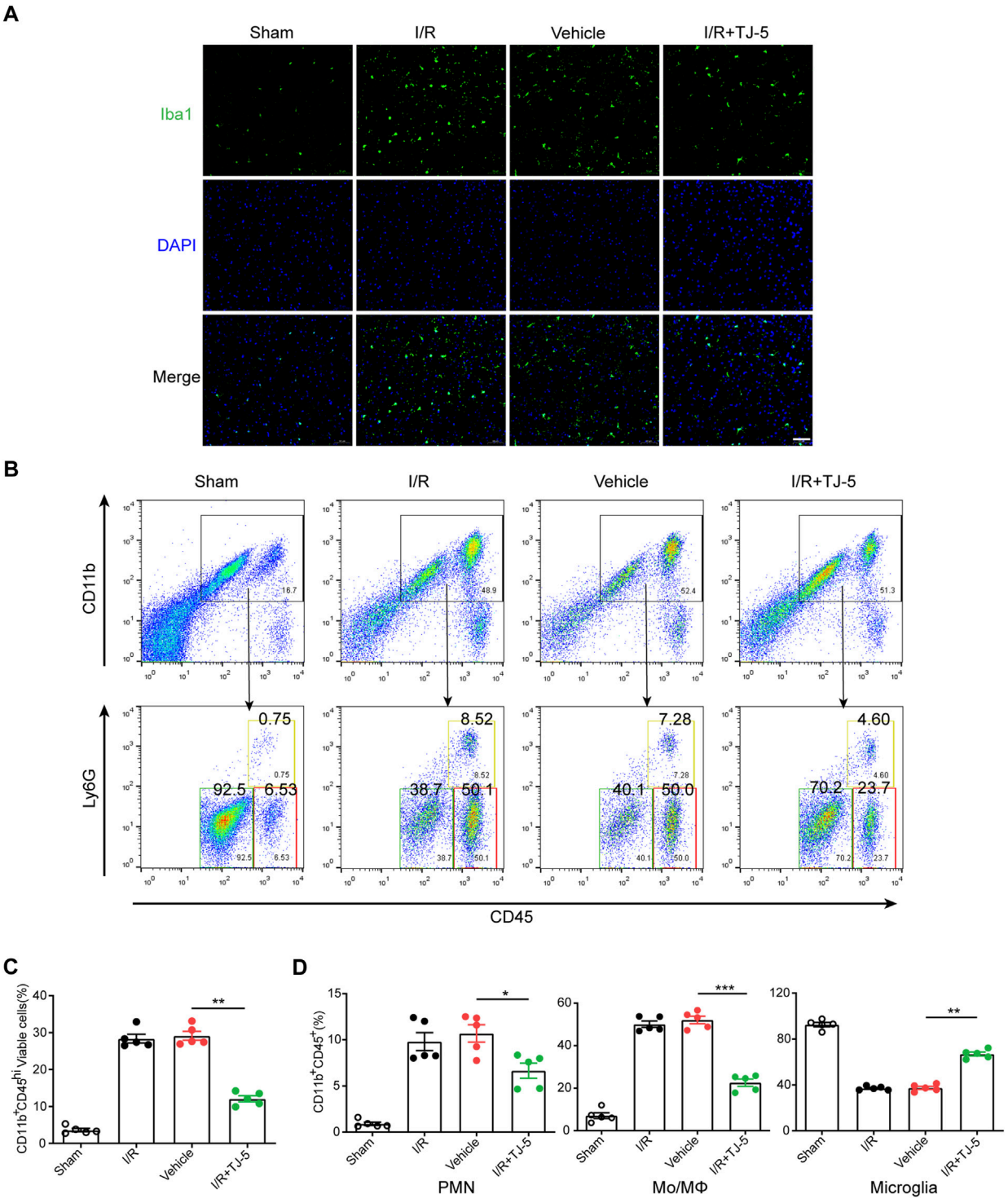

FIGURE 3
Effect of TJ-5 treatment on microglia and peripheral infiltrating myeloid cells in CIRI mice. Mice were injected intravenously with TJ-5 (15 mg/kg) after 1 h ischemia. **(A)** Representative Iba-1 staining image of the cortex (scale bar = 50 μm); the activated microglia were decreased with TJ-5 treatment. **(B)** Immune cells were isolated from the ischemic brain hemisphere after 24 h reperfusion and were stained with CD45, CD11b, and Ly6G. Plots identify $CD11b^{+}CD45^{hi}Ly6G^{-}$ Mo/MΦ, $CD11b^{+}CD45^{int}$ microglia and $CD11b^{+}CD45^{hi}Ly6G^{+}$ PMNs. **(C)** Percentage of brain-infiltrating $CD11b^{+}CD45^{hi}$ myeloid cells was statistically analyzed. **(D)** Percentages of PMNs, Mo/MΦ, and microglia in $CD11b^{+}CD45^{+}$ cells were statistically analyzed. All the experiments were repeated three times. Values are mean ± SEM. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

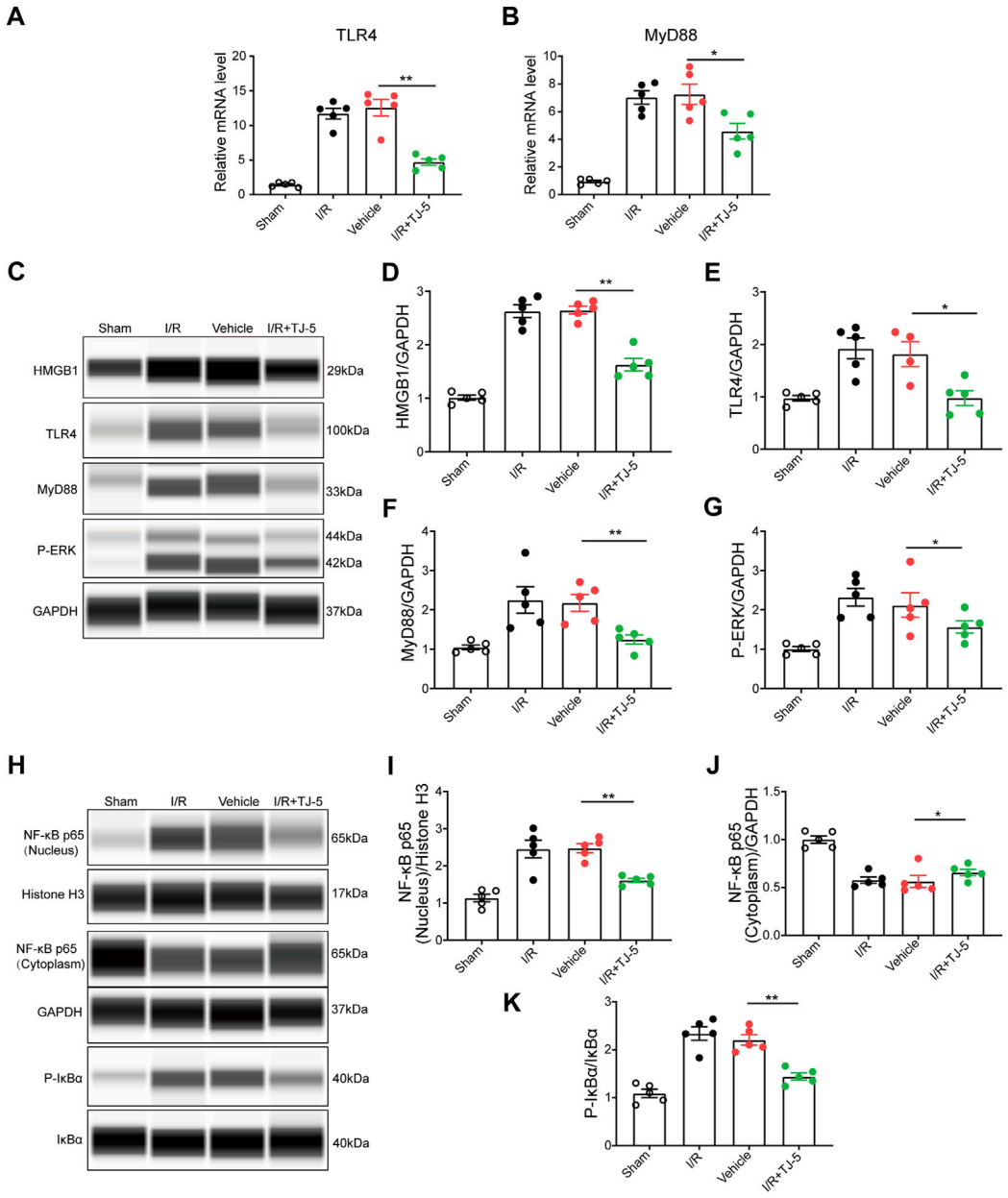

FIGURE 4
TJ-5 downregulates the MyD88/NF-κB and ERK signaling pathway in CIRI mice. **(A,B)** The mRNA levels of TLR4 and MyD88 in the brain were detected. **(C–G)** HMGB1, TLR4, MyD88, and P-ERK protein levels in the brain were assessed and analyzed. **(H–K)** Cytoplasmic and nuclear proteins were extracted from the brain to detect the NF-κB p65 protein levels and analyze phosphorylation of IκBα and NF-κB p65 nuclear translocation. All the experiments were repeated three times. Values are mean ± SEM. (*$p < 0.05$, **$p < 0.01$).

summarized in Table 1 and the calculated PK parameters are summarized in Table 2. The time-concentration curves of TJ-5 in different tissues indicated that TJ-5 was eliminated according to first-order kinetics and that TJ-5 was rapidly distributed from the blood to the brain, heart, and liver after intravenous injection (Figures 5B, C). The TJ-5 concentration ratio of brain tissue to serum at each time point is shown (Figure 5D). It is well known that intense neuroinflammation occurring during the acute phase of cerebral ischemia is associated with BBB breakdown (Candelario-Jalil et al., 2022). Interestingly, the concentrations of TJ-5 in the Sham, IL and CL brains were similar, suggesting that the BBB may have little effect on the diffusion of TJ-5 into the brain. Thus, TJ-5 has good BBB permeability and can be quickly distributed in parenchymal organs with a short half-life.

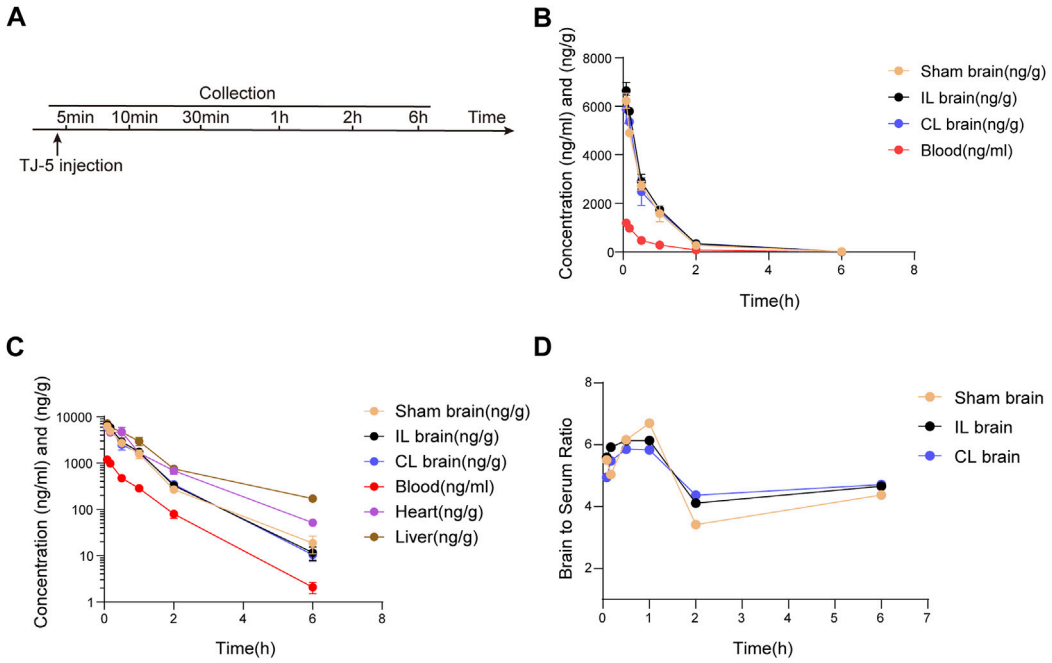

FIGURE 5
Pharmacokinetic profile of TJ-5. The concentrations of TJ-5 were analyzed in the serum, Sham brain, IL brain, CL brain, liver, and heart at specified time points after intravenous injection of TJ-5 at 15 mg/kg. **(A)** Experimental timeline of injection and detection. **(B)** The concentration-time profiles of TJ-5 in the serum and brain. TJ-5 was quickly distributed into the brain after intravenous injection. **(C)** The concentration-time profiles of TJ-5 with expressed in logarithmic ordinate. Elimination of TJ-5 followed the first-order elimination kinetics. **(D)** Concentration ratios of the brain to serum at 5 min, 10 min, 30 min, 1 h, 2 h, and 6 h. IL brain, ipsilateral ischemic hemisphere. CL brain, contralateral non-ischemic hemisphere.

TABLE 1 Distribution of TJ-5 in tissues during the different time points (ng/ml or ng/g, mean ± SD).

Sample	5 min	10 min	30 min	1 h	2 h	6 h
Serum	1187.80 ± 208.63	979.85 ± 202.81	471.74 ± 151.14	284.90 ± 29.10	79.60 ± 26.93	2.08 ± 1.12
Sham brain	6232.40 ± 435.71	4902.53 ± 53.92	2725.04 ± 256.34	1575.86 ± 468.76	267.68 ± 6.25	18.74 ± 11.04
IL brain	6646.07 ± 590.77	5798.80 ± 29.80	2893.70 ± 611.00	1747.40 ± 163.95	327.29 ± 57.19	11.65 ± 6.43
CL brain	5877.53 ± 989.32	5364.30 ± 749.83	2480.87 ± 985.41	1662.40 ± 117.03	347.65 ± 55.25	10.43 ± 5.69
Heart	5914.10 ± 824.68	4591.93 ± 351.85	4729.27 ± 2061.45	1655.33 ± 335.19	684.13 ± 198.89	51.60 ± 11.87
Liver	7162.10 ± 593.97	5890.47 ± 528.302	4713.00 ± 1130.68	2961.40 ± 1031.41	750.37 ± 249.40	169.90 ± 36.06

TJ-5 inhibits the activation of LPS- or OGD/R-stimulated BV-2 cells and apoptosis of OGD/R-induced SH-SY5Y cells

The effects of TJ-5 on microglia and neurons were also evaluated *in vitro*. The CCK-8 experiment confirmed that TJ-5 did not affect the viability of BV-2 and SH-SY5Y cells at concentrations below 20 μM (Figures 6A, B). Observation of cell morphology under the microscope showed that after 24 h of LPS stimulation, BV-2 cells became amoeba-like and showed more protrusions, whereas the cells in the control group were spherical with a small number of protrusions. The activation of BV-2 cells was inhibited by TJ-5 intervention (Figure 6C). The levels of TNF-α and IL-6 in the supernatants of LPS- or OGD/R-stimulated BV-2 cells were markedly reduced by TJ-5 treatment (Figures 6D, E). In addition, an Annexin V/propidium iodide flow cytometry assay indicated that TJ-5 significantly reduced apoptosis of OGD/R-induced SH-SY5Y cells (Figures 6F, G). Meanwhile, ST2825 has the same anti-

TABLE 2 PK parameters of TJ-5 in tissues of mice (mean ± SD).

Sample	Pharmacokinetic parameters	
Serum	$AUC_{0-6\ h}$ (ng/ml*h)	919.3 ± 82.24
	$t_{1/2}$(h)	0.38
Sham brain	$AUC_{0-6\ h}$ (ng/ml*h)[a]	4579 ± 275.7
	$t_{1/2}$(h)	0.65
	K_p	4.98
IL brain	$AUC_{0-6\ h}$ (ng/ml*h)[a]	5136 ± 239.3
	$t_{1/2}$(h)	0.41
	K_p	5.58
CL brain	$AUC_{0-6\ h}$ (ng/ml*h)[a]	4792 ± 352.8
	$t_{1/2}$(h)	0.41
	K_p	5.21
Heart	$AUC_{0-6\ h}$ (ng/ml*h)[a]	6485 ± 768.9
	$t_{1/2}$(h)	0.67
Liver	$AUC_{0-6\ h}$ (ng/ml*h)[a]	8238 ± 852.1
	$t_{1/2}$(h)	0.70

[a]The tissue density was assumed to be 1 g/ml. K_p: Brain-serum ratio was calculated by the mean of $AUC_{0-6\ h}$ ratios. $t_{1/2}$: elimination half-life.

inflammatory effect. These results suggest that TJ-5 has anti-neuroinflammatory and neuroprotective effects on microglia and neurons, respectively.

TJ-5 inhibits NF-κB p65 protein nuclear translocation in BV-2 cells

To further explore the effect of TJ-5 on the nuclear translocation of NF-κB p65 protein in microglia, NF-κB p65 protein in BV-2 cells was estimated by immunofluorescence. First, p65 content in the nucleus was observed at 6, 8, 12, and 24 h after LPS treatment. The results showed that p65 nuclear translocation was most obviofter 12 h of stimulation (Figure 7A). Subsequently, 12 h after stimulation was selected as the observation time point. The results showed that TJ-5 significantly prevented LPS-induced p65 nuclear translocation, which is same as ST2825 (Figures 7B–D). Therefore, the main mechanism by which TJ-5 inhibits excessive microglial activation may be the inhibition of NF-κB p65 nuclear translocation.

Discussion

TJ-5 is a novel MyD88 pharmacological inhibitor. In this study, we evaluated the neuroprotective effects of TJ-5 in both *in vitro* and *in vivo* models of cerebral ischemia-reperfusion injury, explored its underlying mechanisms and investigated the druggability of inhibition of MyD88 in the brain.

Ischemic stroke has become one of the most common causes of disability and death worldwide (Virani et al., 2021). Recanalization as soon as possible is the primary treatment after ischemic stroke, but the ensuing reperfusion injury aggravates the brain injury and expands the infarct size. Unfortunately, there is currently a lack of specific treatment options (Qin et al., 2022). The TLR/MyD88/NF-κB signaling pathway has been found to be involved in the development of neuroinflammation injury in CIRI (Mitsios et al., 2006; Chen et al., 2022). MyD88 is a core transduction protein involved in this signaling pathway (Kawai and Akira, 2010). Considering that MyD88 activation may enhance neuroinflammation caused by ischemic stroke, we hypothesized that TJ-5 might potently inhibit neuroinflammation to protect against CIRI (Figures 8A, B). In CIRI mice model, we compared the neuroprotective effects of edaravone, a medicine currently used clinically for the treatment of ischemic stroke (Li et al., 2021), and different concentrations of TJ-5. We found that TJ-5 at 15 mg/kg was more effective, as the infarction volume was reduced by approximately 80%, achieving better neuroprotective effects than edaravone. Considering 3–4 h reperfusion is a clinically relevant therapeutic window in case of stroke, we performed and found that intravenous TJ-5 at 4 h reperfusion remained effective in reducing infarct volume. Meanwhile, TJ-5 reduced apoptosis of OGD/R-induced SH-

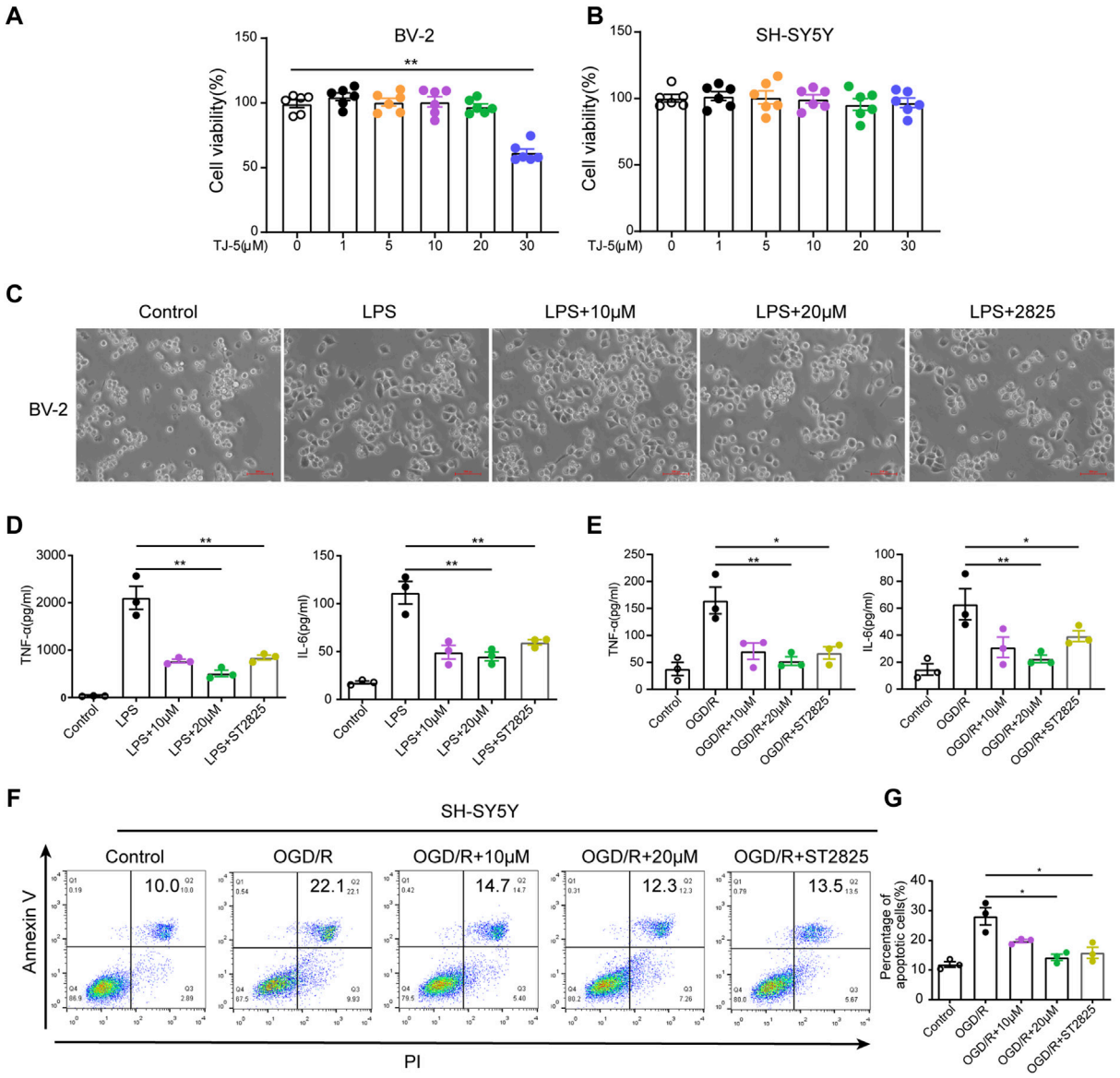

FIGURE 6
Effect of TJ-5 intervention on LPS or OGD/R-stimulated BV-2 cells and OGD/R-induced SH-SY5Y cells. BV-2 cells were pretreated with TJ-5 for 2 h before LPS or OGD/R stimulation for 24 h. SH-SY5Y cells were exposed to OGD for 4 h and then treated with TJ-5 during reperfusion for 24 h MyD88 inhibitor ST2825 was used as positive drug. **(A)** Cell viability of BV-2 cells at 24 h after TJ-5 intervention. **(B)** Cell viability of SH-SY5Y cells at 24 h after TJ-5 intervention. **(C)** The morphology of BV-2 cells was observed under the microscope. TJ-5 inhibited the activation of LPS-stimulated BV-2 cells (original magnification ×200). **(D)** TNF-α and IL-6 secretion in LPS-stimulated BV-2 cells was inhibited by TJ-5 intervention. **(E)** TNF-α and IL-6 secretion in OGD/R-stimulated BV-2 cells was inhibited by TJ-5 intervention. **(F,G)** Apoptosis of OGD/R-induced SH-SY5Y cells analyzed by flow cytometry. All the experiments were repeated three times. Values are mean ± SEM. (*$p < 0.05$, **$p < 0.01$).

SY5Y cells with no neurotoxicity. Many studies have suggested that the inflammatory response of the innate immune cell such as Mo/MΦ, PMN and microglia is essential for CIRI (Petrovic-Djergovic et al., 2016; Liu et al., 2019). Our findings proved that TJ-5 reduced the infiltration ratio of peripheral myeloid cells in the cerebral infarction area, increased the proportion of inactive microglia, and decreased the expression levels of TNF-α, IL-1β, and IL-6 in the infarction areas after 24 h of reperfusion, suggesting that TJ-5 may interrupt the inflammatory cascade and inhibit excessive neuroinflammation. Furthermore, we found that TJ-5 caused a reduction in inflammation of LPS- or OGD/R-stimulated BV-2 cells. These results indicate that TJ-5 has the potential to treat ischemic stroke and other CNS diseases caused by neuroinflammation.

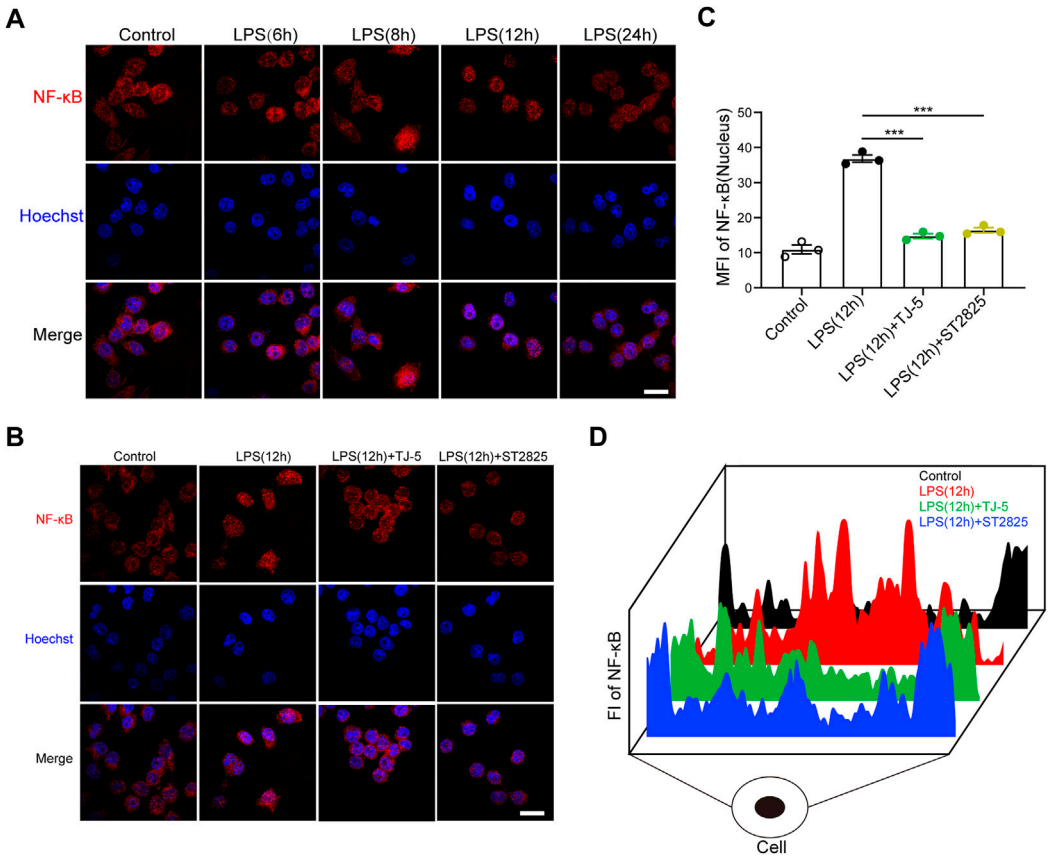

FIGURE 7
(A) Immunofluorescence was used to determine the optimal observation time point for NF-κB p65 nuclear translocation in LPS-stimulated BV-2 cells (scale bar = 50 μm). **(B)** After 12 h of LPS stimulation, immunofluorescence was used to analyze the effect of TJ-5 on NF-κB p65 nuclear translocation in BV-2 cells (scale bar = 50 μm). **(C)** The mean fluorescence intensity (MFI) of NF-κB p65 in the nucleus was statistically analyzed. **(D)** The distribution diagram of NF-κB p65 in cells was obtained by detecting fluorescence intensity (FI). All the experiments were repeated three times. Values are mean ± SEM. (***$p < 0.001$).

Mechanistically, we explored NF-κB and ERK signaling pathway which were relevant to TLR4/MyD88 signaling and ischemia stroke (Zhang et al., 2021). NF-κB p65 protein binds to IκB protein and is present in the cytoplasm. IκB kinases (IKKs) act immediately after TLR4/MyD88 signaling to phosphorylate IκB and NF-κB p65, which results in the degradation of IκB and nuclear translocation of NF-κB p65 (Karin and Ben-Neriah, 2000). Reducing NF-κB p65 activity is associated with reduction in infarct volume after MCAO (Liu et al., 2018). Our findings indicated that TJ-5 inhibited NF-κB p65 signaling in the brains of CIRI mice and nuclear translocation of NF-κB p65 in BV-2 cells. The inhibition of P-ERK could produce a potential neuroprotective effect in ischemic stroke (Zhang et al., 2021). Our data suggested TJ-5 treatment showed lower level of P-ERK when compared to vehicle treatment. HMGB1, one of the major ligands for TLR, are significantly elevated and closely associated with neuroinflammation in CIRI (Singh et al., 2016). We found that TJ-5 reduced the expression of HMGB1, suggesting that the neuroinflammatory injury was alleviated. The results indicate that TJ-5 exerts its anti-inflammatory effect through the MyD88/NF-κB and ERK signaling pathway.

The BBB is an interface that controls the exchange of substances between the CNS and blood, which makes it difficult to develop drugs (Banks, 2016). Currently, many drugs are unable to enter the CNS efficiently through the BBB, which limits the development of therapies for CNS diseases (Chiu et al., 2021; Liu et al., 2021). Microglia are resident macrophages in the brain. The activation of microglia in the CNS is an important factor contributing to the occurrence and development of CIRI (Cheng et al., 2019; Shen et al., 2022). In CIRI mice, activation of endogenous microglia and infiltration of exogenous immune cells promote a cascade of inflammation in the brain and increase the scope of injury. Inhibiting microglial activation is the focus of research on drugs for treating ischemic stroke (Ye et al., 2019; Wang et al., 2020). In CNS drug discovery,

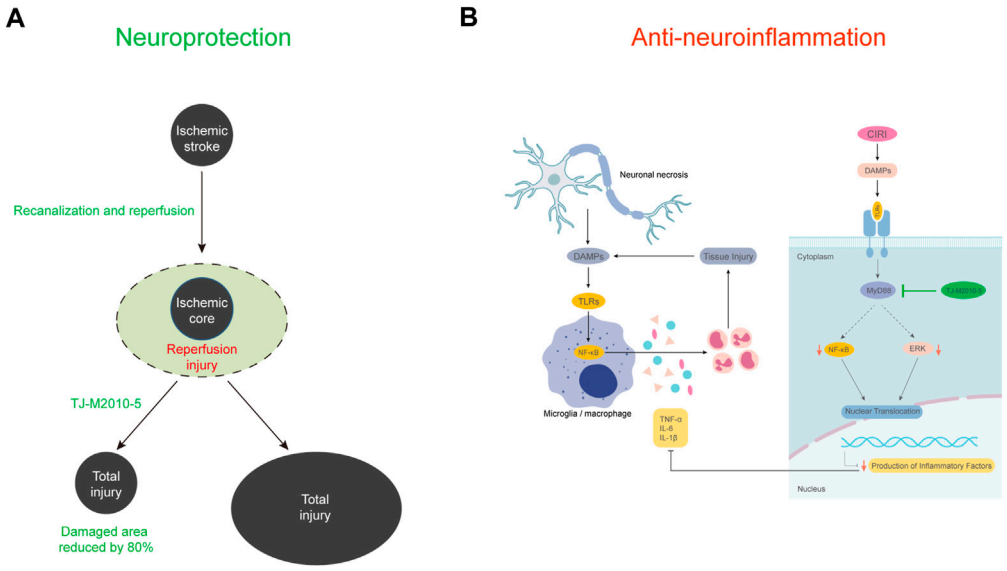

FIGURE 8
Schematic model of the present study. **(A)** The occurrence of reperfusion injury after vascular recanalization in ischemic stroke significantly expands the final necrotic area. TJ-5 reduces the final necrotic area by reversing ischemia-reperfusion injury. **(B)** The activation of microglia and infiltration of myeloid cells promote neuroinflammation and brain tissue injury after acute cerebral I/R. TJ-5 obstructs the vicious circle of neuroinflammatory injury by inhibiting MyD88/NF-κB and ERK signaling pathway.

the BBB permeability of drugs is an essential factor, as it determines whether drugs can directly affect microglia in the brain. Therefore, the inhibitory effects of TJ-5 on microglial activation and BBB permeability were examined in the present study. Pharmacologic evidence demonstrates that TJ-5, a small-molecule compound, can pass through the BBB and directly inhibit the activation of microglia. Moreover, TJ-5 has a short half-life, can only maintain effective concentration for about 6 h with a single intravenous injection of near toxic dose, suggesting that continuous low-dose intravenous infusion should be considered. More PK data with the other dose regimens will be performed in future clinical trials. The simultaneous inhibition of peripheral myeloid cells and microglial activation may be responsible for the excellent efficacy of TJ-5 for abating the negative effect of CIRI. These results indicate that TJ-5 has significant clinical application value in the treatment of CIRI and suggest the druggability of inhibition of MyD88 in the brain.

This study affirms the potency of TJ-5 in treating CIRI, as it demonstrates a better neuroprotective effect in the early stage of cerebral I/R. Moreover, we verified that TJ-5 not only acts on peripheral innate immune cells, but also directly on cells in the brain, which may be an influential factor contributing to its exceptional anti-neuroinflammatory and neuroprotective effects. However, this study had some limitations. The inflammatory response to CIRI is a “double-edged sword”. An excessive inflammatory response causes the injury to expand, but the inflammatory response also promotes the immune cells to devour necrotic tissue, which can promote tissue repair (Wyss-Coray and Mucke, 2002; Xue et al., 2021). TJ-5 regulates the neuroinflammatory response in CIRI and is effective in the acute phase; however, its efficacy in the chronic phase requires further investigation. One study showed that a congenital deficit of MyD88 failed to reduce cerebral infarct size in MyD88 knockout mice, but MyD88-dependent signaling contributes to the inflammatory responses induced by cerebral I/R (Ye et al., 2016). Why there is the difference in efficacy between congenital defects and the acquired short-term inhibition of MyD88? As the downstream of MyD88, the different roles of NF-κB activity in the early and late stages of ischemic stroke may be an explanation (Ridder and Schwaninger, 2009). One report claims that the anti-apoptotic properties of NF-κB may indeed have an effect at late stage of transient cerebral ischemia (Duckworth et al., 2006). Nijboer et al. (2008) found that inhibition of early NF-κB-activity by intraperitoneal administration of the NF-κB inhibitor TAT-NBD at 0/3 h has strong neuroprotection in neonatal hypoxia-ischemia model, whereas inhibition of both early and late NF-κB-activity at 0/6/12 h or only late NF-κB activity at 18/21 h aggravated cerebral damage. They suggest that inhibition of early NF-κB activity is neuroprotective only when late NF-κB activity is maintained. Therefore, short-term inhibition of MyD88 attenuates cerebral damage in ischemic stroke. Considering the rapid diffusion of the drug and its short half-life, the selection of the intensity and duration of intervention with TJ-5 in this study has limitations. Taken together, for aseptic inflammatory reactions like CIRI, it can be concluded that the

key to the treatment of CIRI is to balance the regulation of the immune system and minimize neuron loss. The anti-CIRI effect of TJ-5 should be evaluated in clinical studies.

Conclusion

In summary, we confirmed that MyD88 inhibitor TJ-5 has an impressive therapeutic effect during the acute phase of CIRI as an emergency drug candidate by inhibiting neuroinflammation. Moreover, we clarified for the first time that the druggability of MyD88 in the CNS to TJ-5. We found TJ-5 can cross the BBB to directly inhibit the activation of microglia with no neurotoxicity. TJ-5 attenuates intense neuroinflammation *via* the MyD88/NF-κB and ERK signaling pathway.

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 authors.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at the Tongji Hospital, Wuhan, China.

Author contributions

ZL, DD, and PZ designed the experiments. ZL performed most experiments, analyzed the results, and wrote the manuscript. XZ and YL helped with the experiments *in vivo* and manuscript editing. MZ, LZho, YY, and YX assisted with the experiments *in vitro*. ZZ, LZha, and RS helped with data acquisition and analysis. FJ synthesized the TJ-M2010-5. All authors approved the final manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.1080438/full#supplementary-material

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Dobutamine promotes the clearance of erythrocytes from the brain to cervical lymph nodes after subarachnoid hemorrhage in mice

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Background: Erythrocytes and their breakdown products in the subarachnoid space (SAS) are the main contributors to the pathogenesis of subarachnoid hemorrhage (SAH). Dobutamine is a potent β_1-adrenoreceptor agonist that can increase cardiac output, thus improving blood perfusion and arterial pulsation in the brain. In this study, we investigated whether the administration of dobutamine promoted the clearance of red blood cells (RBCs) and their degraded products *via* meningeal lymphatic vessels (mLVs), thus alleviating neurological deficits in the early stage post-SAH.

Materials and methods: Experimental SAH was induced by injecting autologous arterial blood into the prechiasmatic cistern in male C57BL/6 mice. Evans blue was injected into the cisterna magna, and dobutamine was administered by inserting a femoral venous catheter. RBCs in the deep cervical lymphatic nodes (dCLNs) were evaluated by hematoxylin–eosin staining, and the hemoglobin content in dCLNs was detected by Drabkin's reagent. The accumulation of RBCs in the dura mater was examined by immunofluorescence staining, neuronal death was evaluated by Nissl staining, and apoptotic cell death was evaluated by TUNEL staining. The Morris water maze test was used to examine the cognitive function of mice after SAH.

Results: RBCs appeared in dCLNs as early as 3 h post-SAH, and the hemoglobin in dCLNs peaked at 12 h after SAH. Dobutamine significantly promoted cerebrospinal fluid (CSF) drainage from the SAS to dCLNs and obviously reduced the RBC residue in mLVs, leading to a decrease in neuronal death and an improvement in cognitive function after SAH.

Conclusion: Dobutamine administration significantly promoted RBC drainage from cerebrospinal fluid in the SAS *via* mLVs into dCLNs, ultimately relieving neuronal death and improving cognitive function.

KEYWORDS

dobutamine, subarachnoid hemorrhage, cerebrospinal fluid, erythrocytes, cervical lymphatic nodes

Abbreviations: SAH, subarachnoid hemorrhage; SAS, subarachnoid space; RBCs, red blood cells; LECs, lymphatic endothelial cells; Lyve-1, the lymphatic vessel endothelial hyaluronan receptor 1; PROX1, Prospero homeobox 1; VEGFR3, vascular endothelial growth factor receptor 3; dCLNs, the deep cervical lymphatic nodes; mLVs, meningeal lymphatic vessels; SSS, superior sagittal sinus; TS, transverse sinus; PSS, petrosquamosal sinus; SS, sigmoid sinus; CSF, cerebrospinal fluid; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; ANOVA, one-way analysis of variance; CNS, central nervous system; BBB, blood–brain barrier.

1 Introduction

Subarachnoid hemorrhage (SAH), which is mainly caused by the rupture of an intracranial aneurysm, accounts for 9.7% of all types of strokes (Valery et al., 2021). The high mortality among young adults and the cognitive impairments among survivors make SAH a tremendous burden to society (Macdonald and Schweizer, 2017). Blood pours into the subarachnoid space (SAS) through the ruptured aneurysms after SAH, and the influx of red blood cells (RBCs) and degraded cell debris result in early brain injury (within 72 h) and delayed cerebral ischemia (days to weeks after SAH) (van Gijn et al., 2007).

Louveau and colleagues elaborated on the structural and functional features of meningeal lymphatics in the dura mater (Louveau et al., 2015). Perisinusal lymphatic vessels express classical molecular hallmarks of lymphatic endothelial cells (LECs), including lymphatic vessel endothelial hyaluronan receptor 1 (Lyve-1), the main LEC transcription factor, prospero homeobox 1 (PROX1), podoplanin, and the vascular endothelial growth factor receptor 3 (VEGFR3). Macromolecules and immune cells can drain from the SAS to the deep cervical lymphatic nodes (dCLNs) *via* the meningeal lymph flow (Louveau et al., 2015). Meningeal lymphatic vessels (mLVs) are located within dural folds around the superior sagittal sinus (SSS), transverse sinus (TS), petrosquamosal sinus (PSS), and sigmoid sinus (SS) (Figures 1A–C) (Ahn et al., 2019). The dorsal mLVs run along the SSS and TS, with smaller diameters and no lymphatic valves, making them morphologically more similar to the initial lymphatic vessels (Louveau et al., 2015). The clearance function of mLVs in various diseases has been explored recently. It was reported that RBCs in the SAS could drain to the dCLNs in the SAH mouse model, which could be inhibited by the ablation of mLVs *via* the VEGFR3 tyrosine kinase inhibitor MAZ51 or the photodynamic drug Visudyne (Chen et al., 2020). Further studies are warranted to identify clinically available drugs targeted at mLVs.

Dobutamine is a synthetic catecholamine that functions as an agonist of the β_1 adrenergic receptor. This sympathomimetic agent has inotropic efficacy that could immediately increase cardiac contractility and output, thus improving blood perfusion and arterial pulsation in the brain (Tuttle and Mills, 1975). Cerebral arterial pulsation is one of the main driving forces of fluid exchange in the brain, which may accelerate the clearance of deposited metabolic products (Iliff et al., 2013).

In this study, we explored whether the enhancement of cerebral arterial pulsatility by dobutamine could expedite the drainage of accumulated erythrocytes in the SAS after SAH and whether mLVs play a role in this clearance process. We anticipated that the reinforced drainage function could alleviate the neurological deficits after SAH.

2 Materials and methods

2.1 Experimental design

Part 1: Mice were randomly divided into sham and SAH groups (Figure 2A). The mice in the SAH group were executed 3 h, 6 h, 12 h, 24 h, 3 days, and 7 days after SAH. Cerebrospinal fluid (CSF) was collected before the mice were executed to count the RBCs. The dCLNs and brain tissues were then isolated and collected for hematoxylin–eosin staining (HE staining).

Part 2: Mice were randomly divided into a control group, Evans blue group, Evans blue + saline group, and Evans blue + dobutamine group. Evans blue was diluted using saline to 2.5%, which was injected into the cisterna magna of mice at a dose of 50 μL, while the same amount of saline was injected in the control group. The mice were executed 12 h after the Evans blue injection. The dCLNs were then isolated and collected to detect the Evans blue concentration.

Part 3: We randomly divided the mice into sham, SAH, SAH + saline, ligation + SAH, SAH + dobutamine, and ligation + SAH + dobutamine groups. The ligation procedure was conducted 1 week before SAH. CSF was collected 12 h after SAH to count the RBCs

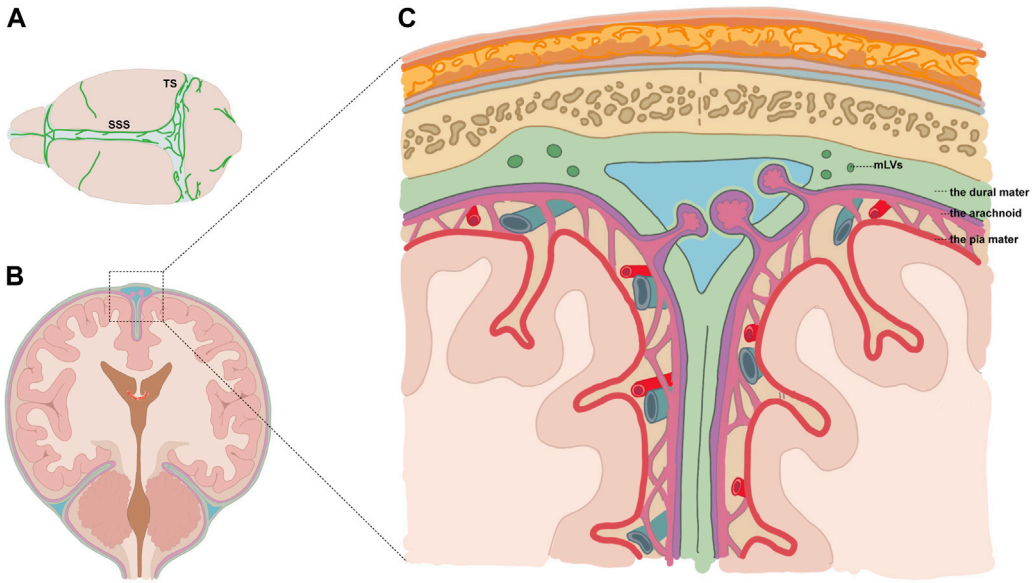

FIGURE 1
Diagram showing the location of mLVs. **(A)** Meningeal lymphatic vessels (mLVs) located around the superior sagittal sinus (SSS), transverse sinus (TS), *etc.* **(B,C)** Vessels were located within dural folds (green) and were adjacent to the subarachnoid space.

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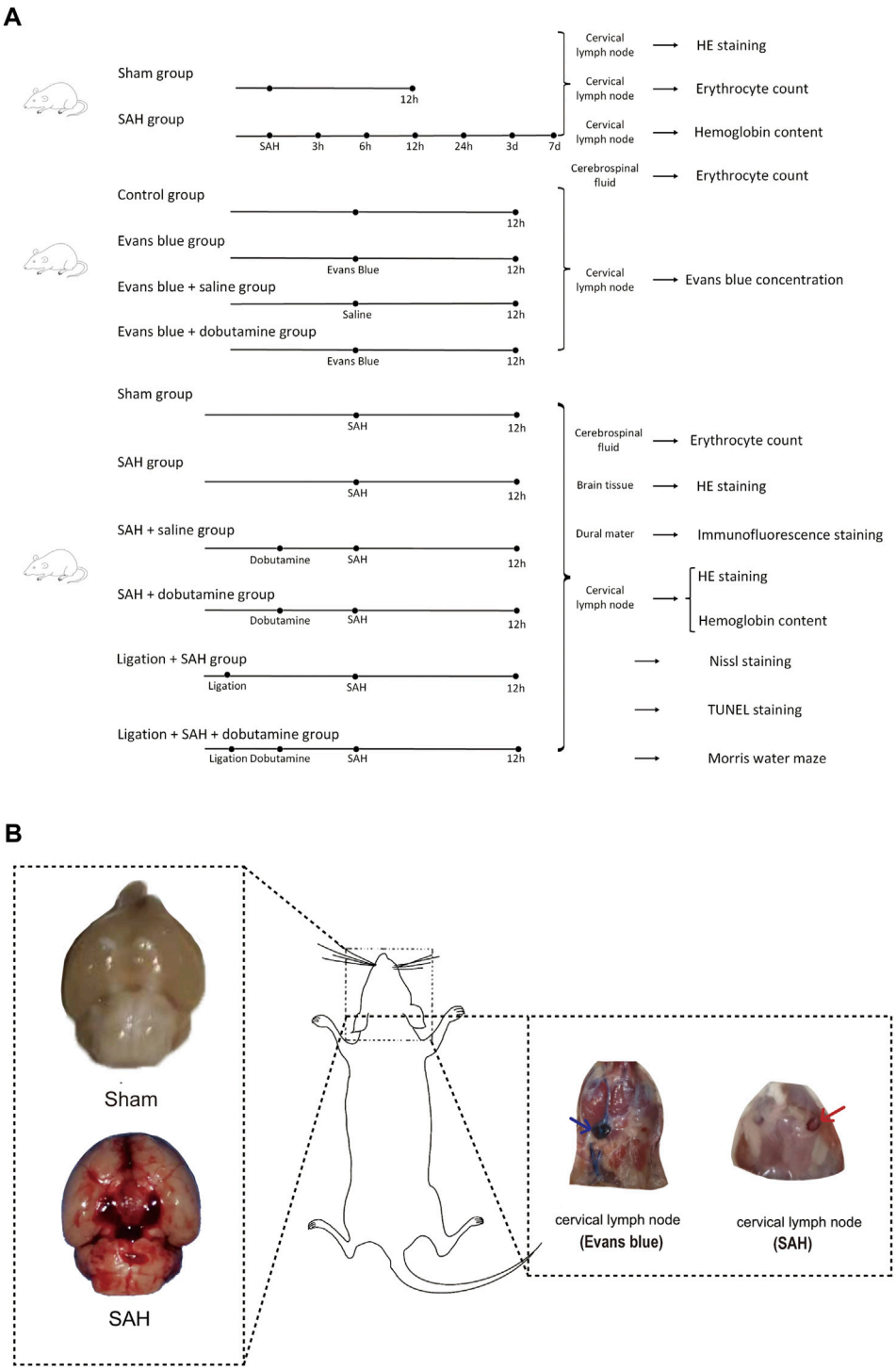

FIGURE 2
Experimental design of the present study and gross appearances of the SAH model and dCLN drainage of Evans blue and erythrocytes. **(A)** We first collected dCLNs from the sham and SAH groups for hematoxylin–eosin staining (HE staining). We then compared the Evans blue concentration of dCLNs among the control, Evans blue, Evans blue + saline, and Evans blue + dobutamine groups. Finally, cerebrospinal fluid, brain tissue, dura mater, and dCLNs were isolated and collected for different analyses of the sham, SAH, SAH + saline, SAH + dobutamine, ligation + SAH, and ligation + SAH + dobutamine groups. **(B)** Gross appearances of the skull base in the sham group (upper left insert) and SAH model (lower left insert). The right panel shows the gross observation of dCLNs after Evans blue staining (blue arrow) and RBC injection (red arrow).

before the mice were executed. The brain tissue was isolated to perform HE staining, and the dura mater was isolated and collected for immunofluorescence staining. We then collected dCLNs for HE staining and evaluation of the hemoglobin content. Nissl staining, TUNEL staining, and the Morris water maze test were processed 1 week after SAH.

2.2 SAH model

In this study, adult male C57BL/6 mice were used (Model Animal Research Center of Nanjing University, Nanjing, China) to establish the SAH model. All of the procedures involving animals were approved by the Institutional Animal Care and Use Committee of Jinling Hospital. The mice were anesthetized with 2% isoflurane in 100% O_2 and maintained with 1% isoflurane. Then, an incision was made to expose the skull. A burr hole was drilled 4.5 mm anterior to the bregma. Then, a 27-gauge needle was tilted at 45° to inject approximately 50 μL of autologous arterial blood from the femoral artery into the prechiasmatic cistern in 30 s using a syringe pump. The needle was maintained in this position for 3 min to prevent CSF leakage and blood reflux. The burr hole was sealed with bone wax, and the incision was sutured immediately. The mice in the sham group underwent the same procedures as the experimental groups, except for blood injection.

2.3 Dobutamine administration

A femoral venous catheter was inserted for systemic dobutamine (40 μg/kg in saline, HY-15746, MCE) administration. The SAH model was established 10 min after dobutamine administration for the first time. The SAH + saline group was infused with saline, and dobutamine was treated every 30 min for the next 3 h. Finally, the mice were executed 12 h after SAH.

2.4 Ligation of dCLNs

The mice were shaved and cleaned with iodine before being anesthetized with 2% isoflurane in 100% O_2 and maintained with 1% isoflurane. Then, a midline incision was made 5 mm superior to the clavicle. We retracted the sternocleidomastoid muscles to expose the dCLNs on each side. Subsequently, 10–0 synthetic and non-absorbable sutures were used to ligate afferent lymphatic vessels on both sides. The other groups underwent the same midline incision and muscle retraction procedures.

2.5 HE staining

HE staining was used to depict the morphological characteristics of dCLNs and SAS. After dissolving all of the wax away with xylene, the tissues were passed through concentration gradient changes of alcohol to remove the xylene before rinsing in water. Subsequently, the tissues were stained with nuclear hematoxylin stain and then treated with a weak alkaline solution to convert the hematoxylin to a dark blue color. A weak acid alcohol was used to remove non-specific background staining before applying the eosin counterstain. Subsequently, the tissues were rinsed, dehydrated, cleared, and finally mounted.

2.6 Hemoglobin content detection

We isolated dCLNs 12 h after SAH and then ground dCLNs to the homogenate. Subsequently, we used Drabkin's reagent (Sigma, United States, Cat# D5941) to detect the hemoglobin content of the dCLN homogenate. The reagent consists of potassium ferricyanide, potassium cyanide, and potassium dihydrogen phosphate. Potassium ferricyanide oxidizes hemoglobin to methemoglobin and then to cyanomethemoglobin, which could be detected at 530 nm.

2.7 Immunofluorescence staining

The dura mater of the mice was isolated and fixed with 4% paraformaldehyde 12 h after SAH. The meninges were then incubated with the primary antibodies against Lyve-1 (1:200, ab14917, Abcam, Cambridge, MA, United States) and RBCs (1:200, GTX01475, GeneTex, United States) overnight at 4°C. The appropriate fluorescently labeled donkey anti-rabbit IgG antibody (1:200, A24221, Abbkine, Wuhan, China) and goat anti-rat IgG antibody (1:200, A23340, Abbkine, Wuhan, China) were added after washing twice with phosphate-buffered saline (PBS) containing Tween 20 (10 min each time). Subsequently, the dura was incubated with 4′,6-diamidino-2-phenylindole (DAPI) solution (C1005, Beyotime, Nantong, China) at room temperature for 4 min before sealing with the anti-fluorescence quenching mounting solution. The fluorescent images were obtained *via* confocal microscopy.

2.8 Nissl staining

Nissl staining was performed to detect neuronal death. Basic dyes were used in Nissl staining to stain basophilic Nissl bodies and cell nuclei. As neurons are active protein-synthesizing cells and the Nissl body is an important site of protein synthesis, it was possible to evaluate neuronal damage *via* the morphological changes in Nissl bodies. Under normal conditions, neurons have multiple large Nissl bodies, which indicates their strong protein synthesis abilities. Regarding neuronal damage, the number of Nissl bodies decreases before they experience lysis and may even disappear. Three fields (400X) were chosen randomly from the temporal lobe, and the average number of counted surviving neurons from 12 fields was calculated in each mouse.

2.9 Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL staining)

TUNEL staining is widely used for detecting apoptotic cell death. When genomic DNA is broken, the exposed 3′-OH can be catalyzed by terminal deoxynucleotidyl transferase (TdT) with fluorescein and biotin-labeled dUTP. First, the deparaffinized brain sections were incubated with proteinase K (20 μg/mL) for 30 min at 37°C before washing three times with PBS for 10 min each time. Subsequently, 2% hydrogen peroxide diluted in PBS was used at room temperature before washing with PBS again. The brain sections were treated with TdT buffer for 2 min before incubating in TdT and UTP for 1 h. Then, the SSC buffer was used to rinse the sections twice for 5 min each time. Horseradish peroxidase streptavidin was diluted in 0.1 M TRIS pH 8.5 and 50 mM NaCl, and 4 mM $MgC1_2$ with 0.5% Tween 20 was then applied for 60 min at room temperature. Then, the chromogen, amino-ethyl-carbazole (Vector Laboratories, Peterborough, United Kingdom), was applied for 10 min before further rinses. The TUNEL positivity was evaluated by two observers who were blind to the grouping.

2.10 Morris water maze test

The Morris water maze test, which includes a navigation training trial and a probe trial, was used to detect the spatial learning and memory ability of mice 1 week after SAH. A relatively small hidden platform was placed in a fixed location. During the place navigation test, mice were sent from different, random locations around the perimeter of the tank, and the time they spent navigating a direct path to the camouflaged platform on the first 4 days was recorded. On the fifth day, the time spent in the target quadrant and the frequency of crossing the platform location were documented after the platform was withdrawn. The Morris water maze test data were collected by ANY-maze software (TOPSCAN G3; ANY-MAZE 6.0).

2.11 Statistical analysis

All statistical analyses were conducted by GraphPad Prism 9.3.1 software, and the data were presented as the mean ± standard deviation. We used one-way analysis of variance (ANOVA) to analyze the statistical differences among three or more groups. Tukey's *post hoc* multiple comparison test was employed when a significant difference was determined by ANOVA. The unpaired Student's *t*-test was used to compare the two groups. The chi-squared test was used to compare the survival rate between two different SAH models. A p-value <0.05 was considered significant.

3 Results

3.1 RBCs in the SAS drained by CSF accumulated in dCLNs after SAH and the hemoglobin content peaked at 12 h

The SAH model was successfully established in mice by injecting autologous blood into the prechiasmatic cistern (Figure 2B). Then, the dCLNs of mice were isolated at different times (3 h, 6 h, 12 h, 24 h, 3 days, and 7 days) after SAH (Figure 2A). The time-course accumulation of RBCs in dCLNs was shown by HE staining. Morphologically intact RBCs or degraded RBC debris was observed in dCLNs as early as 3 h after SAH and gradually accumulated (Figures 3A–C), which confirmed the RBC drainage function of dCLNs after SAH. To quantify the drainage of RBCs from SAS to dCLNs, we used Drabkin's reagent to detect the hemoglobin content of dCLNs. The hemoglobin content increased significantly after SAH and peaked at 12 h ($p < 0.001$, sham *vs.* 12 h; Figure 3D). Additionally, the RBCs in CSF were quantified to evaluate the dynamic change of RBC residue in SAS (Figure 3E).

3.2 Dobutamine promoted the clearance of Evans blue and RBCs from SAS to dCLNs

The brain and dCLNs were isolated 12 h after Evans blue was injected into the cisterna magna to confirm the drainage function of mLVs from CSF. The dCLN slices of the Evans blue group and the Evans blue + saline group showed obviously deeper staining than the control group (Figure 4B). The levels of Evans blue in dCLNs were quantitatively analyzed and showed prominent differences ($p < 0.001$, Con *vs.* EB; $p < 0.001$, Con *vs.* EB + saline; Figure 4C). Moreover, dobutamine significantly accelerated the clearance of Evans blue in CSF ($p < 0.001$, Figure 4C).

To further verify the facilitation role of dobutamine in the clearance of RBCs, dobutamine was systemically administered to SAH animals. Then, we collected CSF and isolated brain tissues and dCLNs at 12 h after SAH. The brain slices showed obvious RBC accumulation in the SAS of the SAH, the SAH + saline, the ligation + SAH, and the ligation + SAH + dobutamine groups, whereas the administration of dobutamine ameliorated this situation (Figure 5A). The CSF RBC counts in different groups demonstrated similar effects of dobutamine (Figure 5C). As for dCLN staining, dobutamine administration increased the infused RBCs in dCLNs (Figure 5B). Hemoglobin contents of the dCLNs in the SAH and SAH + saline groups were dramatically increased compared to that of the sham group ($p < 0.001$), and dobutamine significantly increased the hemoglobin content of dCLNs ($p < 0.001$, Figure 5D).

3.3 Dobutamine promoted the clearance of RBCs after SAH by meningeal lymphatics

To further determine whether dobutamine could promote the clearance of RBCs in the SAS by meningeal lymphatics, we isolated the meninges 12 h after SAH. The anti-Ter-119 antibody was used to label RBCs, and the anti-Lyve-1 antibody was used to visualize meningeal lymphatics. Although no RBCs were observed entering or exiting the meningeal lymphatics in the sham group, the RBCs in the SAH, SAH + saline, ligation + SAH, and ligation + SAH + dobutamine groups showed evident accumulation in the meningeal lymphatics ($p < 0.001$, sham *vs.* SAH; $p < 0.001$, sham *vs.* SAH + saline; $p < 0.001$, sham *vs.* the ligation + SAH group; $p < 0.001$, sham *vs.* the ligation + SAH + dobutamine group; Figures 6A, B). The administration of dobutamine significantly attenuated the accumulation of RBCs in meningeal lymphatics ($p < 0.01$, SAH + saline *vs.* SAH + dobutamine; Figures 6A, B).

3.4 Dobutamine alleviated neuronal damage and improved cognitive function after SAH

Nissl staining was performed to assess the extent of neuronal damage 1 week after SAH. Neurons in the SAH, SAH + saline, ligation + SAH, and ligation + SAH + dobutamine groups showed clearly shrunken cell bodies and condensed nuclei, whereas the number of surviving neurons was significantly declined, with the highest decline in the ligation + SAH group (Figures 7A, B). Additionally, the administration of dobutamine remarkably alleviated neuronal damage after SAH (Figures 7A, B).

TUNEL staining was conducted to evaluate the apoptotic cell death 1 week after SAH. The broken genomic DNA exposed 3′-OH, which was catalyzed by TdT with fluorescein and biotin-labeled dUTP. The fluorescence intensity in the ligation + SAH group was the strongest, whereas that of the SAH and SAH + saline groups also increased significantly compared to that of the sham group (Supplementary Figure S1A, B). Comparatively, the SAH + dobutamine group showed greatly alleviated cell death (Supplementary Figure S1A, B).

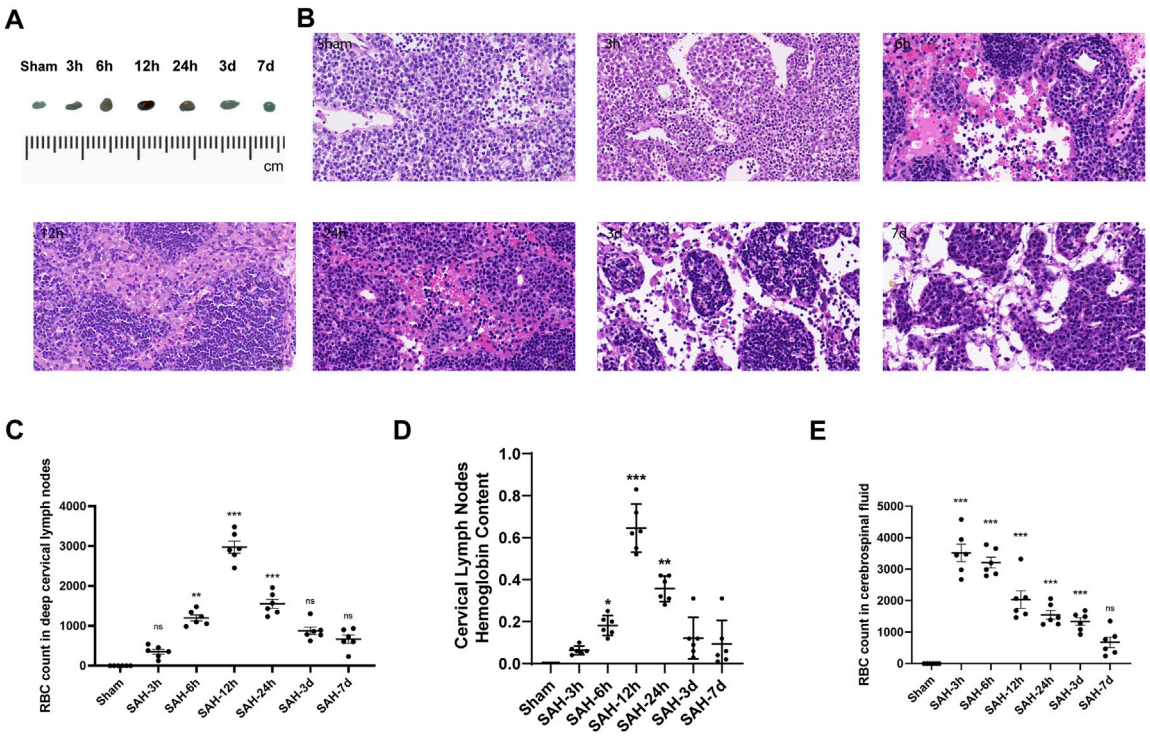

FIGURE 3
Time-course accumulation of RBCs in dCLNs after SAH. **(A)** Gross appearance of dCLNs after SAH at different times. **(B)** HE staining of dCLNs after SAH at different times. The erythrocyte accumulation in dCLNs obviously increased 12 h after SAH. **(C)** Quantitative analysis of the RBC count in deep cervical lymph nodes at different times (n = 6 mice, $^{**}p < 0.01$, and $^{***}p < 0.001$). **(D)** Hemoglobin content analysis of dCLNs after SAH at different times. Data are presented as the mean ± SD (n = 6 mice, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). **(E)** Quantitative analysis of the RBC count in cerebrospinal fluid at different times (n = 6 mice and $^{***}p < 0.001$).

To measure the extent of cognitive function impairment, mice from different groups underwent the Morris water maze test. The SAH + dobutamine group showed a significant decrease in escape latency compared to the SAH, SAH + saline, ligation + SAH, and ligation + SAH + dobutamine groups from days 2–4 (p = 0.0141, day 1; p = 0.0071, day 2; p = 0.0216, day 3; Figure 7D). Additionally, mice in the SAH + dobutamine group tended to cross the platform more frequently (p = 0.0401) and spend more time in the target quadrant (p = 0.0276) after the administration of dobutamine compared to those in the SAH + saline group ($p < 0.01$) (Figure 7E).

4 Discussion

SAH, as a type of severe life-threatening stroke, affects a younger productive life than other subtypes of strokes. Even if the patient survives, the neurological deficits result in a huge decrease in the patient's quality of life (Lawton and Vates, 2017). SAH resulting from the rupture of an intracranial aneurysm accounts for approximately 80% of all types of SAH (Valery et al., 2021). The ruptured aneurysm ejects RBCs into SAS, and then hemoglobin and its breakdown products, which are directly neurotoxic and can trigger the release of inflammation cytokines, which may contribute to the pathogenesis of SAH, eventually causing neurological deficit (Lucke-Wold et al., 2016). The concentrations of hemoglobin and its breakdown products, such as heme and iron, in CSF are markedly increased after SAH (Bulters et al., 2018). Hence, we attached great importance to precipitating the clearance of RBCs and their degraded products to alleviate neuronal damage in the early stage post-SAH.

Blood scavenging pathways in the central nervous system (CNS) include erythrophagocytosis, haptoglobin binding, hemopexin binding, and heme oxygenase (Bulters et al., 2018). Nevertheless, these pathways could be easily saturated in the CNS, having many specialized anatomical structures, such as the blood–brain barrier (BBB), which limits solute drainage (Andersen et al., 2017). Therefore, promoting the clearance of RBCs and breakdown products after SAH is a promising therapy when classic blood scavenging pathways are overwhelmed.

As the CNS lacks a classical lymphatic system, it was long considered to undergo immune privilege (Ransohoff and Engelhardt, 2012). The discovery of mLVs confirmed that the CNS undergoes constant immune surveillance within the dura mater (Aspelund et al., 2015). Lymph flow in the dura appears to start from both eyes and track around the cribriform plate above the olfactory bulb (Louveau et al., 2015). CSF in the SAS exchanged with the interstitial fluid *via* the lymphatic system could be absorbed by mLVs and then transported to deep dCLNs *via* the foramina at the skull base (Aspelund et al., 2015).

Exogenous tracers and immune cells have been demonstrated to be drained from the CSF by mLVs into the peripheral circulation (Louveau et al., 2015; Da Mesquita et al., 2018a). Meningeal lymphatic drainage has also been confirmed to play a key role in the accumulation of β-amyloid[16] (Da Mesquita et al., 2018b; Da Mesquita et al., 2021). The diameters of mLVs were shown to

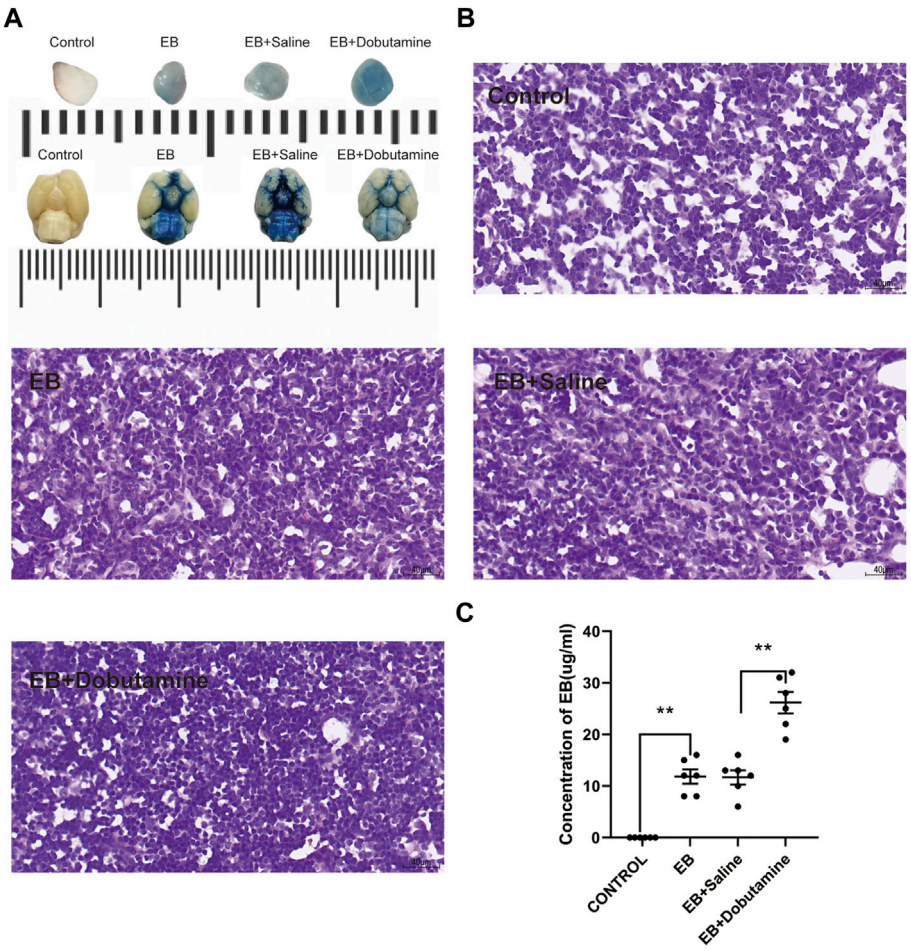

FIGURE 4
Dobutamine promoted the drainage of Evans blue from SAS to dCLNs. **(A)** Gross appearance of dCLNs (upper) and the skull base views of the brains (lower) in the control, Evan blue, Evans blue + saline, and Evans blue + dobutamine groups. **(B)** dCLN sections after Evans blue injection. **(C)** Quantitative analysis of the concentration of Evans blue in dCLNs. Data are presented as the mean ± SD (n = 6 mice and $^{**}p < 0.01$)

increase 1 h after brain hemorrhage, indicating activation of blood component drainage and clearance *via* meningeal lymphatics (Semyachkina-Glushkovskaya et al., 2020). Chen et al. (2020) demonstrated the drainage process from CSF to dCLNs after SAH, and the ablation of mLVs obviously inhibited the drainage of RBCs. Compared to other types of hemorrhagic strokes, RBCs accumulate in the SAS after SAH, and the anatomical characteristics allow them easy access to mLVs in the dura before subsequent drainage into dCLNs. In this study, we confirmed the drainage function of mLVs to dCLNs by injecting Evans blue and autologous blood into SAS. The time-course accumulation of RBCs was shown by HE staining of dCLNs, combined with the quantification of the RBC count in CSF and the content of hemoglobin in CLNs (Figures 3A–E). We chose 12 h as the execution and tissue harvest time because the changes in the hemoglobin content of dCLNs were most evident at that time (Figures 3C–E).

On this basis, our further study focused on the β_1-adrenoreceptor agonist dobutamine, which is widely used as a potent positive inotropic agonist due to its rapid action and short half-time (Levy et al., 1999). Dobutamine could consistently increase heart output, which was associated with the heart rate and the volume of blood ejected with each beat by targeting cardiac β_1 receptors (Annane et al., 2018; King and Lowery, 2022). The blood perfusion in the brain is higher than that in other peripheral vital organs to guarantee neurotrophic effects (Sweeney et al., 2019). Additionally, the vascular system in the adult brain includes more than 600 km of blood vessels, making it the anatomic basis of exerting substantial forces on intracranial structures surrounding the vessels (Rasmussen et al., 2021). All the aforementioned phenomena have made cardiovascular dynamics one of the major driving forces of pumping CSF exchange and ensure that the brain parenchyma can easily access the necessary nutrients and drain solutes in a timely manner.

In this study, we explored the acceleration function of dobutamine in brain fluid exchange. We observed an obvious promoting effect of dobutamine on Evans blue clearance. The dobutamine-treated group showed a significantly increased Evans blue concentration in dCLNs (Figures 4A–C), which indicated that there was relatively less residual dye in the brain.

Herein, we used the prechiasmatic cistern injection model of SAH rather than the endovascular perforation model to control the blood volume injected into the prechiasmatic cistern. Hence, we could compare the RBC drainage without the bias of the amount of

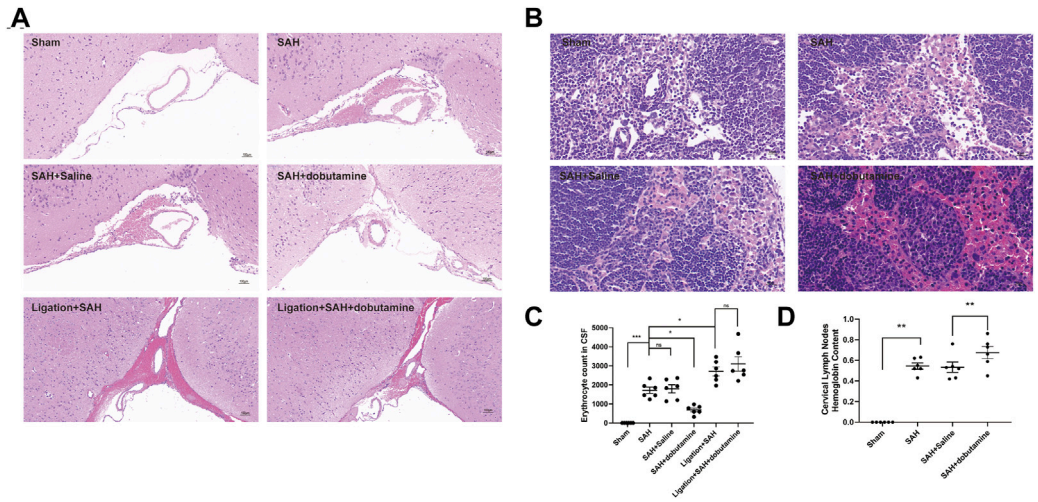

FIGURE 5
Dobutamine promoted the drainage of RBCs from SAS to dCLNs. **(A)** HE staining of RBC accumulation in the SAS in the sham, SAH, SAH + saline, SAH + dobutamine, ligation + SAH, and ligation + SAH + dobutamine groups. **(B)** HE staining of dCLNs showed decreased RBC accumulation in dCLNs after dobutamine administration. **(C)** Quantitative analysis of erythrocyte count in CSF. Data are presented as the mean ± SD (n = 6 mice, $^{**}p < 0.01$, and $^{***}p < 0.001$). **(D)** Hemoglobin content of dCLNs after dobutamine administration was detected. Data are presented as the mean ± SD (n = 6 mice and $^{**}p < 0.01$).

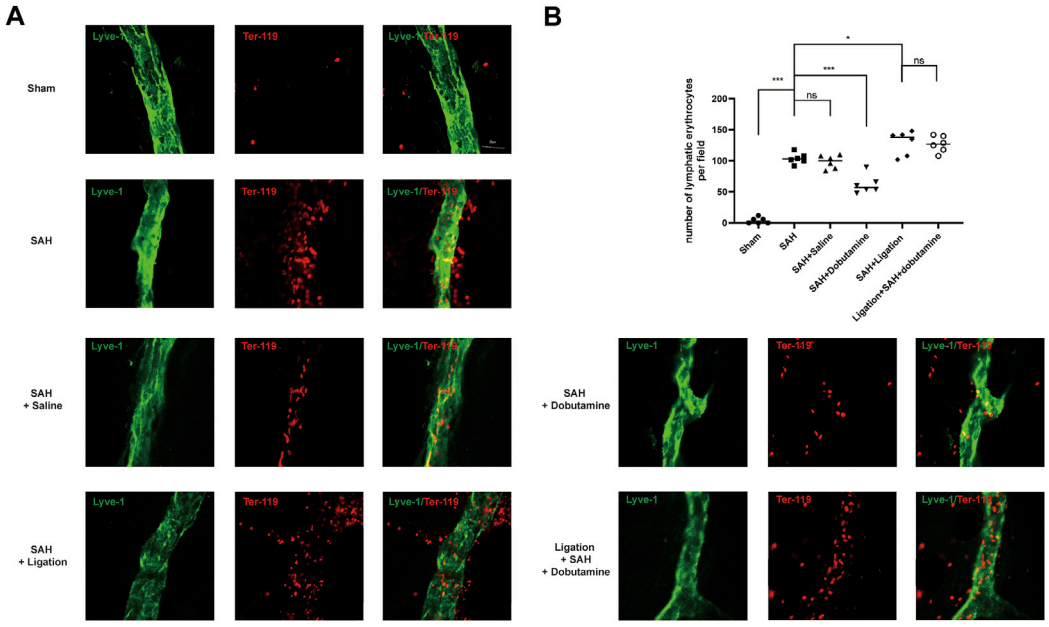

FIGURE 6
Dobutamine promoted the drainage of RBCs from the SAS to dCLNs *via* mLVs. **(A)** Representative immunofluorescence staining of RBCs and mLVs in different groups. **(B)** Quantitative analysis of the number of lymphatic erythrocytes per field in mLVs. Data are presented as the mean ± SD (n = 6 mice, $^{**}p < 0.01$, and $^{***}p < 0.001$).

blood ejected into SAS. Given that dobutamine can increase blood pressure as a β_1-adrenoreceptor agonist, we conducted the filament perforation SAH model and the prechiasmatic cistern injection SAH models before dobutamine administration to compare the mortality caused by increased blood pressure (Supplementary Table S1). The dobutamine-treated SAH animals appeared to have less RBC residue in the SAS (Figure 5A) and more RBC accumulation in dCLNs (Figure 5B). The quantitative analysis of RBC counts and the hemoglobin content showed consistent results (Figures 5C, D). We used an anti-Lyve-1 antibody targeting lymphatic endothelial cells to visualize the morphology of meningeal lymphatics and an anti-Ter-119 antibody, a lineage marker for erythroid cells from early proerythroblast to mature RBC stages, to verify the RBC drainage function of mLVs. We observed an apparently decreased

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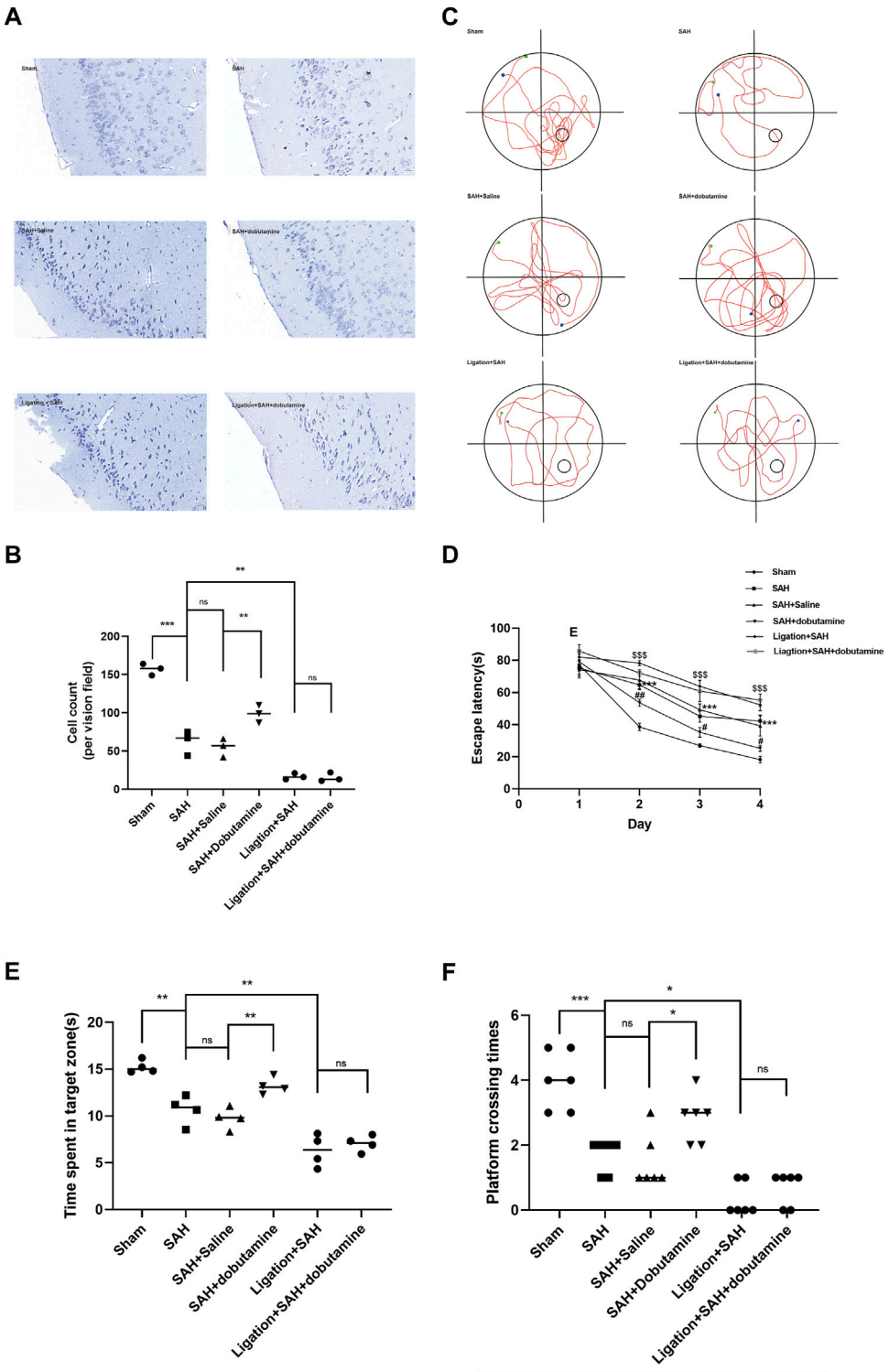

FIGURE 7
Dobutamine alleviated neuronal damage and improved cognitive function after SAH. **(A)** Representative Nissl staining of the dobutamine-treated SAH model. **(B)** Quantitative analysis of the morphologically normal neuron count. Data are presented as the mean ± SD (n = 3 mice, *p < 0.05, and ***p < 0.001). **(C)** Representative swimming tracks of mice in the Morris water maze test. The green point indicates the starting point, and the blue point indicates the end point. The small circle in the lower right quadrant shows the hidden platform. **(D)** Escape latencies in the first 4 days were recorded. Data are presented as the mean ± SD (n = 4 mice, ***p < 0.001 when the SAH + saline group was compared to the sham group, #p < 0.05 when the SAH + dobutamine group was compared to the SAH + saline group, ##p < 0.01 when the SAH + dobutamine group was compared to the SAH + saline group, and $$$ p < 0.001 when the SAH group was compared to the ligation + SAH group). **(E)** Then, the time spent in the target quadrant was analyzed. Data are presented as the mean ± SD (n = 4 mice, *p < 0.05, and **p < 0.01). **(F)** Frequency of mice platform crossing was recorded (n = 4 mice and *p < 0.05).

RBC count in mLVs in the dobutamine-treated group, which suggested that the administration of dobutamine reinforced the brain fluid exchange, thus promoting RBC clearance from the CSF to dCLNs *via* mLVs (Figures 6A, B). Nissl and TUNEL staining also confirmed that dobutamine treatment could reduce neuronal damage (Figures 7A, B; Supplementary Figure S1A, B). This was consistent with the Morris water maze test results, which demonstrated that the impaired learning and memory ability post-SAH were obviously attenuated after dobutamine treatment (Figures 7D–F).

Systemic dobutamine administration has been confirmed to facilitate the paravascular influx of intracisternal injection of subarachnoid CSF tracers (confirm, which further confirmed the role of dobutamine in enhancing brain fluid exchange). The lymphatic system was found to serve a lymphatic role in clearing the extracellular metabolites of the brain parenchyma (Iliff et al., 2012; Rangroo Thrane et al., 2013). The lymphatic system is a low-resistance peri-arterial fluid flow pathway, which can be driven by the cardiac pulse (Mestre et al., 2018). Paravascular influx promoted by systemic dobutamine administration suggests that the cardiovascular pulse plays a key role in pumping the supply of fresh CSF to the lymphatic system (Mestre et al., 2018; Hablitz et al., 2020). Dobutamine administration functioned in CSF perfusion in the lymphatic system and the subsequent drainage to dCLNs, which might assist with further understanding the relationship between the lymphatic system and mLVs. Lymphatic inhibition was observed after mLV ablation *via* the photodynamic drug verteporfin (Aspelund et al., 2015; Louveau et al., 2015; Ahn et al., 2019; Hauglund et al., 2020). Lymphatic efflux has also been confirmed to present around the TS and straight sinus (Iliff et al., 2012; Rangroo Thrane et al., 2013; Iliff et al., 2014). These findings suggest that the lymphatic function may be directly linked to mLVs, or it might serve as a sink for the perivenous efflux, draining CSF, and extracellular fluid to dCLNs (Hauglund et al., 2020; Ringstad and Eide, 2020). However, the specific anatomical connections between the lymphatic system and mLVs were not fully demonstrated due to the current limitations to the experimental technique, which should be explored in the future. The clearance-promoting function of dobutamine could not only be achieved by accelerating meningeal lymph flow but also enhances the brain fluid exchange between CSF and interstitial fluid *via* the glymphatic system, thus providing a possible therapy for other types of hemorrhagic strokes, such as intracerebral hemorrhage.

In summary, dobutamine administration provides a promising treatment for the early clearance of RBCs and its breakdown products, such as hemoglobin, after SAH, which suggests that the changes in the arterial pulsatility contribute to alleviating long-term complications, such as cognitive impairment post-SAH.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Jinling Hospital.

Author contributions

M-LZ, WX, H-JD, and Y-LH designed and interpreted experiments. WX, H-JD, S-QG, J-YQ, S-HM, and Y-SZ performed experiments. M-LZ, TL, and C-CG analyzed the data. WX and H-JD wrote the manuscript. All authors critically read the manuscript.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.1061457/full#supplementary-material

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Memantine as a neuroprotective agent in ischemic stroke: Preclinical and clinical analysis

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The primary mechanism for neuron death after an ischemic stroke is excitotoxic injury. Excessive depolarization leads to NMDA-mediated calcium entry to the neuron and, subsequently, cellular death. Therefore, the inhibition of the NMDA channel has been proposed as a neuroprotective measure in ischemic stroke. The high morbimortality associated with stroke warrants new therapies that can improve the functional prognosis of patients. Memantine is a non-competitive NMDA receptor antagonist which has gained attention as a potential drug for ischemic stroke. Here we analyze the available preclinical and clinical evidence concerning the use of memantine following an ischemic stroke. Preclinical evidence shows inhibition of the excitotoxic cascade, as well as improved outcomes in terms of motor and sensory function with the use of memantine. The available clinical trials of high-dose memantine in patients poststroke have found that it can improve patients' NIHSS and Barthel index and help patients with poststroke aphasia and intracranial hemorrhage. These results suggest that memantine has a clinically relevant neuroprotective effect; however, small sample sizes and other study shortcomings limit the impact of these findings. Even so, current studies show promising results that should serve as a basis to promote future research to conclusively determine if memantine does improve the outcomes of patients' post-ischemic stroke. We anticipate that future trials will fill current gaps in knowledge, and these latter results will broaden the therapeutic arsenal for clinicians looking to improve the prognosis of patients poststroke.

KEYWORDS

memantine, acute stroke, ischemia-reperfusion, NMDA, dementia, neuroprotection

1. Introduction

Acute stroke is the acute onset of focal neurological findings in a vascular territory due to underlying cerebrovascular disease (Katan and Luft, 2018). Acute stroke remains a significant cause of morbidity and mortality worldwide, currently the second leading cause of death worldwide, accounting for 6–7 million deaths in 2019 (Katan and Luft, 2018; Feigin et al., 2021). Stroke can be classified etiologically into ischemic stroke, which accounts for 62 percent of stroke cases; intracerebral hemorrhage, representing 28 percent; and subarachnoid hemorrhage, representing 10 percent of all global incident cases of stroke (Krishnamurthi et al., 2013; Feigin et al., 2021). Even though both ischemic and hemorrhagic are highly prevalent, ischemic stroke

remains far more common, representing up to 87 percent of stroke cases in the U.S. (Roger et al., 2011). The most common consequence of a stroke is neurologic deficits. Stroke is a leading cause of chronic disability worldwide, with up to 30% of stroke survivors becoming permanently disabled. The most common disabilities are hemiparesis, difficulty walking, aphasia, and depression (Roger et al., 2011). Without medical intervention, it is estimated that 62% of stroke patients become dependent or die after 6 months (Heller et al., 2000). Current best medical therapy results in improved functionality, decreased recurrence, complications, and mortality (Phipps and Cronin, 2020). In the last few decades, stroke mortality has been consistently decreasing. According to the CDC, from the year 2000 to 2015, the annual stroke death rate decreased by 47.56% (Centers for Disease Control and Prevention NC for HS, 2016), while the overall burden of disease in that same period, measured by the World Health Organization in Disability Adjusted Life Years (DALYs), just decreased 15.95% (World Health Organization, 2022). An important cause of reduction in morbimortality of stroke has been the advancement of medical therapies available, that while having had a significant benefit on mortality, also result in patients living for longer with disabilities, which is reflected in the minor change in DALYs relative to mortality. The data further emphasizes the drastic consequences of stroke-related disabilities not only in the quality of lives of patients but also in the burden of their caregivers. Hence the importance of striving to keep improving the overall management of patients with stroke.

The gold standard for reduction of mortality and morbidity in ischemic stroke, the most common cause of stroke, is reperfusion therapy, either through thrombolysis or thrombectomy, and despite their benefits, in actual practice, only around 1–3% of stroke patients receive reperfusion therapy (Fisher et al., 2005), and of those who receive it, a majority are still left with disabling neurologic deficits (Grefkes and Fink, 2020). Despite the significant advances made in the treatment of acute stroke over the years, the available therapeutic options for stroke patients are minimal, further emphasizing the need for more research, focusing not only on the acute treatment of stroke but also exploring the recovery options of patients after suffering the acute event. It is crucial to comprehensively analyze any potential therapeutic measure in an unbiased manner to determine potential benefits in the prognosis and functionality of stroke patients. The current search for therapies for ischemic stroke has been based on modifying the natural timeline of events following a stroke. This review aims to examine the currently available evidence supporting the use of one of these potential therapies in ischemic stroke, the drug memantine, presenting evidence supporting its utility and its setbacks. There is solid preclinical evidence supporting the use of memantine for ischemic stroke, yet clinical evidence supporting this drug has been lacking (Seyedsaadat and Kallmes, 2018). In the last few years, there has been a growing amount of evidence supporting the use of memantine in the setting of ischemic stroke, which will be analyzed and presented throughout this review.

2. Current stroke treatment

The reduction of mortality in stroke has been partly thanks to the control of risk factors known to increase the risk of death in stroke. The control of isolated systolic hypertension by antihypertensive therapy has been shown in clinical trials to be an essential measure associated with reductions in risk, incidence, and mortality of stroke (Lackland et al., 2014). Atrial fibrillation is also a well-documented risk factor for stroke and systemic embolism; warfarin and oral anticoagulant, including the non-vitamin K antagonist oral anticoagulants (NOAC) as factor Xa inhibitors and direct thrombin inhibitors, are all effective in preventing atrial fibrillation-related stroke; NOAC has been shown to correlate with a significantly lower risk of intracranial hemorrhage than vitamin K antagonist in patients with atrial fibrillation without prior intracranial hemorrhage (Liu et al., 2022). Otherwise, observational studies demonstrated that anticoagulation with vitamin K antagonists correlated with a lower rate of ischemic stroke and no significantly increased intracranial hemorrhage recurrence compared with antiplatelet agents or no antithrombotic medication (Korompoki et al., 2017). The most common side effects caused by the current standard of care are bleeding complications, even more so with vitamin K antagonists than with NOACs (Tsai et al., 2020). Current lines of treatment are based on managing the vascular factors that underlie ischemic stroke. However, there are currently no therapies that act upon the cellular mechanism of neuron death behind ischemic neurotoxicity (Grefkes and Fink, 2020; Matei et al., 2021).

3. Role of NMDA in neuron death in ischemic stroke

The current understanding of the cause of neuron death in stroke is due to an excessive excitatory transmission in the setting of ischemia, and one of the main drivers of these events is excitatory cation channels. The NMDA (*N*-methyl-D-aspartate) ionotropic receptor is a major mediator for excitatory transmission in our brain (Rezvani, 2006). It is expressed in 80% of cortical neurons and is involved in many physiological processes, such as memory formation and synaptic long term-potentiation (Conti, 1997). The NMDA receptor can induce metabolic and transcriptional changes in neurons by regulating calcium entry following an excitatory stimulus (Sossa et al., 2006). The NMDA channel becomes permeable to calcium ions when a neuronal depolarization is coupled to glutamate binding in its synapse. The latter phenomenon allows neurons to have a graded response to stimulus, with an initial depolarization mediated by other glutamatergic receptors, such as AMPA. When a neuron is depolarized enough, the NMDA receptor allows for metabotropic changes in a neuron mediated by increased calcium permeability (Kandel et al., 2014).

This receptor can also become pathologically hyperactive when an increased extracellular accumulation of glutamate occurs in ischemic conditions (Zhou et al., 2013). The increased glutamate release, coupled with an inability to repolarize neurons, results in an excessive calcium entry through the hyperactive NMDA channel. The high intracellular calcium concentration results in the activation of various pathways, which results in neuronal death. *In vitro* studies have identified that it is mainly through the NMDA channel that calcium enters the neuron, increases reactive oxygen species (ROS) production, increases mitochondrial membrane permeability, and induces neuron death (Lai et al., 2014). The excessive glutamate release and the increased calcium influx are the main drivers of

excitotoxicity, which is thought to be the primary mechanism of neuron death in the early stages of ischemic stroke (Zhou et al., 2013).

4. Pathophysiology of ischemic stroke

Understanding the events that follow an ischemic stroke allows us to make sense of the current therapeutic endeavors to treat stroke patients. The occlusion of a cerebral vessel can be of embolic or thrombotic origin. In both cases, the resulting decrease in blood flow will cause dysfunction of normal neuronal activity, followed by ischemic injury and irreversible cell death (Moskowitz et al., 2010). The neurons most proximal to the occluded vessel will die most rapidly, thus forming an ischemic core. At the same time, most distal to the occlusion, there will be an area of electrically and functionally stunned neurons, called the penumbral zone (Şekerdağ et al., 2018). These neurons can follow one of two paths: recover their function through perfusion restoration, or die if the ischemia persists (Moskowitz et al., 2010).

In neurons, the initial decrease in oxygen causes a reduction in ATP concentration and hence a dysfunction of the Na, K-ATPase (Moskowitz et al., 2010). The transmembrane electrical imbalance results in neuronal depolarization, which subsequently impairs the neuronal capacity to transmit action potentials (Campbell et al., 2019). This causes a sizeable excitatory neurotransmitter release (mainly glutamate) in the depolarized ischemic core, which causes a wave of self-propagating electrical activity through the areas surrounding the infarction zone (Moskowitz et al., 2010). The release of glutamate, coupled with neuronal depolarization, results in the opening of AMPA and NMDA cation channels, further worsening the electrolyte imbalance and resulting in excitotoxic cell injury (Pál et al., 2020). The unregulated entry of calcium through the NMDA receptor activates intracellular proteases, endonucleases, and lipases, among other enzymes that trigger apoptotic pathways and result in cell death (Pál et al., 2020). This excitotoxic pathway is the primary mechanism for cell death in the early stages of stroke (Zhou et al., 2013). Ischemia by itself also causes an increased free radical production, which results in cellular necrosis and a disruption of the blood–brain barrier in the ischemic core (Moskowitz et al., 2010). The neurons in the penumbra, which have not undergone cell death, but have ceased electrical activity due to the decreased blood flow, will remain at high risk for irreversible injury (Moskowitz et al., 2010). These neurons initially survived thanks to collateral blood flow, which allowed cells to maintain more than 20 percent of baseline perfusion. Still, the continued hypoperfusion and the subsequent neuroinflammatory response can further hinder their viability (Moskowitz et al., 2010; Campbell et al., 2019).

Microglial activation, which occurs the first hours following ischemia, will create a proinflammatory environment by releasing TNFα and IL1β (Moskowitz et al., 2010; Jayaraj et al., 2019). Other inflammatory cells, such as neutrophils, monocytes, and IL-17-producing lymphocytes, will also migrate and promote debris cleanup (Moskowitz et al., 2010; Jayaraj et al., 2019). In the first 48–96 h after ischemia, astrocytes undergo reactive astrogliosis, becoming hypertrophic and creating a glial scar (Campbell et al., 2019). The coupling of the ongoing neuroinflammatory response, and the antiproliferative effect of reactive astrocytes, will induce the neurons in the penumbral zone to undergo apoptosis and autophagy (Şekerdağ et al., 2018), which will be most pronounced at the 3rd-day day post-ischemia (Angelo et al., 2009). The viability of the penumbral neurons will depend on their environment; in the days following a stroke, angiogenesis will take part in increasing the perfusion of the peri-infarct zone (Adamczak and Hoehn, 2015), and at the same time, there will be a sizeable synaptic rewiring, which will be vital to the process of stroke recovery (Hara, 2015). Current therapies for acute stroke aim to increase the survivability and functionality of the peri-infarct neurons; through reperfusion or experimental methods, such as attenuation of the neuroinflammatory response; and a blockade of proapoptotic second messengers (Liu et al., 2010).

5. Blockage of NMDAR as a neuroprotective mechanism

The inhibition of the main pathway of neuronal damage is an attractive target for stroke treatment, but many inhibitors of the excitotoxic cascade have not been successful in clinical trials. Following the occlusion of a vessel, the ischemic changes in brain tissue depend on the degree of hypoxia (Sodaei and Shahmaei, 2020). The site which suffers the most significant decrease in blood flow is the ischemic core, where neurons rapidly undergo cell death (Phan et al., 2002). While in the penumbra, the reduction in blood flow has caused the complete cessation of electrical activity but has not induced the morphological changes of cell death (Phan et al., 2002). The neurons in the penumbral zone will undergo excitotoxic neuron death in the following 4–24 h unless perfusion improves (Li and Wang, 2016; Fifield and Vanderluit, 2020). Despite the attractive idea of inhibiting the excitotoxic cascade through the blockade of the NMDA receptor, there have previously been many clinical trials with NMDAR blockers that have failed to show improvement in the morbidity or mortality of stroke patients. NMDA channel blockers have been demonstrated to be neuroprotective in preclinical models of ischemic stroke. However, these results have not been able to be replicated in a clinical setting (Lai et al., 2014). The reasons for the unsuccessful clinical transition of these NMDAR antagonists are unclear. However, proposed reasons include, but are not limited to, inappropriate dosage for neuroprotection, intolerable side effects, administration out of their neuroprotective window, poor experimental design, and variable patient populations, among others (Lai et al., 2014). The pharmacodynamic differences between NMDAR antagonists are partly responsible for the varying results in clinical effectiveness, highlighting the importance of both efficacy and tolerability.

6. Memantine

Memantine (3,5-dimethyltricyclo[3.3.1.$1^{3,7}$]decan-1amine or 3,5-dimethyladamantan-1-amine) is a primary aliphatic amine. It is a member of the adamantanes, in the same class as amantadine (Lee et al., 2016). Memantine is an *N*-methyl-D-aspartate (NMDA) receptor antagonist. The FDA approved memantine in 2003 for its use in moderate to severe Alzheimer's disease. Continuous activation of the NMDA receptors in the central nervous system caused by glutamate is thought to be partially responsible for the symptoms of

Alzheimer's disease. The pharmacological effect of memantine occurs via its activity as a non-competitive (open-channel) rapid off-rate NMDA receptor antagonist, which prevents the action of glutamate on its receptor. Memantine preferentially binds hyperactive NMDA receptors without disrupting the normally functioning NMDA cation channels (Chen and Lipton, 2006). This property allows it to act mainly in pathologically depolarized brain regions that inhibit calcium influx into cells, normally caused by chronic NMDA receptor activation by glutamate. Despite these antagonist effects, memantine has not been proven to prevent or delay neurodegeneration in patients diagnosed with Alzheimer's (Robinson et al., 2006). Due to the unique kinetics of memantine, it has excellent clinical tolerability, in contrast to other high-affinity NMDA channel blockers (Chen and Lipton, 2006).

The interest in memantine for vascular diseases follows many years of its use for dementia. Memantine has been shown to improve behavioral disturbances (decreased aggression and liability), delays cognitive decline, and improve overall mood when given at a dose of 20 mg a day as monotherapy, or alongside a Cholinesterase Inhibitor (rivastigmine, galantamine, or donepezil), in patients with moderate to severe Alzheimer's disease (Wilcock et al., 2008; Kishi et al., 2017; McShane et al., 2019). Adverse effects are rare, with a low rate of dizziness and headaches. Despite this, there are no differences between rates of treatment discontinuation between memantine and placebo groups (Kishi et al., 2017; McShane et al., 2019). There is a lack of evidence to support the use of memantine for mild Alzheimer's disease, with reviews showing that it provides no benefits in this group of patients (Schneider et al., 2011). The mechanism through which memantine improves symptoms in dementia is not fully understood, with current evidence supporting that NMDA blockade decreases the progressive death of cholinergic neurons. Through the reduction of oxidative stress, there is a decrease in the expression of amyloid precursor protein and tau proteins (Rogawski and Wenk, 2003; Dominguez et al., 2011). Evidence supports the use of memantine in other neurodegenerative diseases, such as vascular dementia, in which the NMDA blockade in the setting of chronic cerebrovascular disease improves cognitive function. The neuroprotective effect of memantine in the setting of ischemia encouraged research in other vascular diseases, such as stroke.

Memantine at high doses can reduce neuronal synaptic plasticity, which is involved in learning and memory processes. At lower concentrations, typically used in the clinical setting, memantine can enhance neuronal synaptic plasticity in the brain, improve memory, and act as a neuroprotectant against the destruction of neurons caused by excitatory neurotransmitters (Rogawski and Wenk, 2003). Memantine has a minimal activity for voltage-dependent K^+, Ca^{2+}, and Na^+ channels, benzodiazepines, dopamine, adrenergic, histamine, GABA, and glycine receptors. This drug has shown antagonist activity at the serotonin $5HT_3$ receptors. Memantine does not affect the reversible acetylcholinesterase inhibition normally caused by tacrine, galantamine, or donepezil (Kishi et al., 2017).

Memantine has a bioavailability close to 100%, reaching C_{max} within 3–8 h and a half-life between 60 and 70 h. Memantine and its metabolites are mainly excreted via the kidneys, contributing to tubular secretion. About 80% of the circulating memantine dose is present in humans as the parent compound. Memantine undergoes hydroxylation and oxidation, but CYP does not catalyze these reactions, hence a low risk for drug interactions (Noetzli and Eap, 2013).

7. Preclinical evidence of neuroprotection by memantine

Preclinical studies have shown that memantine post-stroke can decrease infarction size, increase peri-ischemic vascularity, inhibit neuronal apoptosis in the penumbral zone, decrease brain edema formation, and improve post-ischemic neurological function (**Figure 1**; Görgülü et al., 2000; Culmsee et al., 2004; Chen et al., 2017). Memantine has been proven to provide post-ischemic neuroprotection via multiple mechanisms, including inhibition of apoptosis (Tuo et al., 2021), NMDA inhibition mediated excitotoxicity (Wu and Tymianski, 2018), preserving intracellular ATP stores (Tuo et al., 2021), and increasing tissue concentration of neuron-specific growth factors (Wang et al., 2017). The magnitude of the benefit of memantine administration post-ischemia depends on how early it is administered (Seyedsaadat and Kallmes, 2018). It has also been shown that memantine can decrease the neuronal death caused by reperfusion injury; during *in vitro* studies and a preclinical model when co-administered with Recombinant Tissue Plasminogen Activator (rtPA) (Montagne et al., 2012; Liu et al., 2018). The preclinical models of the effects of memantine on ischemic stroke are mainly murine and *in vitro* cell cultures, as summarized in **Supplementary Table 1**.

This review mainly focuses on the effects of memantine administered post-stroke. Still, various *in vitro* trials have given us a more in-depth look into the molecular mechanisms of neuroprotection. *In vitro* models of ischemia have identified a dose-dependent neuroprotective effect following ischemia (Chen et al., 2017), with no neuroprotection at low dosages (memantine at 0.1 μM); and a significant attenuation of cell death following hypoxia at higher concentrations (memantine at 10 and 50 μM) (Seif el Nasr et al., 1990). The neuroprotective effects of memantine in hypoxia models are augmented when coupled with other therapies, such as memantine with an *in vitro* model of hypothermia (Landucci et al., 2018).

7.1. Memantine post-ischemic use

Culmsee et al. (2004), in a mouse model, showed that administering 20 mg/kg of memantine 5 min after stroke reduced cortical infarction size by 10%, an effect that did not carry over when administering memantine 30 min after stroke (Culmsee et al., 2004). Even so, there is evidence that memantine can decrease infarction volume when coupled with reperfusion (Kilic et al., 2013). The administration of memantine can protect against the earliest effects of NMDA-mediated excitotoxic neuronal death; this effect, however, has repeatedly failed to carry over when administering memantine 2 h after the onset of ischemia. The benefits after this time frame occur mainly in motor, sensory, and behavioral function (Seyedsaadat and Kallmes, 2018).

López-Valdés et al. (2014), showed that the administration of memantine 2 h after stroke in a mice model for 28 days did not change the volume of infarction but significantly increased forepaw sensory perception (through sensory brain mapping) and motor function (through motor cylinder test) when compared to a control. There was also an increase in peri-infarct vascularity and decreased reactive astrogliosis at 28 days post-stroke (López-Valdés et al., 2014), the latter of which, studies have suggested, can reduce inflammation

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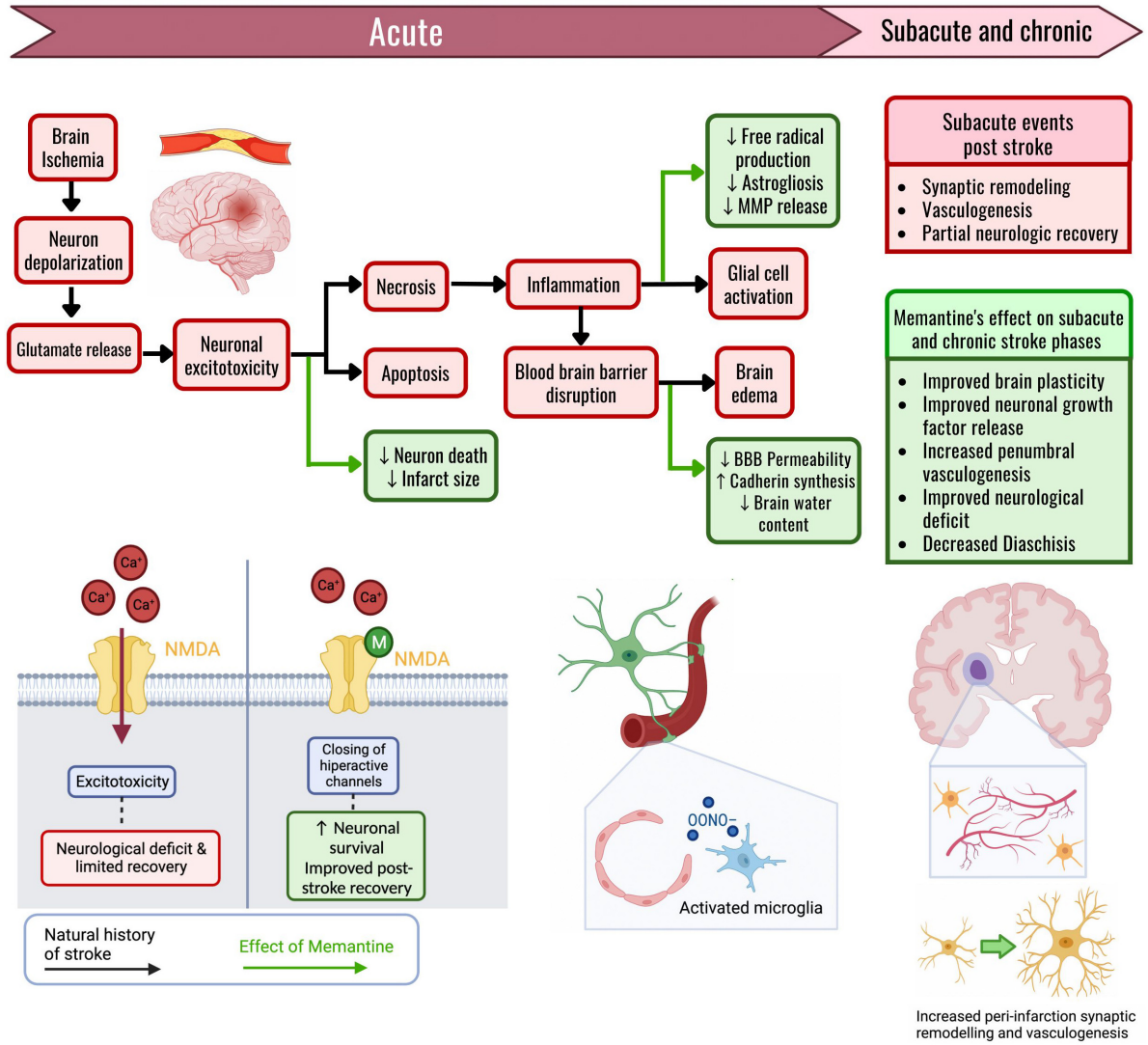

FIGURE 1
The effects of memantine on the pathophysiologic cascade of an ischemic stroke. The effects of memantine administration are marked through green arrows. When a region of the brain suffers ischemia, the neurons initially depolarize. The wave of depolarizations triggers glutamate release, and neuronal permeability to calcium increases due to the NMDA channel opening. Prolonged ischemia with increased intracellular calcium thus causes excitotoxicity, with cellular death through necrosis and apoptosis. The main effect of memantine is through the blockade of the hyperactive NMDA channels that drive excitotoxicity. Memantine given in these acute stages of ischemic stroke results in an inhibition of neuron death and causes a reduction in stroke size. Tissue necrosis triggers inflammation, disrupts the blood–brain barrier (BBB) through endothelial damage, and activates both microglia and astrocytes, causing brain edema. Memantine can reduce the activation of reactive cells, thus reducing peroxynitrite formation and MMP release, consequently helping maintain the impermeability of the BBB. The preservation of BBB permeability results in an attenuated brain edema formation. Following a stroke, in the subacute and chronic stages, there is a partial functional recovery mainly due to synaptic remodeling and peri-infarct vasculogenesis. If memantine is given during these latter stages, there is an increase in the concentration of neuronal growth factors, along with an improved capacity for synaptic remodeling. Memantine induces an increase in axonal sprouting, and an increase in peri-infarct vasculogenesis, thus giving further support to the recovering neurons around the ischemic core. Through these mechanisms, memantine improves the functional recovery that follows an ischemic stroke.

and promote functional recovery (Shen et al., 2021). Other studies have found that starting memantine 2 h after a stroke can result in a more significant improvement of neurological function at 72 h post-stroke (Aluclu et al., 2014). At this time frame, memantine use also provides a considerable mortality benefit (Kalemenev et al., 2012). It is well established that following the initial deficits in a stroke, patients tend to improve during the first few months, resulting in a partial recovery (Grefkes and Fink, 2020). When comparing neurological improvement post-stroke, it is noticeable that the initiation of memantine, 3 h after stroke, has been shown to enhance motor function when evaluated at 24 h post-stroke while decreasing anxious behavior on the 7th day after ischemia (Babu and Ramanathan, 2009).

In addition, a study by Wang et al. (2017), it was demonstrated that the use of memantine at 20 mg/kg/day for 28 days, starting at 72 h after stroke, resulted in an improvement in motor coordination (through Rotarod and tight rope tests), an increase in peri-infarct vascularity, and decreased astrogliosis in mice (Wang et al., 2017). The reduction of astrogliosis and other results that show a reduced edema formation, demonstrated that memantine attenuates the inflammatory response to stroke (Kilic et al., 2013). It was also shown that the administration of memantine resulted in fibers

from the contralesional corticospinal tract sprouting and decussating toward various ipsilesional motor nuclei, showing improved post-stroke brain plasticity. Functional motor improvement post-stroke has been shown to occur partly thanks to the formation of new tracts and synapses from neurons related to the infarcted territory (Grefkes and Fink, 2020). In this study, memantine, beginning at 72 h, did not decrease the cortical volume of infarction but reduced the striatum's secondary atrophy (Wang et al., 2017). Kim et al. (2021), also reported that memantine did not reduce the size of primary infarction but reduced secondary atrophy of the ipsilesional thalamus. The secondary atrophy of a site, following its disconnection from a territory that has suffered ischemia, is termed "diaschisis" (Zhang et al., 2012). Secondary neurodegeneration has been associated with limited recovery and the worst outcomes post-stroke (Ni et al., 1998). The beneficial effects of memantine administration are most significant when administered early, the neuroprotective effect decreases as time goes on, yet there is still a significant benefit if memantine is begun even at 72 h post-stroke (Seyedsaadat and Kallmes, 2018).

7.2. Memantine on brain edema

The loss of integrity of the blood–brain barrier (BBB), and the formation of brain edema from cytotoxic and vasogenic sources, are critical pathologic events in stroke (Michinaga and Koyama, 2015). The cerebral edema is initially cytotoxic due to transmembrane ion imbalance of Sodium and Calcium. Still, after 4–6 h, there is an increase in the permeability of the BBB, particularly in the areas around the ischemic core. A loss of tight junction proteins causes an increase in permeability, membrane damage by free radicals, and enzymatic digestion of barrier proteins (particularly the MMP family of enzymes), among others.

Memantine has been shown to reduce the formation of brain edema and decrease the levels of inflammatory mediators that increase BBB permeability (Görgülü et al., 2000). The earliest evidence of how memantine can modify the development of edema in stroke was developed by Görgülü et al. (2000). They showed that if memantine was administered 15 min after the onset of ischemia, the rat models developed less edema at the infarct periphery compared to control (measured with cerebral water content), while also decreasing BBB permeability (Görgülü et al., 2000). The decreased peri-infarct edema was also coupled with a decreased infarct volume and decreased post-stroke neurologic deficit. Kilic et al. (2013), also demonstrated that memantine decreased BBB permeability 90 min after ischemic stroke when administered alongside melatonin. By itself, memantine also decreases DNA fragmentation, and neuroinflammatory response proteins at the ischemic core; p38, MAPK, and p21 (Kilic et al., 2013). *In vitro* trials on brain endothelial cells have also shown that following ischemia, memantine helps maintain the impermeability of the endothelial monolayer, mainly through the downregulation of proinflammatory cytokines (IL-1β and TNFα) and by upregulation of the KLF2 transcription factor, which maintains the integrity of BBB through increased synthesis of occludin proteins between endothelial cells (Liu et al., 2018). Following ischemia, the release of Matrix metalloproteinases (MMPs) can acutely increase BBB permeability; Memantine has been found to decrease the amounts of MMP2 and MMP9 (Chen et al., 2016; Liu et al., 2018), preventing the breakdown of BBB collagen fibers, and also decreasing the activation of microglia. Other models of acquired brain injury have also found that memantine can ameliorate the development of cerebral edema through decreased peroxynitrite formation, increased occludin proteins, and decreased inflammatory cytokines (Chen et al., 2021).

7.3. Memantine on neurotrophic actors

A growing topic of stroke research are neurotrophic factors, and their effects on synaptic plasticity and post-stroke rehabilitation. Neurotrophins, such as BDNF (brain derived neurotrophic factor), NGF (nerve growth factor), GDNF (glial cell line-derived neurotrophic factor), among others, are responsible for synapse maturation, preservation of normal cognitive function, neurite arborization, and overall neuronal maintenance (Liu et al., 2020). Following a stroke, neurotrophins tend to decrease, in a manner correlated with stroke severity (Chaturvedi et al., 2020). The role of BDNF post-stroke has been of particular interest, having been shown to attenuate stroke-induced apoptosis, improve synaptic remodeling, stimulate neurogenesis, and improve post-stroke sensorimotor recovery (Schäbitz et al., 2007; Liu et al., 2020). Considering all of this, it is very interesting to analyze how memantine has been shown to increase BDNF in the area surrounding an infarct zone (Martínez-Coria et al., 2021); the rise in neurotrophins occurring both in ipsilesional and contralesional zones of the brain (Wang et al., 2017). These findings suggest that the neuroprotective properties of memantine and its effects on post-stroke functional recovery are due, in part, to increases in the endogenous synthesis of neurotrophic factors.

7.4. Memantine on reperfusion injury

Reperfusion injury is an essential mechanism of neuron death in both the natural history of stroke and after therapeutic reperfusion (Nour et al., 2013). An *in vitro* study by Liu et al. (2018), demonstrated that the use of memantine in a model of reperfusion results in an inhibition of the release of cytotoxic cytokines (IL-1b and TNF-a) and, at the same time, reducing both endothelial permeability and, expression of matrix metalloproteinases (Liu et al., 2018). These enzymes are involved in the neuroinflammatory response after stroke (Rosell et al., 2005). The coupling of memantine with reperfusion has been shown to decrease infarct size, improve neurological function, and improve recovery at 1 week (Aluclu et al., 2014). It has also been shown to improve safety and reduce neuronal death when co-administered with rtPA up to 4 h after stroke (Montagne et al., 2012). Reperfusion injury is an inevitable consequence of therapeutic revascularization (Cowled and Fitridge, 2011), but these results open the possibility of memantine as an adjunct drug to help ameliorate neuron injury when used along rtPA.

7.5. Memantine effects at distinct dosages

The pharmacologic effects of memantine vary between dosages and the time frame of the neurological insult in which it is administered. The unique kinetic properties of memantine result in the predominant blockade of pathologically overactive NMDA channels (Lipton, 2006). Despite the preclinical and *in vitro* evidence

supporting its neuroprotective effect at dosages between 20 and 30 mg/kg/day following an ischemic event, it should be noted that this dosage has also been documented to be neurotoxic in distinct conditions. Trotman et al. (2015), described the dose-dependent effects of memantine when given before an ischemic event, showing that low-dose memantine (0.2 mg/kg/day) can significantly reduce stroke volume and improve behavior scores, while on the contrary, if memantine is administered at high dose (20 mg/kg) for 24 h before ischemia, the size of ischemic injury is increased (Trotman et al., 2015). These findings are consistent with Creeley et al. (2006), who described how administering early memantine high dose can result in neurological functional deficits (Creeley et al., 2006). Current evidence suggests that the high-dose neurotoxic effects are not NMDA mediated but could be due to the interaction of memantine with other channels, such as serotonin 5-HT receptors (Reiser and Koch, 1989; Trotman et al., 2015). This evidence supports the idea that the inhibitory effect of memantine is potentiated when administered for more extended periods and how this effect can be deleterious at higher dosages (Bakiri et al., 2008). The non-competitive binding to NMDA channels makes it so high doses provide significant benefits in pathological conditions while also having the potential to be neurotoxic in a dose-dependent manner (Trotman et al., 2015).

8. Memantine's clinical use in stroke?

Even though little evidence is available in the literature about the impact of memantine and its specific use on stroke, some preliminary studies have started to illuminate its potential use. It is not the first time memantine has demonstrated some efficacy in vascular-related brain diseases, such as Vascular Dementia. Since memantine has been approved for moderate to severe cases of Alzheimer's, many patients with Vascular Dementia have been treated with memantine, mainly because Alzheimer's Disease cannot be ruled out and often because both conditions are comorbid (Kapasi et al., 2017). Up to date, two studies have compared memantine 20 mg/day vs. placebo in patients with mild to moderate Vascular Dementia during 28 weeks (Orgogozo et al., 2002; Wilcock et al., 2002). Results showed improved performance on cognitive scales but not on functional outcomes, such as activities of daily living (Kavirajan and Schneider, 2007; McShane et al., 2019).

Even though Vascular Dementia and stroke are both vascular diseases, their natural history of disease is different. This difference brings up the question: ¿Can memantine have any clinical benefit in patients with stroke?

Regarding patients with ischemic stroke, a small clinical trial explored the short-term outcomes of 53 patients with mild-to-moderate ischemic stroke. The control arm (29 patients) was treated based on the standard care guidelines by the American Heart Association and American Stroke Association (AHA/ASA) and compared with patients who were additionally treated with high-dose memantine (20 mg/kg three times a day) in the first 24 h after ischemic stroke disease onset, and for the following 5 days (24 patients). The outcome was measured by comparing both groups' changes in the National Institute of Health Stroke Scale (NIHSS). The memantine-treated group showed a significant improvement in the NIHSS ($p = <0.05$), hence suggesting a possible improvement in neurologic function (Kafi et al., 2014). An additional clinical trial done in 2020, that showed similar results has yet to be published (Clinical Trials.gov Identifier: NCT02535611), while another trial of memantine on stroke recovery is currently ongoing (ClinicalTrials.gov Identifier: NCT02144584). Furthermore, another interesting clinical trial also studied 77 patients with mild-to-moderate ischemic stroke and randomized into the intervention (24 patients) or control group (29 patients). Both groups were treated with standard care; the intervention group was additionally treated with 20 mg memantine every 8 h for 5 days and 20 mg once daily for the following 3 months. The measured outcomes were estimated with serum concentrations of neuronal damage biomarkers, matrix metalloproteinases (MMP)-2 and MMP 9; neurologic function was evaluated with the NIHSS and Barthel Index (BI). Five days after the intervention, results showed a significantly smaller increase in serum MMP-9 in the intervention group ($p = <0.05$), but not in the MMP-2 ($p = >0.05$). The memantine group also showed significant clinical improvement, based on NIHSS ($p = <0.05$) and BI ($p = <0.05$) during inpatient hospital care and the following days (Beladi Moghadam et al., 2021). These findings suggest that memantine may improve neurologic function and reduce brain damage by working as a neuroprotective drug.

Aphasia is a loss of the capacity to produce or understand language; cerebrovascular diseases most commonly cause it, and it is also one of the most dreaded consequences of cerebral infarction. Aphasia is a common complication of ischemic strokes, representing up to 15–38 percent of said complications (Wade et al., 1986; Pedersen et al., 1995; Berthier, 2005; Inatomi et al., 2008). Over the years, an effort has been made to target language therapy techniques that help the recovery of patients with aphasia, including constraint-induced aphasia therapy (CIAT, a high-intensity therapy that mainly focuses on restricting non-verbal communication) (Cherney et al., 2008; Szaflarski et al., 2008). In a study of 27 patients with chronic post-stroke aphasia, CIAT was beneficial when used alone and combined with memantine 10 mg twice daily. The benefit of memantine was enhanced when combined with CIAT (Berthier et al., 2009).

Unfortunately, the evidence of the use of memantine in stroke complications and recovery is minimal. Even though many questions are yet to be answered, more research is needed to provide conclusive evidence. There is currently one ongoing clinical trial exploring if memantine can enhance stroke recovery (Clinical Trials.gov Identifier: NCT02144584).

On the other hand, in an alternative randomized, double-blind clinical trial, they compared the use of Memantine and Placebo on the clinical outcome of 64 patients with Intracranial Hemorrhage (ICH). This study assessed several neurological functional scales on admission, on the seventh day, upon discharge, and 3 months after the ICH onset; this was achieved by measuring the NIHSS, BI, modified Rankin scale (mRs), and Glasgow Coma Scale (GCS). The memantine group was treated with 10 mg/day in the first month and 20 mg/day in the second and third months. Results showed a significant increase of the mean in the BI and a decrease in the mRs in the memantine-treated group compared to placebo; these results were measured from admission time until the 3 months ($P = <0.05$). No significant differences were demonstrated when analyzing mortality rate, GCS, or NIHSS score ($P = >0.05$) (Bakhshayesh-Eghbali et al., 2015). This further increases the potential therapeutic landscape suggesting that the benefits of memantine may also extend to other etiological types of stroke; however, further research needs to be done before any conclusions can be drawn. Even though fascinating

clinical evidence exists for memantine in vascular-related diseases, evidence still needs to be compelling. Further research needs to be done to discover if memantine can provide definitive clinical benefits for patients in the future.

9. Discussion

A solid amount of preclinical evidence backs up memantine as a neuroprotective agent in the setting of ischemic stroke. However, these results have not been directly replicated in a clinical setting. There have been few but significant clinical trials showing that memantine can improve the prognosis of patients post-stroke, but these trials have been limited by their small sample size. As an inhibitor of the excitotoxic cascade, memantine has been found to have excellent tolerability compared to other NMDA channel blockers, with only 2% of patients having significant side effects compared to placebo at the standard dose of 20 mg/day (Alva and Cummings, 2008). Results from clinical trials using high-dose memantine (60 mg/day) in the setting of mild to moderate stroke (NIHSS <17) show a significant benefit in the neurological function and measures of independence of patients post-stroke. The most common side effects at these dosages were nausea, with a 10–25% prevalence in intervention groups (Kafi et al., 2014; Beladi Moghadam et al., 2021). These studies support high-dose memantine as being safe and tolerable. While promising, the utility of memantine as a neuroprotective agent in conditions other than Alzheimer's disease still requires further research. More extensive clinical trials using the appropriate and effective dosages are needed before determining if memantine improves the outcomes of patients following an ischemic stroke.

10. Conclusion

Stroke is a main cause of morbimortality worldwide, and research into therapies that can improve functionality and independence post-stroke is of the utmost importance. The death of neurons in an ischemic stroke is mainly driven by excitotoxicity, resulting in an ischemic core and a penumbral zone with viable neurons. The inhibition of the excitotoxic cascade through a blockade of the NMDA channel can be achieved using memantine. This drug is a well-studied neuroprotective agent for Alzheimer's disease. The use of memantine as a therapeutic agent in ischemic stroke has been studied in various preclinical models and has shown consistent benefits. The sooner memantine was administered, the greater the benefit in preclinical experiments. On the other hand, the clinical evidence on the use of memantine in ischemic stroke is scarce but significant, showing an improvement in NIHSS and BI in patients post-stroke. Current evidence should serve as the basis for future large-scale studies to investigate if memantine indeed improves the neurological function of patients post-stroke while determining the appropriate dosage, administration route, treatment duration, and the time window in which it could be effective.

Author contributions

DP-R: conceptualization and writing—original draft preparation. PP-R and JC-B: validation and formal analysis. AS-M and JC-B: writing—review and editing. All authors read and agreed to the published version of the manuscript.

Conflict of interest

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

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Analysis of risk factors and treatment strategies for lumbar cistern blockage after craniocerebral surgery

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Objective: Lumbar cistern blockage is a common complication of continuous lumbar cistern drainage. This paper analyzes the risk factors for lumbar cistern blockage drainage due to various causes and proposes a series of prevention and intervention measures to reduce blockage or improve recanalization after blockage.

Methods: The clinical data of 637 patients with various lesions who underwent lumbar cistern drainage in our hospital were retrospectively collected and analyzed. Perioperative clinical and imaging data were assessed. Variates were analyzed using univariate and multivariate logistic regression analyses.

Results: A total of 13.7% (87/637) of patients had lumbar cistern blockage. Multivariate analysis revealed that drainage time (≥7 days), CSF volume <200 (mL/d), CSF leakage, and abnormal CSF properties were predictors of lumbar cistern blockage. Reducing the probability of lumbar cistern blockage can be achieved by repeatedly flushing, increasing the drainage flow and shortening the drainage time. The recanalization rate after blockage was 67.8% (59/87). After the drainage tube was removed, no complications related to the drainage tube occurred during the 1-week follow-up.

Conclusion: Lumbar cistern blockage is the main reason for poor drainage. Prevention or early intervention can effectively reduce the probability of blockage and achieve the purpose of drainage of cerebrospinal fluid.

KEYWORDS

lumbar cistern drainage (LCD), lumbar cistern blockage, risk factors, treatment strategies, surgery

Introduction

Lumbar cistern drainage (LCD) is used to treat intracranial infection, vasospasm after hemorrhage, intractable intracranial hypertension after craniocerebral trauma, cerebrospinal fluid leakage, and other related diseases (Wolf, 2015; Chen et al., 2017; Zwagerman et al., 2018; Badhiwala et al., 2021). It is widely used in the field of neurosurgery because of its reliable effect and obvious shortening of the course of disease. The premise for achieving therapeutic effects of lumbar cistern drainage is to maximize the drainage volume of cerebrospinal fluid and avoid complications such as headache and even cerebral herniation caused by excessive drainage volume (Sugrue et al., 2009).

The study confirmed that the drainage volume of cerebrospinal fluid (250–300 ml/d) is safe and effective (Ma et al., 2021). Insufficient drainage volume may affect the treatment effect and prolong the hospital stay. The most important reason for insufficient drainage is lumbar cistern blockage. After blockage, the cerebrospinal fluid cannot be drained smoothly or cannot flow out, which increases the treatment time. Replacing the lumbar cistern tube will increase cost for patients, and even more seriously, it may lead to intracranial infection and aggravate the condition of patients. Therefore, the goal is to prevent lumbar cistern blockage or recanalization after blockage to ensure continuous lumbar cistern drainage, and to avoid many complications after lumbar cistern drainage.

Although lumbar cistern drainage is a common and minimally invasive neurosurgery procedure, there is no systematic study on the risk factors for lumbar cistern blockage, and there are few reports on the methods of recanalization after blockage. This paper retrospectively analyzed 637 patients who required lumbar cisterna drainage after craniocerebral surgery for various reasons in our department, determined the risk factors for lumbar cisterna blockage and introduced the treatment experience of recanalization after blockage to provide guidance and reference values for clinical prediction.

TABLE 1 Demographic and clinical data.

Variable	Value (%)
No. of patients	637
Sex	
Female	329 (51.6%)
Male	308 (48.4%)
Age	
<60	277 (43.5%)
≥60	360 (56.5%)
BMI	
<24	230 (36.1%)
≥24	407 (63.9%)
Primary diagnosis	
Traumatic brain injury	187 (29.4%)
Vascular hemorrhage	213 (33.4%)
Intracranial tumor	219 (34.4%)
Brain abscess	18 (2.8%)
Causes (LD)	
Intracranial infection	307 (48.2%)
Intraventricular hemorrhage	149 (23.4%)
Subarachnoid hemorrhage	82 (12.9%)
Increased intracranial pressure	52 (8.2%)
CSF rhinorrhea/otorrhea	47 (7.3%)
Complications (LD)	175 (27.5%)
Drainage tube blockage	87 (13.7%)
CSF leakage (puncture point)	67 (10.5%)
Excessive drainage	17 (2.7%)
Drainage tube prolapse	4 (0.6%)

Materials and methods

Patient selection

A retrospective study was performed on 637 patients who underwent lumbar drainage for various reasons in the Department of Neurosurgery, Tangdu Hospital of the Air Force Medical University (Xi'an, China) from July 2012 to November 2022. We collected patient data from radiology systems and electronic medical records. The criteria for inclusion were as follows: (1) Fisher grade 3 and

TABLE 2 Baseline characteristics of the patients.

Variable	Overall	Non-blockage	Blockage	*P*
	(*n* = 637)	(*n* = 550)	(*n* = 87)	
Sex				0.306
Female	308 (48.4%)	261 (47.5%)	47 (54.0%)	
Male	329 (51.6%)	289 (52.5%)	40 (46.0%)	
Age (years)				0.876
<60	277 (43.5%)	238 (43.3%)	39 (44.8%)	
≥60	360 (56.5%)	312 (56.7%)	48 (55.2%)	
BMI				0.057
<24	230 (36.1%)	207 (37.6%)	23 (26.4%)	
≥24	407 (63.9%)	343 (62.4%)	64 (73.6%)	
Drainage time (day)				0.015
<7	351 (55.1%)	314 (57.1%)	37 (42.5%)	
≥7	286 (44.9%)	236 (42.9%)	50 (57.5%)	
CSF volume (mL/d)				<0.001
≥200	420 (65.9%)	392 (71.3%)	28 (32.2%)	
<200	217 (34.1%)	158 (28.7%)	59 (67.8%)	
CSF leakage (puncture point)				0.021
No	220 (34.5%)	200 (36.4%)	20 (23.0%)	
Yes	417 (65.5%)	350 (63.6%)	67 (77.0%)	
CSF properties				0.014
Normal	142 (22.3%)	132 (24.0%)	10 (11.5%)	
Abnormal	495 (77.7%)	418 (76.0%)	77 (88.5%)	
Causes				0.053
Others	179 (28.1%)	164 (29.8%)	15 (17.2%)	
Infection	306 (48.0%)	258 (46.9%)	48 (55.2%)	
Hemorrhage	152 (23.9%)	128 (23.3%)	24 (27.6%)	
Lesion location				0.208
Supratentorial	457 (71.7%)	400 (72.7%)	57 (65.5%)	
Subtentorial	180 (28.3%)	150 (27.3%)	30 (34.5%)	
Position				0.748
Supine	301 (47.3%)	258 (46.9%)	43 (49.4%)	
Lateral	336 (52.7%)	292 (53.1%)	44 (50.6%)	
Manufacturers				0.391
Medtronic	389 (61.1%)	340 (61.8%)	49 (56.3%)	
Branden	248 (38.9%)	210 (38.2%)	38 (43.7%)	

4 subarachnoid hemorrhage; (2) partial ventricular hemorrhage; (3) antimicrobial therapy for central nervous system infections; and (4) adjuvant treatment of cerebrospinal fluid leakage. Exclusion criteria were as follows: (1) severe increase in intracranial pressure; (2) puncture site lumbar deformity or bone destruction, resulting in lumbar puncture or catheterization difficulty; (3) dying individuals with severe systemic infections (e.g., severe sepsis), shock or on the verge of shock, and unstable vital signs; (4) cerebrospinal fluid circulatory pathway incomplete obstruction; and (5) patients with restless or abnormal mental behavior who cannot cooperate with the diagnosis and treatment. All the study procedures were approved by the ethics committee of Tangdu Hospital and followed the guidelines of the Helsinki Declaration.

Variables and data collection

All patient data were collected from the hospital electronic medical records. Follow-up data were obtained *via* telephone interviews. Clinical data, such as age, sex, BMI primary diagnosis, causes of lumbar drainage, and complications of lumbar drainage, were retrieved. Drainage time was classified as <7 days and ≥7 days. CSF volume was divided into ≥200 ml/d and <200 ml/d. CSF properties were divided into normal and abnormal. Normal CSF was defined as CSF protein levels between 120–800 mg/L, colorless, and WBC of 0–50 × 10^6/L. Abnormal CSF was defined as CSF protein ≥800 mg/L, turbid/bloody/yellow, and WBC >50 × 10^6/L. The patient's position after lumbar drainage is mainly prone or supine. Drainage tube choice manufacturer was either Medtronic or Branden.

Statistical analysis

The categorical variables were expressed in numbers (percentages), and differences were evaluated using chi square tests or Fisher's exact tests, the statistical significance level was set at $p < 0.05$. To detect risk factors associated with the incidence of lumbar drainage blockage, a univariate regression analysis was used. The risk factors with $p < 0.05$ in univariate logistic regression analysis were selected for further multiple regression analysis. The odds ratio (OR) and 95% confidence interval (CI) were calculated. All statistical analyses were performed using R software version 4.0 (R Core Team, R Statistical Computing Foundation, Vienna, Austria)[1].

Results

Overview of lumbar drainage

This study analyzed the medical records of 637 patients who underwent lumbar drainage at our institution. Table 1 shows the clinical and demographic information of these patients. The main reasons for lumbar drainage included intracranial infection (48.2%, 307/637), intraventricular hemorrhage (23.4%, 149/637), subarachnoid hemorrhage (12.9%, 82/637), increased intracranial pressure (8.2%, 52/637) and CSF rhinorrhea/otorrhea (7.3%, 47/637). Complications of lumbar drainage included drainage tube blockage (13.7%, 87/637), CSF leakage (puncture point, 10.5%, 67/637), excessive drainage (2.7%, 17/637) and drainage tube prolapse (0.6%, 4/637).

Among the 87 drainage tube blockage cases, drainage was achieved in 59 cases after recanalization by reducing the drainage height, adjusting the body position, repeatedly flushing with normal saline, achieving suction under negative pressure and pulling out part of the drainage tube, and the recanalization rate was 67.8% (59/87). Thirteen patients underwent lumbar cistern drainage again. Sixty-seven cases of cerebrospinal fluid leakage were combined with drainage tube blockage, which was pressurized and sutured through local wounds. After the drainage tube was opened, 54 cases had no cerebrospinal fluid leakage. The drainage tube was removed, and the wound was sutured in 13 cases. Excessive drainage (13 cases) is not the total amount of cerebrospinal fluid drained daily, but there is no guarantee of a continuous average drainage

1 http://www.R-project.org/

TABLE 3 Clinical risk factors for prediction of drainage tube blockage.

Variable	Univariable analysis		Multivariable analysis	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Sex (female vs. male)	0.77 (0.49–1.21)	0.306		
Age (years) (<60 vs. ≥60)	0.94 (0.60–1.49)	0.876		
BMI (≥24 vs. <24)	1.67 (1.02–2.83)	0.057		
Drainage time (day) (<7vs. ≥7)	1.79 (1.14–2.85)	0.015	1.92 (1.18–3.11)	0.008
CSF volume (mL/d) (≥200 vs. <200)	5.20 (3.22–8.57)	<0.001	5.29 (3.23–8.69)	<0.001
CSF leakage (puncture point) (no vs. yes)	1.90 (1.14–3.31)	0.021	1.96 (1.12–3.42)	0.016
CSF properties (normal vs. abnormal)	2.40 (1.26–5.08)	0.014	2.38 (1.17–4.87)	0.017
Causes (others vs. infection)	2.02 (1.12–3.85)	0.053		
Causes (others vs. hemorrhage)	2.04 [1.03;4.14]	0.053		
Lesion location (supratentorial vs. subtentorial)	1.41 (0.86–2.26)	0.208		
Position (supine vs. lateral)	0.90 (0.57–1.43)	0.748		
Manufacturers (Medtronic vs. Branden)	1.26 (0.79–1.98)	0.391		

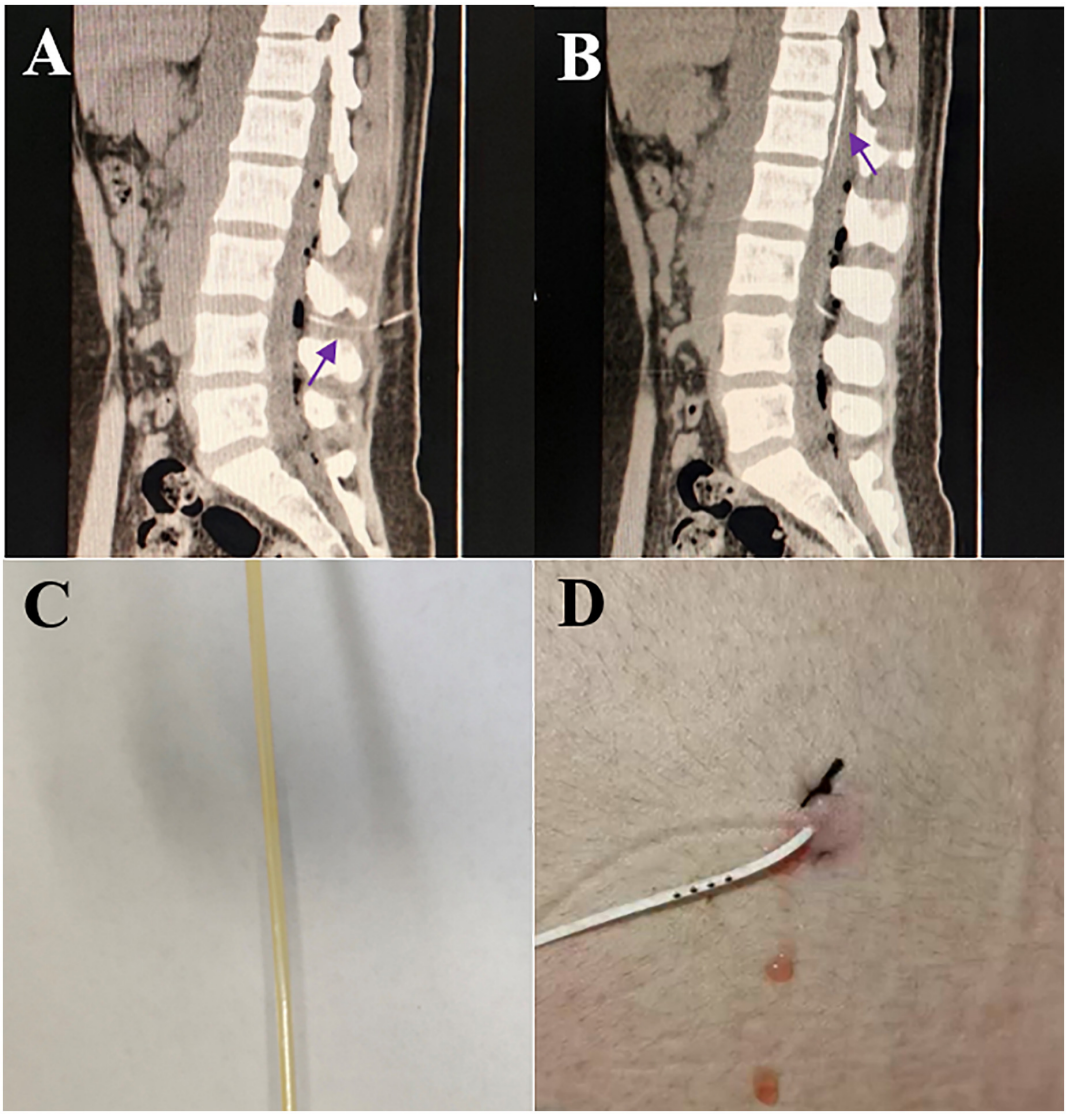

FIGURE 1
(A) The routine puncture site of lumbar cistern is between L3–4 (purple arrow); **(B)** the length of the drainage tube in the spinal canal is about 20 cm (purple arrow); **(C)** yellow, purulent cerebrospinal fluid flowing out of lumbar cistern canal; **(D)** intermittent bloody cerebrospinal fluid outflow at puncture site.

volume of cerebrospinal fluid per hour (average drainage speed <10–15 ml/h) (Nanidis et al., 2014). In some periods, the drainage speed of cerebrospinal fluid is too fast, which leads to intracranial hypotension symptoms (headache, occasional nausea, and vomiting) (Manley and Dillon, 2000; Açikbaş et al., 2002). After clamping the drainage tube, the patient improved after being placed in a supine position and infusion of normal saline. Drainage tube prolapse (4 cases) occurs when the drainage tube in the spinal canal is partially or completely pulled out of the body. After removal of the lumbar drainage tube, the patient was followed up for 1 week, and no symptoms related to the drainage tube were found.

Predictors of lumbar drainage blockage

Lumbar drainage blockage was the most important complication of lumbar drainage (13.7%, 87/637), and possible risk factors for lumbar drainage blockage were analyzed.

Univariate analysis: The incidence of lumbar drainage obstruction was higher in patients with drainage duration ≥7 days than in patients with drainage duration <7 days (57.5 vs. 42.5%, $P = 0.015$, OR 1.79, 95% CI 1.14–2.85). CSF volume <200 mL/d was significantly more often associated with lumbar drainage blockage than CSF volume ≥200 mL/d (67.8 vs. 32.2%, $p < 0.001$, OR 5.20, 95% CI 3.22–8.57). Patients undergoing CSF leakage had lumbar drainage blockage significantly more often than those without CSF leakage (77.0 vs. 23.0%, $p = 0.021$, OR 1.90, 95% CI 1.14–3.31). Patients with abnormal CSF properties had lumbar drainage blockage significantly more often than those with normal CSF properties (88.5% vs. 11.5%, $p = 0.014$, OR 2.40, 95% CI 1.26–5.08) (Table 2).

Multivariate analysis: we performed a multivariate logistic regression analysis to identify potential predictors of lumbar drainage blockage. Drainage time ≥7 days ($p = 0.008$, OR 1.92, 95% CI 1.18–3.11), CSF volume <200 mL/d ($p < 0.001$, OR 5.29, 95%

CI 3.23–8.69), CSF leakage (p = 0.016, OR 1.96, 95% CI 1.12–3.42), and abnormal CSF properties (p = 0.017, OR 2.38, 95% CI 1.17–4.87) were identified as independent and significant predictors of lumbar drainage blockage (**Table 3**).

Discussion

Continuous drainage of cerebrospinal fluid from lumbar cistern drainage is one of the most commonly used treatment techniques in neurosurgery (**Figures 1A, B**). Its main purpose is to drain bloody or contaminated cerebrospinal fluid outside the skull. It is also sometimes used to monitor and control intracranial pressure and promote wound healing of cerebrospinal fluid rhinorrhea/otorrhea. Drainage tube blockage is a common complication of continuous lumbar cistern drainage. Once the tube is blocked, the daily drainage volume of cerebrospinal fluid decreases significantly or is completely absent, which hinders the purpose of treatment. At the same time, the existence of the drainage tube increases the possibility of infection (Fried et al., 2016). We retrospectively analyzed the risk factors for lumbar cistern blockage. (1) Cerebrospinal fluid characteristics [infection (**Figure 1C**) or blood] are the main reason for lumbar cistern blockage. The protein content in the infected cerebrospinal fluid is increased, viscous infectious secretion can be seen in the cerebrospinal fluid with the naked eye, and the bloody cerebrospinal fluid is mixed with blood clots, a large amount of hemoglobin and inflammatory factors after red blood cell disintegration. These abnormal impurities are easily attached to the inner wall of the drainage tube. With the accumulation of impurities, the diameter of the drainage tube gradually narrows or is even blocked completely. (2) The leakage of cerebrospinal fluid at the lumbar cistern puncture point is an early sign of lumbar cistern blockage, which indirectly indicates that the lumbar cistern is blocked, leading to increased pressure in the spinal canal and forcing cerebrospinal fluid to flow out of the puncture point (**Figure 1D**). We compared BMI, and the thickness of subcutaneous fat at the puncture point prevented cerebrospinal fluid leakage to some extent, but there was no significant difference. The most fundamental reason is that the drainage tube is blocked, which leads to an increase in local cerebrospinal fluid accumulation in the spinal canal, and when the pressure reaches a certain point, the cerebrospinal fluid exudes from the puncture point. (3) Daily drainage volume: lumbar cistern drainage can achieve maximum drainage while ensuring safety. Studies have shown that continuous drainage of 200 ml every day is safe (Wang et al., 2013), and some studies have confirmed that the maximum drainage volume should not exceed 300 ml (Ma et al., 2021), and this needs to be comprehensively evaluated according to the characteristics of individual conditions and clinical manifestations. Ensuring continuous drainage can reduce the probability of drainage blockage by continuously flushing and pushing CSF, drainage wall-attached infectious substances, blood clots, or proteins out. (4) As the duration of drainage increased (≥7 days), the probability of drainage tube blockage increased significantly, and the probability of infection caused by catheterization also increased significantly.

The risk factors for drainage tube blockage are identified, and corresponding disposal measures should be taken according to the risk factors. In the case of infection or bloody cerebrospinal fluid, clogging is prevented by prophylactic repeated extrusion of the drainage tube or by injecting saline through a syringe and with intermittent suction. Ensuring continuous drainage of 200 ml of cerebrospinal fluid every day and a catheterization duration <7 days can effectively avoid the possibility of cerebrospinal fluid leakage and infection at the puncture point. If the tube is blocked for unknown reasons, and to ensure safety, one should reduce the height of the drainage tube (low intracranial pressure), adjust the body position (the punctured intervertebral space is forced to compress the drainage tube), pull out part of the drainage tube in the spinal canal (the side hole of the drainage tube is adsorbed to the arachnoid), and the drainage tube may recanalize.

Lumbar cisterna drainage is a routine operation in neurosurgery. At the same time, it is also a double-edged sword that can be used to shorten the recovery time of patients. If used improperly, it will cause many adverse consequences. Therefore, after catheterization of the lumbar cisterna, the daily drainage volume (200–300/ml/day) and retention time (<7 days) should be maintained in strict accordance with the guidelines or existing research conclusions. This should be performed with a relatively sterile operation and careful disposal to prevent lumbar cisterna blockage and ensure continuous drainage and achieve the purpose of disease treatment.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Tangdu Hospital Ethics Committee. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

MZ: conceptualization and writing—original draft. QT: methodology. XW, LL, and XD: writing—original draft. YQ: supervision. QC: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Interventional strategies for ischemic stroke based on the modulation of the gut microbiota

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The microbiota-gut-brain axis connects the brain and the gut in a bidirectional manner. The organism's homeostasis is disrupted during an ischemic stroke (IS). Cerebral ischemia affects the intestinal flora and microbiota metabolites. Microbiome dysbiosis, on the other hand, exacerbates the severity of IS outcomes by inducing systemic inflammation. Some studies have recently provided novel insights into the pathogenesis, efficacy, prognosis, and treatment-related adverse events of the gut microbiome in IS. In this review, we discussed the view that the gut microbiome is of clinical value in personalized therapeutic regimens for IS. Based on recent non-clinical and clinical studies on stroke, we discussed new therapeutic strategies that might be developed by modulating gut bacterial flora. These strategies include dietary intervention, fecal microbiota transplantation, probiotics, antibiotics, traditional Chinese medication, and gut-derived stem cell transplantation. Although the gut microbiota-targeted intervention is optimistic, some issues need to be addressed before clinical translation. These issues include a deeper understanding of the potential underlying mechanisms, conducting larger longitudinal cohort studies on the gut microbiome and host responses with multiple layers of data, developing standardized protocols for conducting and reporting clinical analyses, and performing a clinical assessment of multiple large-scale IS cohorts. In this review, we presented certain opportunities and challenges that might be considered for developing effective strategies by manipulating the gut microbiome to improve the treatment and prevention of ischemic stroke.

KEYWORDS

ischemic stroke, gut microbiome, fecal microbiota transplantation, probiotics, traditional Chinese medication

Introduction

Stroke is a devastating cerebrovascular disease characterized by high morbidity, disability, recurrence, and mortality. The data provided by the Global Burden of Disease (GBD) 2019 suggested that stroke is the second most common reason for death and the third leading reason for disability across the world. Also, the absolute number of first-ever stroke and stroke-related deaths has increased considerably over the last decade (GBD 2019 Stroke Collaborators, 2021). China has a greater burden of stroke, considering that the country has the highest prevalence of stroke in the world. Additionally, most of the years of life lost and disability-adjusted life years among Chinese adults are because of stroke

(Wu et al., 2019; Ma et al., 2021; Wang Y. J. et al., 2022). Stroke can be broadly classified into ischemic and hemorrhagic stroke, with ischemic stroke (IS) contributing to more than 70% of total incidences of stroke worldwide (GBD 2016 Lifetime Risk of Stroke Collaborators et al., 2018; Tuo et al., 2022). It primarily occurs due to a cerebral arterial occlusion caused by a thrombus or embolus (Tian et al., 2019; Mistry and Dumont, 2020). Besides damaging the brain parenchyma surrounding the ischemic areas, IS also triggers complex neuropathophysiological and neuropathological events followed by neuroinflammation and immune response (Pluta et al., 2021; Zhang S. R. et al., 2021). Many recent studies have suggested that post-stroke immunosuppression and intestinal barrier damage can increase the risk of opportunistic infections after IS, which can seriously worsen the outcomes of IS (Ghelani et al., 2021). These findings indicate that effective treatment of IS and the extension of the therapeutic window are challenging, and new therapeutic strategies need to be developed.

Recanalization and neuroprotection are the main approaches for treating IS in the clinic. Performing intravenous/intra-arterial thrombolysis and mechanical thrombectomy for effective reperfusion following recanalization are necessary for a positive prognosis of IS patients (Prabhakaran et al., 2015; Wu et al., 2019). The Food and Drug Administration (FDA) has only approved intravenous recombinant tissue plasminogen activator (IV rtPA) for treating IS (National Institute of Neurological Disorders and Stroke rt-Pa Stroke Study Group, 1995). Endovascular reperfusion therapy can partially improve the overall likelihood of a good IS outcome (Prabhakaran et al., 2015; Wu et al., 2019; Saver and Adeoye, 2021). However, the overall safety and efficacy are limited by a narrow treatment window (Yeo et al., 2013) of 4.5 h from the onset of the symptoms, the challenges of cerebral ischemia-reperfusion injury (Eltzschig and Eckle, 2011; Sun et al., 2018), and the tendency of hemorrhagic transformation (Gauberti et al., 2018) during the treatment course. Therefore, many researchers are investigating novel approaches for treating IS. In the past two decades, more than 1,000 potential neuroprotectants have been found to attenuate ischemic brain injury by promoting neuronal survival, neural plasticity, neurogenesis, and synaptogenesis (Liberale et al., 2018; Shen et al., 2023). However, the studies were mainly conducted on experimental IS animal models, and only a few agents targeting these molecules could be administered in the clinic (Gauberti et al., 2018; Gan et al., 2020; Mani et al., 2023). Stem cell therapy and neural progenitor cell transplantation therapy provide a regenerative strategy for protecting neural tissue in the acute phase and the replacement of lost tissues in the sub-acute or chronic phase of IS (Wei et al., 2017; Yu et al., 2019). However, this technique has numerous challenges, including identifying suitable neural progenitors, low overall survival of the neurons, and insufficient neuronal differentiation (Wei et al., 2017; Wang S. N. et al., 2020; Mani et al., 2023). Thus, the technique needs to be further improved before clinical application.

Along with the typical neurological deficit in the acute phase (Powers, 2020), more than half of the patients with IS suffer from gastrointestinal complications, including gut motility and absorption dysfunction, intestinal bleeding, gut leakiness, and enteropathogenic sepsis (Wen and Wong, 2017). After the concept of the microbiota-gut-brain axis (MGBA) was proposed, many studies confirmed the presence of a bidirectional MGBA and the potential of microbiota-directed interventions to improve stroke outcomes (Zhao et al., 2018). Detailed studies on the underlying mechanisms might provide a theoretical basis for developing novel interventions and therapeutic strategies for IS based on microbes (Cryan et al., 2019). With the advancement of high-throughput and "-omics" technologies, especially the integration of metagenomics and metabolomics techniques, a strong correlation was found between the gut microbiota and potential risk factors for the onset, progression of pathological changes and the prognosis and recovery of IS patients (Benakis et al., 2016; Nam, 2019; Pluta et al., 2021). Several studies have shown that the gut microbiota and their metabolites might play a dual role in IS (Peh et al., 2022). As the gut microbiome is less diverse in IS patients, modulating the composition of the gut microbiome might improve the prognosis of IS patients. On the other hand, consuming foods rich in choline and L-carnitine increases the occurrence of IS due to the generation of trimethylamine-N-oxide. Meanwhile, consuming dietary fiber improves the outcomes in IS patients due to the action of short-chain fatty acid metabolites containing butyrate and propionate, derived from gut microbes (Chen et al., 2019b; Battaglini et al., 2020; Peh et al., 2022).

Several effective strategies have been proposed for treating disorders related to gut microbiota in IS patients. The gut microbiota can be modulated using two ways: (1) By identifying keystone taxa in the gut microbiome and performing interventions; (2) By altering the composition of the intestinal microbiota by single or combined use of dietary interventions, antibiotics, probiotics, fecal microbiota transplantation (FMT), or traditional Chinese medication (TCM). Several studies have also suggested that repairing the damaged intestinal mucosal barrier by gut-derived stem cell transplantation might be a new treatment strategy, which could prevent the occurrence of endotoxemia and secondary infections. Therefore, in this review, we discussed intestinal microbiota as an intervention technique for treating IS to gain further insights into the emerging field of IS therapy.

Dietary interventions in IS

Diet directly affects the composition of the gut microbial communities and the production of metabolites. Cellular stress caused by unhealthy diets, such as a high intake of high-fat foods, animal byproducts, and processed foods, may influence abnormal lipid metabolism and cerebral small vessel disease, which can trigger the neuroinflammatory process and, as a result, activate a neurodegenerative cascade (Nassir et al., 2021; Flaig et al., 2023). Foods high in choline and L-carnitine, such as red meat, can be metabolized by intestinal microbiota to produce trimethylamine N-oxide (TMAO), which has been shown in experimental and clinical studies to promote the occurrence of atherosclerosis and stroke (Koeth et al., 2013; Zhu et al., 2021). Reduced reverse cholesterol transport induced by TMAO *via* gut flora-related pathways is one possible mechanism (Zhu et al., 2021; Peh et al., 2022). Meanwhile, the presence of specific bacterial species in human feces has been linked to TMAO plasma concentration and diet pattern (Peh et al., 2022). TMAO may also promote platelet hyperreactivity and thrombosis by increasing Ca^{2+} release from intracellular stores during submaximal agonist stimulus-dependent platelet activation (Zhu et al., 2016). Clinical trials

confirmed that plasma TMAO levels could independently predict the risk of thrombosis, including heart attack and stroke (Tang et al., 2013; Zhu et al., 2016; Wang M. et al., 2022). Furthermore, TMAO-mediated pathogenesis is associated with the activation of multiple inflammatory signaling pathways, which may result in oxidative stress, mitochondrial dysfunction, neuronal aging, synaptic compromise, and cognitive impairment (Praveenraj et al., 2022).

Consumption of dietary fiber and polyphenols, on the other hand, may improve stroke outcomes *via* gut flora-associated SCFAs such as butyrate and propionate (Fraga et al., 2019; Peh et al., 2022). Long-term consumption of short-term fermented soybeans (chungkookjang) containing specific Bacillus species in animal models of stroke could influence host metabolism, particularly inflammation and insulin resistance, through regulation of gut microbiota composition (increase in *Lactobacillus*, *Bacillus*, and *Akkermansia*) and metabolites (increase in propionate and butyrate), and further prevent neuronal cell death and memory dysfunction from the artery occlusion (Zhang T. et al., 2021). Nonetheless, the underlying mechanisms are unknown. In a recent study, sodium butyrate was shown to reduce neuronal apoptosis by activating PI3K/Akt *via* the G protein-coupled receptor GPR41/Gβγ in a rat model (Zhou et al., 2021).

Collectively, dietary intervention may be an appealing and valuable way to influence the course of IS.

Dietary patterns in the prevention of IS

Many studies have shown the importance of overall dietary patterns in the prevention and reduction of the occurrence of IS. Diet quality and unbalanced nutrition are risk factors that strongly increase the chances of the incidence of a first-ever stroke (O'Donnell et al., 2016), as well as the relapse of stroke and other vascular events (Amarenco et al., 2016; Yusuf et al., 2020). A study found that compared to not consuming vegetables, consuming 306–372 g of vegetables can reduce the risk of IS by 23.2%. The results indicated that vegetable consumption could effectively protect people from IS (Stanaway et al., 2022). Additionally, long-term dietary habits and the intensity of systemic inflammation were found to be strongly correlated, suggesting that the diet can modulate carotid plaque vulnerability in IS patients (Peng et al., 2020). In that study, Peng et al. (2020) calculated the dietary inflammatory index (DII) of 32 food components with a detailed questionnaire on food frequency. They found that IS patients who consumed foods with lower anti-inflammatory properties, including fruits, vegetables, and nuts, had a higher DII score and were vulnerable to plaques.

In the IS population, evaluating whole dietary patterns is more promising than evaluating individual nutrients or food components. The EAT-Lancet Commission proposed an integrated framework related to a health-reference diet based on a sustainable food system to achieve better overall health outcomes and to conform to food culture in most parts of the world. Individualizing energy intake based on body size, body composition, and physical activity levels was recommended (English et al., 2021; Wu and Anderson, 2021). English et al. (2021) evaluated the information related to the dietary patterns affecting primary and secondary stroke prevention, and they recommended that the most effective dietary strategies include following the Mediterranean diet, low sodium intake, and intake of folic acid supplements in regions with low folate. To address the complexities and the insufficient evidence directly relevant to clinical implications, well-designed randomized controlled trials need to be conducted based on appropriate dietary interventions, especially for people who have suffered a stroke.

The effects of drinks have also been investigated. According to a 16-year follow-up study, drinking water with a high concentration of calcium and magnesium (magnesium ≥10 mg/L or calcium ≥50 mg/L) is related to a lower risk of IS. The study also showed that drinking water enriched with calcium and magnesium, especially magnesium, can significantly reduce the risk of IS in postmenopausal women (Helte et al., 2022). Coffee and tea are extremely popular beverages globally and possess health benefits. A large prospective cohort study conducted with 365,682 participants from the UK Biobank showed that drinking 2–3 cups of coffee or tea per day decreased the risk of stroke by 32% during the median follow-up of 11.4 years for new onset IS. People who consumed both coffee and tea, particularly up to 3–6 cups daily, had the lowest risk of IS and vascular dementia after a stroke (Zhang Y. et al., 2021).

Dietary alteration accompanied by shifts in the intestinal metabiome

The gut microbiota encodes many carbohydrate-active enzymes. Dietary fiber and carbohydrates in the diet can be fermented to produce short-chain fatty acids (SCFAs) through these enzyme systems. Many studies have shown that SCFAs can regulate immune responses, maintain gut barrier integrity, suppress the activity of histone deacetylases, and block the cascade of inflammatory reactions (Kasahara and Rey, 2019). Sodium butyrate (NaB; an SCFA) is a histone deacetylase inhibitor generated by butyrate-producing bacteria (BPB). NaB can cross the blood-brain barrier (BBB) and lower oxidative stress in the brain, subsequently increasing the expression level of the neuroprotectant IGF-1 in peripheral tissues (Park and Sohrabji, 2016), reducing the expression of proinflammatory cytokines in the serum (Wang H. et al., 2022), and ultimately effectively decreasing brain injury after a stroke. Therefore, it can aid in neurological recovery and treat cognitive impairment following a stroke (Wang H. et al., 2022). Furthermore, when a moderate amount of fiber, butyrate, or probiotic-producing butyrate is added to the diet, the leaky gut can be repaired in IS patients (Boivin et al., 2016), and the consolidated integrity of the epithelial barrier can provide neuroprotection during stroke recovery. Also, consuming fermented dairy foods, including cheese and yogurt, which contain beneficial probiotics (Aryana and Olson, 2017), can help in the prevention and treatment of IS (Zhang K. et al., 2020) by improving the overall intestinal microbiota (Carr et al., 2021) after the living microorganisms reach the intestine.

Additionally, moderate restriction in dietary proteins and energy can provide neuroprotection by modulating the gut microbiota. In the mouse model of middle cerebral artery occlusion, the effects of a moderately low protein diet on decreasing the cerebral infarction volume and restoring neuroplasticity

were associated with higher antioxidant reactions, lower neuroinflammation, and rebalanced commensal gut microbiota in the post-acute phase (Silva de Carvalho et al., 2022). Calorie restriction was also reported to enhance post-stroke rehabilitation, which might correlate with the dramatically altered composition of the gut microbiota and its metabolism, in which *Bifidobacterium* was enriched (Huang et al., 2021). These findings might provide novel strategies for stroke rehabilitation in the clinic based on diet control and gut microbiota.

Enteral nutrition (EN) in IS

The stress status during the acute phase of stroke is characterized by high decomposition and high metabolism. It can trigger hyperglycemia, acidosis, hypoproteinemia, and negative nitrogen balance, leading to serious malnutrition, weakening the immune system, and increasing complications (Xu C. Y. et al., 2021). Many studies have proposed the concepts of immune and microecological nutrition, and the latter's role was found to be especially important. For stroke patients, early EN combined with probiotics can help in improving the nutritional status, reconstructing the gut microbiota, stabilizing intestinal barrier function, improving immune tolerance, and decreasing the complications of infection and nutritional diarrhea, thus, facilitating a more effective therapeutic intervention (Xu and Shao, 2015; Mao et al., 2022). Furthermore, systematic reviews and meta-analyses of randomized controlled trials have confirmed the efficacy of EN in IS patients (Chen et al., 2022; Savigamin et al., 2022). On the other hand, additional high-quality and well-designed randomized controlled trials are required to provide more reasonable theoretical guidance for clinical practice (Chen et al., 2022).

Administration of antibiotics in IS

Many studies have investigated the application of antibiotics to prevent post-stroke infections and improve stroke outcomes. According to some studies, post-stroke immunodepression and stress can disrupt the intestinal epithelial barrier and facilitate the spread of commensal bacteria from the host gut microbiota, causing systemic infections (Kumar et al., 2010). Infections, particularly pneumonia, commonly occur after a stroke and might contribute to neurological deficits and an increase in the mortality rate (Faura et al., 2021). Therefore, antibiotics are currently used in clinical practice to prevent infections following stroke; a common approach involves the use of broad-spectrum antimicrobial agents or combinations (Westendorp et al., 2015, 2018). Antibiotics are often administered for the early prevention and control of IS, and for patients with severe IS, broad-spectrum antibiotics are usually administered for 1 week (Meisel and Smith, 2015). However, the safety and efficacy of prophylactic antibiotics used for treating IS remain unclear. Besides their role in antimicrobial prophylaxis, antibiotic intervention can also change the composition of the intestinal microbiota and disturb the homeostasis of the microbiota for several months or even years (Langdon et al., 2016; Rizzatti et al., 2018). This might, in turn, increase the risk of infection, particularly pneumonia, as the disturbance or even eradication of the commensal bacterial communities might lead to the production of bacterial fragments, which can act as toxins and co-stimulants (Winek et al., 2016). Several studies have evaluated the necessity of administering prophylactic antibiotics to IS patients in intensive care units. Early prophylactic antibiotic treatment with ceftriaxone (cephalosporin), levofloxacin (fluoroquinolone), penicillin, and minocycline (tetracycline), most of which were prescribed within 24 h, could not reduce the occurrence of post-stroke pneumonia or the mortality rate in a longer follow-up, despite decreasing the incidence of urinary tract infections and other post-stroke complications (Zheng et al., 2017; Rashid et al., 2020; Wang Q. et al., 2022).

However, preventive antibiotic therapy at the onset of a stroke is still important. For example, the prophylactic use of antibiotics is highly efficient in specific subgroups of IS patients (Vermeij et al., 2018). Liu C. et al. (2022) showed that broad-spectrum antibiotics could decrease systemic and brain cytokine levels, decrease infarct size and perilesional cortex apoptosis, improve long-term behavioral recovery, and strongly affect the gut microbiota in rats after cerebral ischemia. Their study showed that antibiotic prophylaxis has neurorestorative benefits after IS. Their findings indicated that oral administration of non-absorbable antibiotics might strongly affect stroke pathophysiology by altering commensal gut bacteria. Benakis et al. (2020) also showed that a cocktail of antibiotics significantly decreased the infarct volume of IS mice in the acute phase. In contrast, the neuroprotective effect was abolished with the re-colonization of a wild-type gut microbiota in the model mice. They also discovered that antibiotic treatment with ampicillin or vancomycin as monotherapy, rather than neomycin, was sufficient for reducing infarct volume and improving sensory and motor function 3 days after the stroke. Furthermore, specific microbial populations, particularly Bacteroidetes S24.7, and microbial metabolites primarily containing aromatic amino acids, exerted this neuroprotective effect. These findings highlighted the preventive effects on the short-term and long-term outcomes of IS patients due to the targeted modification of the microbiome related to specific microbial enzymatic pathways following the administration of specific antibiotics.

However, further studies are needed to determine whether the administration of antibiotics can improve the outcomes of IS patients and whether antibiotics affect post-stroke infections through the intestinal flora. Also, as non-infectious inflammation comprises a significant portion of stroke-associated pneumonia due to the risk factors of dysphagia and stroke-induced immunodepression (Eltringham et al., 2020), combination therapy using antibiotics and targeted immunomodulatory agents might more effectively improve the prognosis of IS patients (Meisel and Meisel, 2011; Meisel and Smith, 2015).

Probiotics and prebiotics in IS

According to the World Health Organization (WHO), probiotics are live microbial food supplements or components of bacteria that are beneficial to humans when administered in adequate amounts (Hill et al., 2014). Several recent studies

have shown the beneficial effects of specific probiotic strains or a mixture of strains at particular life stages or disease stages. Some studies investigated the mechanism of action of probiotics in IS to elucidate how probiotics strengthen the gut epithelial barrier function, inhibit pathogen adhesion to the intestinal wall by adhering to the intestinal mucosa, suppress bacterial translocation, produce bioactive compounds, including bacteriocins, organic acids, vitamins, and neurotransmitters, reduce certain biomarkers of oxidative stress and inflammatory cytokines, produce anti-inflammatory compounds to modulate the immune system, and upregulate the expression of opioid and cannabinoid receptors in intestinal epithelial cells; thus, activating calcium-dependent potassium channels in intestinal sensory neurons (Sánchez et al., 2017; Martínez-Guardado et al., 2022). Additionally, SCFAs produced by probiotics can counteract neuroinflammation after IS (Sadler et al., 2020; Zhang W. et al., 2022) and help in repairing cognitive dysfunction and brain injury. Probiotics can also improve the negative emotions of IS patients, including anxiety and depression, 3 months after stroke (Bailey and Cryan, 2017; Liu et al., 2020). Probiotic treatment not only alters the microenvironment to limit pathological progress but also plays a complementary role by promoting the pharmaceutical management of calcium-channel blockers and statins (Liu W. et al., 2022). Combinatorial therapy with regenerative medicine, such as stem cell therapy, has also been found by some researchers to increase the level of the neurotrophic factor brain-derived neurotrophic factor (BDNF) through symbiotic treatment to enhance neurogenesis and post-stroke cognitive function. Therefore, this treatment strategy is promising and warrants further investigation (Romo-Araiza et al., 2018; Xu H. et al., 2021).

Lactobacillus and *Bifidobacterium* are probiotics that can hinder the overgrowth of opportunistic pathogens and the invasion of foreign pathogens, and thus, help in maintaining the intestinal microecological balance, lowering the apoptosis of intestinal epithelial cells due to pathogens, protecting the intestinal mucosal barrier, and improving the intestinal and systemic immune functions (Chen et al., 2022). Studies on rodent models have shown the beneficial effects of probiotic strains such as *Bacillus licheniformis* (Li Y. et al., 2021), *Lactobacillus* (Wanchao et al., 2018), and *Clostridium butyricum* (Sun et al., 2016) on stroke. The beneficial effects of prebiotics on IS have also been studied extensively (Hill et al., 2014; Gibson et al., 2017). Lactulose is an important prebiotic, which can elevate the levels of SCFAs in the intestine and serum (Bothe et al., 2017; Chen X. et al., 2020), aggravate post-stroke inflammation, and improve the functional prognosis of stroke (Yuan et al., 2021). Some studies have also shown that intragastric administration of indole-3-propionic acid (IPA) to mice with middle cerebral artery occlusion (MCAO) can restore the alterations in the structure of the gut microbiome with elevated probiotics and reduce the number of harmful bacteria, repair the integrity of the intestinal barrier, inhibit A1 reactive astrogliosis by regulating the activities of regulatory T cells (Tregs)/Th17 cells in gut-associated lymphoid tissue, and thus, efficiently alleviate the effects of neuritic impairment and brain infarction (Xie Y. et al., 2022). Prebiotics like functional barley can increase the number of butyrate-producing bacteria and promote the production of intestinal butyrate (Akagawa et al., 2021). Therefore, to better apply the synergistic and beneficial effects of probiotics and prebiotics on therapy, "synbiotics," which is a mixture of active microorganisms (probiotics) and a matrix (prebiotics), was developed (Swanson et al., 2020). Some studies have also found that the effects of probiotics on the host are not directly associated with the active microorganisms but instead are indirectly mediated by the metabolites or bacterial components of certain probiotics (Klemashevich et al., 2014; Salminen et al., 2021), such as SCFAs, which are plant polysaccharide products that are broken down by the gut microbiota (Fang et al., 2022). A study found a synergistic effect between SCFA-producing bacteria and inulin which can improve neurological deficit and behavioral outcomes post-stroke (Lee et al., 2020).

Probiotics and prebiotics are the most extensively studied biotherapeutic strategies to maintain and improve brain function *via* the MGBA (Dinan et al., 2013; Cryan et al., 2019; Martínez-Guardado et al., 2022). Probiotics and prebiotics are strong candidates for treating and preventing IS as they can reshape the gut microbiota, inhibit oxidative stress, and maintain the regular pathways related to microbial metabolism and brain functions. However, most findings and inferences in this field are based on animal studies, and only a few probiotics and prebiotics have been studied (Sarkar et al., 2016) in different combinations for their commercial availability or other physiological beneficial effects, but no study has investigated their specific properties related to the modulation of the MGBA. Therefore, future studies should focus on the mechanisms and targeted effects to improve the brain function of specific probiotic strains and prebiotics.

Fecal microbiota transplantation (FMT) in IS

Fecal microbiota transplantation is the most efficient intervention to reconstruct the gut microbiota and might be an effective therapeutic strategy for IS. A study found that FMT attenuated cerebral ischemic injury and improved neurological deficit in obese rats, which was probably mediated by the lowering of oxidative stress and apoptosis in the brain (Xie T. et al., 2022). FMT also ameliorated and/or protected transient MCAO mice from transient cerebral ischemic injury (Benakis et al., 2016). *Lactobacillus helveticus* and *Lactobacillus brevis* are the most affected microbiota in ischemia and reperfusion brain injury. Restoration of the *L. helveticus* and *L. brevis* colonies had strong neuroprotective effects. It significantly alleviated the accumulation of branched-chain amino acids (BCAAs), which aggravated microglia-induced neuroinflammation through the AKT/STAT3/NF-kB signaling pathway in the development of IS (Shen et al., 2023). Additionally, as an aged biome can increase the systemic proinflammatory cytokine levels (Spychala et al., 2018), which in turn contributes to the pathogenesis of IS, replenishing the gut microbiome with fresh microorganisms can reverse age-related poor stroke recovery through host immunologic, microbial, and metabolomic modulation.

As a key player in the MGBA, SCFAs can protect against neurodegenerative diseases by regulating the release of hormones and neurotransmitters mediated by G-protein-coupled receptors to further regulate inflammation and the mood of the patient (Fang et al., 2022). Among the known SCFAs, butyric acid showed the highest negative correlation with IS. A recent study reported that

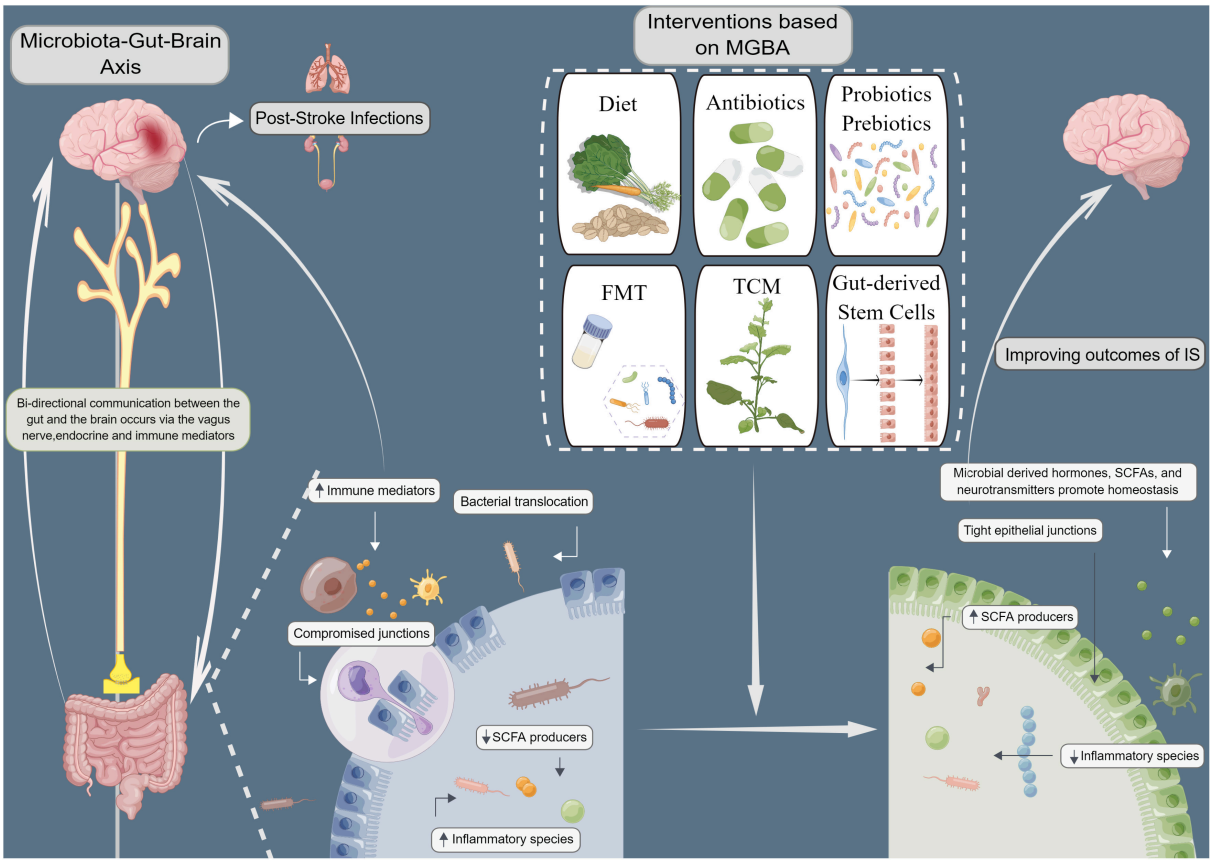

FIGURE 1
Improving ischemic stroke outcomes (IS) with microbiota-gut-brain axis (MGBA)-based interventions. Microbiological interventions, including dietary interventions, antibiotics, probiotics and prebiotics, fecal microbiota transplantation (FMT), traditional Chinese medicine (TCM), and intestinal stem cell transplantation can improve MGBA by altering microbial communities. The gut microbiome is known to be highly involved in the biosynthesis and release of various hormones, neurotransmitters, and numerous active metabolites and agents that may directly or indirectly regulate MGBA *via* neurobiological networks, immunological processes, and/or microbial metabolic signaling pathways, thereby affecting brain function and systemic inflammation. Modulation of gut microbiota composition and microbiota-derived metabolites may prevent infectious complications and improve neurological outcomes in IS patients by increasing short-chain fatty acids (SCFAs) and neurochemicals, decreasing gut permeability, reducing bacterial translocation, and alleviating immunosuppression.

administering butyrate decreased exacerbated cerebral infarction in IS associated with type 2 diabetes. The mechanisms related to this effect might include improvements in the functions of the gut barrier and the blood-brain barrier and a decrease in the serum levels of lipopolysaccharides (LPSs), LPS-binding protein (LBP), and proinflammatory cytokines (Wang H. et al., 2022). Interfering with the gut microbiota by transplanting fecal bacteria rich in SCFAs and supplementing with butyric acid could thus be an effective strategy for treating IS (Chen et al., 2019b). In a study, the researchers performed direct enrichment of selective SCFA-producing bacteria, which included *Bifidobacterium longum*, *Clostridium symbiosum*, *Faecalibacterium prausnitzii*, and *Lactobacillus fermentum*. The results showed that these SCFA-producing bacteria alleviated post-stroke neurological deficits and inflammation and increased the concentrations of SCFAs in the gut, brain, and plasma of aged mice after a stroke (Lee et al., 2020). These findings confirmed the effects of a more targeted and refined microbiome therapy.

These studies showed the beneficial effects of FMT on patients with neurological disorders. However, almost all studies were conducted on animal models. Additionally, one study conducted with an animal model for stroke also recorded an increase in mortality after FMT (Vendrik et al., 2020). As the beneficial effects of FMT are not clear, whether positive findings from animal studies can be verified in treating human diseases needs to be ascertained. Large double-blinded randomized controlled trials need to be conducted to further explain the impact of FMT in IS. In recent years, many novel therapeutic strategies targeting specific bacteria have been developed, such as phage therapeutics or multi-phage cocktail therapy, cytokine modulators, and gene therapy. These techniques are more applicable than FMT.

Traditional Chinese medicine (TCM) in IS

Besides strategies directly modulating the intestinal microbiota, drugs that influence the intestinal microbiota might be more convenient in clinical practice. TCM emphasizes the holistic concept, which is consistent with the modern view of the MGBA in stroke. In China, since the Han Dynasty period, TCM practices

TABLE 1 The summary of pharmacological effects of herbal ingredients and natural products in IS based on the intestinal microbiota.

Natural products and botanical herbal components	Effects in IS based on intestinal microbiota
Active ingredients of herbs	
Anthraquinones (Guo Y. et al., 2021)	*Lactobacillus, Bifidobacterium*↑ *Escherichia coli, Enterococcus*↓
Saponins	
Astragaloside IV (Yin et al., 2020)	*Clostridium, Blautia, Bifidobacterium, Holdemanella, Megamonas* ↑
Ginsenosides (Chen H. et al., 2020)	*Lactobacillus helveticus*↑
Panax notoginseng saponins (Li et al., 2018)	*Bifidobacterium longum*↑
Herb pair	
Chuanxiong-Pueraria (Chen et al., 2019a)	*Ruminococcaceae_UCG_004, Ruminococcaceae_UCG_005, Ruminococcaceae_NK4A214_group, Lachnospiraceae_NK4B4_group, Akkermansia, Alloprevotella, Oscillospira, Megasphaera*↑
TCM prescription	
Angong Niuhuang Pill (Zhang H. et al., 2022)	*the family Prevotellaceae, the genus Alloprevotella, the phylum Bacteroidota*↓ *the family Lachnospiraceae, the genera Lachnoclostridium, the phylum Firmicutes, Enterorhabdus, Colidextribacter, Roseburia, Lachnospiraceae_UCG-006*↑ prostaglandin I2 and uridine↑
Buyang Huanwu decoction (Liu W. et al., 2022)	*Lactobacilli, Bifidobacteria*↑ *Escherichia coli, Actinobacterium*↓
Dihuang Yinzi (Wang X. et al., 2022)	*Firmicutes, Bacteroidetes, Proteobacteria*↑
Huangqi-Honghua (Wang K. et al., 2022)	*Ruminococcaceae, Bacteroides, Phascolarctobacterium, Desulfovibrionaceae*↓ *Blautia, Lachnospiraceae, Oscillibacter, Bifidobacterium*↑ bile acid receptor FXR activated
Huazhuo Jiedu Huoxue Tongluo Prescription (Ni et al., 2022)	*Firmicutes, Bacteroidetes, Lactobacillus, Prevotella*↑ *Enterobacteriaceae, Clostridium, Enterococcus*↓
Tanhuo decoction (Guo Q. et al., 2021)	*Anaerostipes, Bifidobacterium, Blautia, Coprococcus, Gemmiger, Ruminococcus, Streptococcus*↑ *Lachnospira, Odoribacter, Eubacterium, Phascolarctobacterium*↓
Tong-Qiao-Huo-Xue Decoction (Zhang F. et al., 2020)	*Bacteroidetes, Isobacillus, Bifidobacteria*↑ intestinal barrier repaired
Xinglou Chengqi Decoction (Gao et al., 2021)	*Verrucomicrobia, Akkermansia*↑ *Paraprevotella, Roseburia, Streptophyta, Enterococcu, Bacteroidetes*↓ short chain fatty acids (SCFAs)↑
Zhilong Huoxue Tongyu capsule (Wang R. et al., 2022)	*Proteobacteria, Prevotella*↑ *Firmicutes, Bacteroidota, Lactobacillus*↓

have been passed down and evolved over thousands of years, and many classic and effective medicines have been developed for treating IS (Sun et al., 2015). Recent studies have shown that many TCM formulae and monomers exert therapeutic effects by modulating the intestinal microbiota and improving the secretion of gastrointestinal hormones (Zhai et al., 2023).

Traditional Chinese medicine can be used to effectively modulate intestinal homeostasis based on the concept of "homology of medicine and food" and the typical hepatoenteric characteristics of the pharmacokinetic profiles. Terpenoids, glycosides, flavonoids, steroids, polyphenols, and polysaccharides, among other bioactive substances found in TCM, can play distinct roles in multiple gut microbial metabolic pathways (Li X. et al., 2021). These active ingredients in the gut can reshape the structure of the intestinal microflora by increasing beneficial bacteria and decreasing harmful bacteria, thereby facilitating metabolic processes that reduce oxidative stress and inflammation after a stroke (Wang Y. X. et al., 2021).

Here, we briefly summarized the pharmacological effects of natural botanical active ingredients, TCM monomers, and compounds in the pathological state of IS based on the intestinal microbiota and their metabolites, as shown in **Table 1**. The orally administered TCM primarily interacts with the intestinal microbiota in three ways in IS patients. (1) TCM modulates gut microbiota composition; (2) TCM regulates intestinal metabolites; (3) Intestinal microbiota transforms the components of TCM and improves their metabolism, absorption, and synergism. Specifically, TCM can change the composition and structure of the gut microbiota and affect the production of gut microbiota-associated metabolites. Thus, it exerts anti-inflammatory, anti-oxidative, and immune regulatory effects, which can improve the outcome of IS. Additionally, the intestinal microbiota exerts strong effects on the metabolism of TCM through oxidation, reduction, hydrolysis, and hydroxylation reactions, which are important for improving the absorption of TCM and exerting pharmacological effects (Chen et al., 2016). These findings provide new information that might help elucidate the mechanisms through which TCM affects IS.

The benefits of TCM for treating IS based on gut microbiota may be associated with reshaping the gut microenvironment, weakening of bacterial flora translocation, and an increase in probiotics to reduce cerebrovascular damage (Zhang H. Y. et al., 2021). To develop more effective TCM for treating IS, novel gut microbiota sequencing technologies must be used to investigate the gut microbiota for more accurately and precisely assessing the regulatory impact of TCM, as well as to establish more standardized and unified stable IS animal models for determining TCM impact. Furthermore, in various IS models, including rodents and large mammals, the long-term protective effects of TCM on the brain and survival rate and the mechanism of regulating intestinal flora must be determined. Also, the current pharmacokinetics, pharmacodynamics, and toxicological characteristics of TCM require more attention.

Acupuncture treatment at different acupoints, such as Quchi and Zusanli (Ke et al., 2022), is an efficient therapy for IS. It is extensively practiced in China and has also been accepted in other countries and regions in recent years. The mechanism of action of acupuncture might be associated with its effects on intestinal microecology and plasma metabolism. It might influence

Turicibacter, isoflavones, phytoestrogen metabolites (Xian et al., 2022), and IPA levels (Li et al., 2022). Additionally, the combination of acupuncture and TCM might have synergistic effects, which might further enhance the recovery of IS when administered together.

Intestinal epithelial stem cell transplants (gut-derived stem cells) in IS

Several studies have shown an association between a leaky gut and alterations in gut microbiota in patients with IS (Huang and Xia, 2021; Zhang W. et al., 2022). The leaky gut hypothesis suggests that the increase in gut permeability might cause inflammatory cytokines and toxic gut metabolites to pass through the compromised intestinal epithelial barrier. The resultant endotoxemia and bacterial translocation can aggravate gut hemorrhage, gut dysmotility, intestinal paralysis, bowel incontinence, and even gut-origin sepsis, along with neurological impairment and a series of secondary injuries after IS (Larochelle et al., 2022; Zhang W. et al., 2022). Therefore, the intestinal epithelium needs to be repaired for the recovery of the patient after a stroke. Stem cell therapy and organoid techniques are novel strategies for gut remediation (Shaker and Rubin, 2012). Mani et al. (2023) showed that the gut is a critical therapeutic target for stroke. They engrafted organoids containing intestinal epithelial stem cells (IESCs) from young rats into older model rats that suffered a stroke. They found that the transplanted IESCs incorporated into the gut restored gut dysbiosis caused by the stroke and decreased intestinal permeability, which reduced the circulating levels of endotoxin LPS and the inflammatory cytokine IL-17A. They also discovered that IESC transplantation improved stroke-induced acute (4 day) sensory-motor disability as well as chronic (30 day) cognitive-affective function. The findings emphasized the importance of early intervention in the acute stage of stroke and transplantation of IESCs from young people. However, no clinical studies on the efficacy of gut-derived stem cells in the treatment of IS have been reported in the literature to date. In the future, it will be critical to investigate donor selection, the mechanisms underlying cell engraftment, and regimens to maximize transplant efficiency. Therefore, further investigation is needed to optimize the transplantation time, dose, and route to apply gut stem cell therapy in the clinic.

Summary

The gut shows an early response to stroke, and changes in the gut occur simultaneously with stroke-induced hyperpermeability of the BBB. After the concept of MGBA was proposed, several studies showed the high clinical application value of the approaches targeting intestinal microbiota in the treatment of IS. The gut microbiota can influence the metabolic status of the body besides exerting strong effects on blood pressure, blood glucose, and atherosclerosis, all of which are risk factors for IS (Wang J. et al., 2022). A detailed study of the physiological functions of the gut microbiota and gut microbiota disorders associated with the central nervous system might provide new ideas for preventing and treating IS. Additionally, several studies have also investigated the development of the dietary intervention, antibiotics, probiotics and prebiotics, FMT, TCM, as well as gut-derived stem cells for the microbiome-based treatment of IS (**Figure 1**). However, intestinal microbiota-targeted treatment of IS needs further improvement. Large-sample multicenter studies with long-term follow-up need to be conducted to verify the benefits. Identifying specific species of pathogenic bacteria, optimizing targeted regimens, and combining therapies can greatly contribute to the advancements in treating IS.

Author contributions

QL and XL contributed to the conception and design of the study. JW wrote the first draft of the manuscript. JW, QL, and XL wrote sections of the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Research hotspots and frotiers of stem cells in stroke: A bibliometric analysis from 2004 to 2022

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Background: Stroke is one of the leading causes of mortality and permanent disability worldwide. However, the current stroke treatment has a limited effect. Therefore, a new treatment is urgently needed. Stem cell therapy is a cutting-edge treatment for stroke patients. This study aimed to gain better understanding of global stem cell trends in stroke *via* a bibliometric analysis.

Methods: We used the Web of Science Core Collection to search pertinent articles about stem cells in stroke published between 2004 and 2022. Analysis was conducted using CiteSpace, VOSviewer, and the R package "bibliometrix" to identify publication outputs, countries/regions, institutions, authors/co-cited authors, journals/co-cited journals, co-cited references, and keywords.

Results: A total of 6,703 publications were included in the bibliometric analysis. The total number of citations significantly and rapidly increased between 2004 and 2022, with the most pronounced growth pattern observed in the period of 2008–2009. In terms of authoritarian countries, the USA had the most publications among the countries. As for institutions and authors, the most prolific institution was the University of South Florida, followed by Oakland University and then Shanghai Jiao Tong University, and Chopp, M. and Borlongan, Cesario V, had the most output among the authors. Regarding the journals, *Cell Transplantation* had the highest publication, followed by *Brain Research*. As for references, "Mesenchymal stem cells as trophic mediators" was the most frequently cited (2,082), and the article entitled *Neuronal replacement from endogenous precursors in the adult brain after stroke* had the strongest burstiness (strength = 81.35). Emerging hot words in the past decade included "adhesion molecule," "mesenchymal stromal cell," "extracellular vesicle," "pluripotent stem cells," "signaling pathway," "plasticity," and "exosomes."

Conclusion: Between 2004 and 2022, the terms "neurogenesis," "angiogenesis," "mesenchymal stem cells," "extracellular vesicle," "exosomes," "inflammation," and "oxidative stress" have emerged as the hot research areas for research on stem cells in stroke. Although stem cells exert a number of positive effects, the main mechanisms for mitigating the damage caused by stroke are still unknown. Clinical challenges may include complicating factors that can affect the efficacy of stem cell therapy, which are worth a deep exploration.

KEYWORDS
stem cells, stroke, citespace, VOSviewer, web of science, bibliometrics

1 Introduction

Stroke is currently the third major cause of adult disability and the second leading cause of mortality worldwide (Owolabi et al., 2022). Following a stroke, the brain may be damaged by neuronal apoptosis, oxidative stress, and cytotoxic cascade reactions (Kuriakose and Xiao, 2020). Stem cell therapy, an emerging treatment option for stroke, has the potential to improve neurological outcomes and functions by promoting neurogenesis, reducing oxidative stress, and decreasing cytotoxicity (Zhao et al., 2022). At present, many stem cell types have been shown to be effective in treating stroke, such as pluripotent stem cells (Duan et al., 2021), neural stem cells (NSCs) (Zhang et al., 2017), embryonic stem cells (Xia et al., 2021), and mesenchymal stem cells (MSCs) (Bedini et al., 2018). In addition, many studies have demonstrated the capacity of these stem cells for brain rewiring (Pluchino and Peruzzotti-Jametti, 2013), neoangiogenesis (Nam et al., 2015), inflammatory inhibition (He et al., 2021a), and nerve regeneration (Ould-Brahim et al., 2018). Scholars have published a plethora of basic research and clinical trials on stem cell therapy in stroke, but new and comprehensive quantitative evidence to support the direction and research hotspots in this field is limited. Thus, it is necessary to review the development of research on stem cells in stroke from 2004 to 2022 and to present an objective analysis based on data from publications as a foundation for future study.

Bibliometric analysis is a statistical method for forecasting knowledge structure and hotspots within a certain field of study through visual representations (Ninkov et al., 2022). By reading this kind of study, readers may be able to obtain quantitative information on how journals are distributed by nation, organization, author, and journal in a specific field (Zhang L. et al., 2022). Bibliometric analysis provides unambiguous insights into many medical areas (Kokol et al., 2021). However, bibliometric studies conducted in the field of stem cells in stroke are scarce. As a result of the dramatic increase in stem cell research and publications over the past several years, the necessity to integrate and renew research data in a bibliometric analysis on stem cells in stroke has arisen.

As a response to the paucity of quantitative analysis of research regarding stem cells in stroke, the present study acquired global scientific research on stem cells in stroke between 2004 and 2022 with quantitative information on the publication outputs, countries/regions, institutions, authors/co-cited authors, journals/co-cited journals, co-cited and burst references, keywords, and burst keywords. This study aimed to highlight hotspots for study in this area by synthesizing research direction and emergent themes from these investigations.

2 Methods

2.1 Search strategy and data acquisition

The Web of Science (WoS) contains 20,000 reputable academic publications that span 250 different fields worldwide (Zhong and Lin, 2022). Other academic researchers in the field of bibliography have used the WoS as the most trustworthy data source for data extraction in bibliometric analysis (Dong et al., 2022).

We conducted a comprehensive literature search using the Web of Science Core Collection (WoSCC) database from 1 January 2004 to 11 August 2022. To obtain as comprehensive and accurate results as possible, the search strategies we used were TS=(stroke OR apoplexy OR "cerebrovascular accident" OR "cerebral hemorrhage" OR hematencephalic OR encephalorrhagia OR "cerebral ischemia") AND TS=("Stem Cells" OR "Cell, Stem" OR "Cells, Stem" OR "Stem Cell" OR "Progenitor Cells" OR "Cell, Progenitor" OR "Cells, Progenitor" OR "Progenitor Cell" OR "Mother Cells" OR "Cell, Mother" OR "Cells, Mother" OR "Mother Cell" OR "Colony-Forming Unit" OR "Colony Forming Unit" OR "Colony-Forming Units" OR "Colony Forming Units"). Only articles and reviews were included. Furthermore, letters, commentaries, meeting abstracts, and other types of documents were excluded. Finally, 6,703 records were included for analysis. The specific literature screening process is presented in Figure 1.

2.2 Data analysis

The original data downloaded from the WoSCC were firstly imported into Microsoft Excel 2016, and then two authors (QZh and YZ) independently screened the final included articles and collected all data from the final papers that were included, such as titles, authors, keywords, institutions, countries/regions, citations, journals, and publication dates. Subsequently, the processed data was imported to VOSviewer (version 1.6.15), CiteSpace (version 5.8), and R package "bibliometrix" for bibliometric analysis.

CiteSpace is a bibliometric software that enables the analysis and visualization of trends and patterns in a research area (Pan et al., 2018; J; Zhang and Lin, 2022). It also creates a knowledge map of connected fields, clearly presents the panoramic information of a particular knowledge field, and identifies the critical studies, hot research, tendency, and frontiers of a specific scientific field using a variety of dynamic network analysis techniques (Godfrey et al., 2018; Y; Chen, Lin, and Zhuang, 2022). CiteSpace was used in this study to conduct co-occurrence and cluster analyses of authors, research institutions, nations, and discipline features. The parameters of CiteSpace were set as follows: in the Time Slicing column time settings 2004.01–2022.08, each year is a time slice.

The Leiden University Center for Science and Technology Studies (CWTS) created VOSviewer, a software for creating and analyzing bibliometric networks (Netherlands). VOSviewer can extract bibliographic networks (co-authorship, co-occurrence, and citation-based) from bibliographic data (Lin, Chen, and Chen, 2020; Moral-Muñoz et al., 2020; Luo and Lin, 2021). In this study, co-occurrence and cluster analyses of authors, research institutions, countries, and discipline features were conducted using CiteSpace.

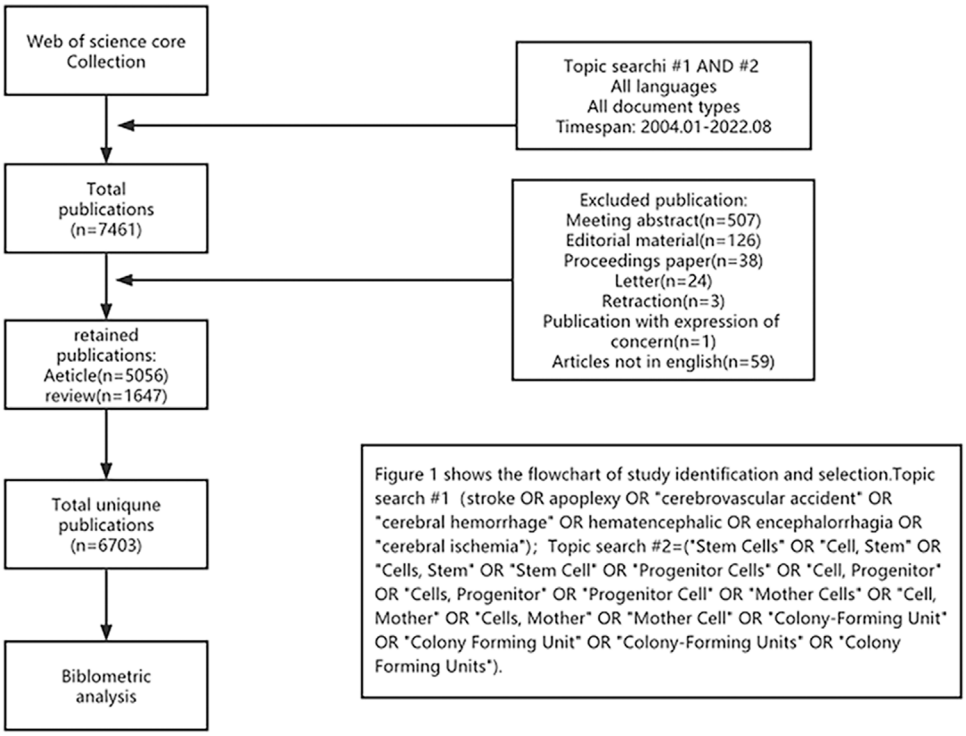


FIGURE 1
Flow chart of the screening process for research on stem cells in stroke.

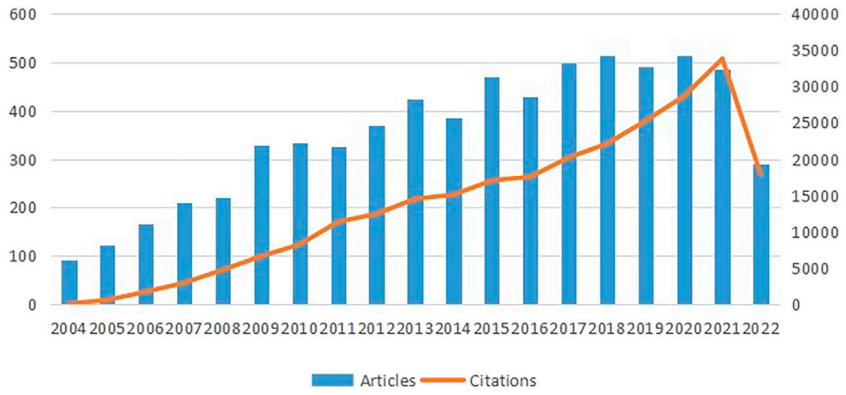


FIGURE 2
Trends of annual publications on research of stem cells in stroke. The data for 2022 is not complete.

Bibliometrix (https://www.bibliometrix.org) is an open-source R package developed by Dr. Massimo Aria and Corrado Cuccurullo from Naples University in Italy. It is capable of conducting comprehensive bibliometric and scientometric analyses (Moral-Muñoz et al., 2020). In this study, bibliometrix was used to create a global distribution network of articles on stem cells in stroke and to analyze the thematic evolution of those publications (Aria and Cuccurullo, 2017).

3 Results

3.1 Temporal trend of publication outputs

As can be seen from Figure 2, the histogram and curves exhibit two trends: the total number of papers published and citations per year. Both trends grow throughout time, illustrating the direction in which research in this area is moving. The number of citations

TABLE 1 Top 10 countries/regions with highest publications on stem cells in stroke.

Rank	Countries/Regions	Total link strength	Count (%)
1	United States	1220	1555 (25.61%)
2	China	585	738 (12.15%)
3	Japan	497	714 (11.76%)
4	Germany	426	342 (5.63%)
5	South Korea	303	280 (4.61%)
6	England	271	271 (4.46%)
7	Canada	244	249 (4.10%)
8	Italy	243	224 (3.69%)
9	Spain	238	220 (3.62%)
10	Taiwan	216	216 (3.56%)

significantly and rapidly increased between 2004 and 2021, suggesting that research on stem cells in stroke has attracted interest. From 2004 to 2007, the number of literature grew rapidly, and in 2009, it dramatically increased. However, the number of articles remained relatively stable from 2010 to 2017. The year 2020 had the most number of publications in recent years, which peaked at 515. Although the data for 2022 have not yet been completed, it is predicted that they will exhibit a moderate trend compared with those in the previous year.

3.2 Contributions of countries/regions

As for the geographical distribution, 6,703 documents were published from 94 different countries and regions; Table 1 presents the top 10 countries/regions in this category. As can be seen from Figure 3B and Supplementary Figure S1, we also used VOSviewer for the visual analysis of countries or regions. The USA published the most papers (1,555papers, 25.61%), followed by China (738 papers, 12.15%) and then Japan (714papers, 11.76%), indicating that these three countries play a crucial role in this field. The quantity and connections among publications in each nation were then used to create a collaborative network (Figure 3A). A country collaboration analysis was conducted on the 30 countries with the highest number of publications in this area (Figure 3C). According to the total link strength, the top five countries/regions were the USA, China, Japan, Germany, and South Korea.

3.3 Contributions of institutions

In Table 2, the top 10 institutions with the highest productivity are ranked by their productivity. China and the USA were the only two countries where the highest productivity were located. The most prolific institution was the University of South Florida (164 publications, 2.70%), followed by the Oakland University (151 publications, 2.49%) and then the Shanghai Jiao Tong University (129 publications, 2.12%). The clustering analysis of institutions is presented in Figure 4A. A tight and continuous interaction between institutions can also be observed. Among them, the institutions with more collaborations were Oakland University, Henry Ford Hospital, and Henry Ford Health Science Center, followed by Sapporo Medical University and Yale University. In Figure 4B, we analyzed the data of articles published in the last 5 years using VOSviewer. For instance, Harvard University started research in the field earlier and had published significantly more articles in the past than it had recently. Contrarily, Capital Medical University entered the field later and has recently published a higher number of articles. As can be seen from the cluster analysis figure, the red and light blue circles indicate mainly Chinese institutions. Combined with the visual timeline, it can be seen that Chinese institutions are predominantly yellow, indicating that they entered the field late or have recently published a high number of articles.

Figure 4C presents the publication trend in this field by different institutions over time. The University of South Florida accounted for more than half publications from 2004 to 2006 and then gradually declined; however, the University of South Florida remained in the leading position, demonstrating the remarkable contributions of this institution to this sector. The rest of the institutions exhibited an upward trend in the publication quantity. Interestingly, Shanghai Jiao Tong University has made the most significant progress. Since 2018, Shanghai Jiao Tong University has, unsurprisingly, ranked first among the 10 institutions in terms of publication ratio. We also used CiteSpace for the visual analysis of institution clusters and marked them with keywords (Figure 4D). Consequently, the Johns Hopkins University, Massachusetts General Hospital, and National University of Singapore exhibited high centrality. The largest cluster in Supplementary Figure S1 was designated “ischemic stroke” (cluster #0), indicating that various institutions are most concerned with this word. It was followed by “clinical trials” (cluster #1), “primate” (cluster #2), and “extracellular matrix” (cluster #3), respectively. Other important clusters were “mesenchymal stem cell,” “neurological function,” and “hypothermia.”

3.4 Authors and Co-cited authors

Approximately 174 writers contributed to a total of 6,703 articles. The most prolific author was Chopp, M. who produced 160 articles (2.63%), closely followed by Borlongan, Cesario, V who produced 143 publications (2.35%). Three authors published 50 and more articles (Zhang Zheng Gang, Yang Guo-Yuan, and Kokaia Zaal), and five authors published 40 and more articles (Hermann Dirk M. Chen Jieli, Lindvall Olle, Sanberg P. R. and Kaneko Yuji) (Table 3). Information on co-authors and co-cited authors was also analyzed using VOSviewer (Figures 5A, B). Figure 5A shows that there are primarily two research teams involved in author collaboration, led by Chopp, M. and Borlongan, Cesario, V, who frequently and closely collaborate with other authors. Co-cited authors are those who have had two or more of their names concurrently mentioned in one or more subsequent articles and who are therefore considered to have a co-citation connection. A total of more than 1000 citations have been received by the top six authors among the top 10 co-cited

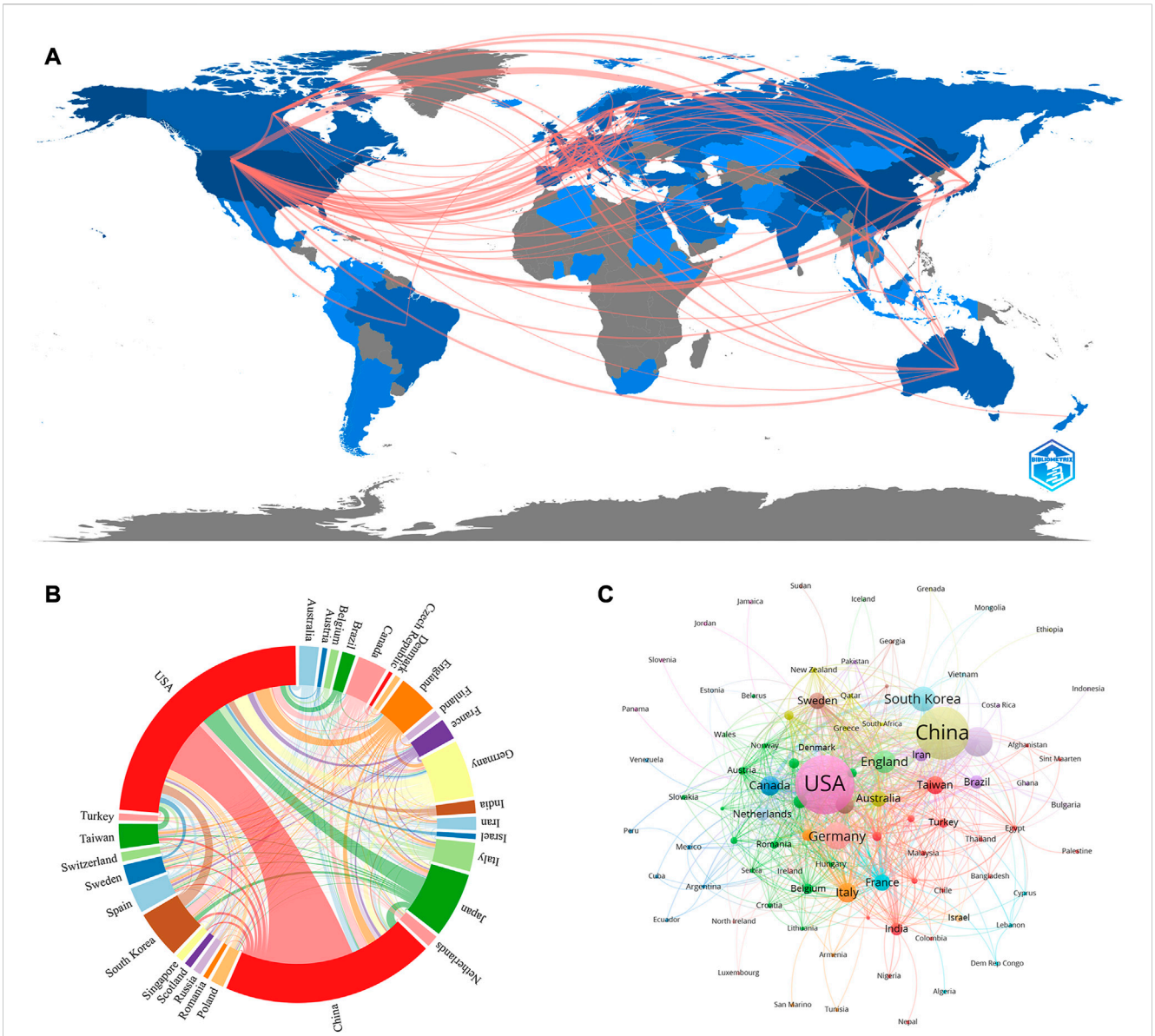


FIGURE 3
(A) Country/region collaboration map. The Darker blues indicated higher collaboration rates, the wider the link line, the higher the rate of collaboration between the two countries. **(B)** Distribution of countries/regions in the field of stem cells in stroke. The size of the circle represents the number of articles issued by countries or regions, and the line means cooperation among countries or regions **(C)** Distribution and international cooperation of countries that are involved in stem cells in stroke research. The thickness of the line reflects the frequency of the cooperation. The thicker the line, the stronger the cooperation.

writers (Table 3). The most frequently referenced author was Chen Jl. (n = 2,214), followed by Jin, Kl (n = 1,473), Li, Y (n = 1,367), Zhang, RI (n = 1,313), Borlongan, Cv (n = 1,144), and Avidsson, A (n = 1,003).

3.5 Journals and Co-cited academic journals

We found that 252 journals published 6,703 papers regarding stem cells in stroke. As can be seen from Table 4, it is clear that the journal *Cell Transplantation* has the most papers (172, 2.83%), followed by *Brain Research* (160, 2.63%). Among the top 10 journals, *Stroke* (10.17) has the greatest impact factor (IF). The number of times the top 10 most co-cited journals are cited determines their influence. As presented in Table 4, the publication with the most citations is *Stroke* (19,776), indicating that it has a significant impact in this category, followed by the *Journal of Neuroscience* (14,061) and *Proceedings of the National Academy of Sciences of the United States of America* (11,769).

Using VOSviewer, we conducted a visual analysis of the published journals and obtained details about journal collaboration through Figures 6A, B. We could see that the journals of *Stroke*, *Archives of Physical Medicine and Rehabilitation*, and *Neurorehabilitation and Neural Repair* had

TABLE 2 Top 10 institutions related to stem cells in stroke.

Rank	Institution	Count (%)	Country	Institution	Total link strength
1	Univ S Florida	164 (2.70%)	United States	Oakland Univ	182
2	Oakland Univ	151 (2.49%)	United States	Henry Ford Hosp	133
3	Shanghai Jiao Tong Univ	129 (2.12%)	China	China Med Univ	114
4	Henry Ford Hosp	107 (1.76%)	United States	Harvard Univ	102
5	Fudan Univ	102 (1.68%)	China	Univ S Florida	101
6	China Med Univ	97 (1.60%)	China	Shanghai Jiao Tong Univ	91
7	Capital Med Univ	96 (1.58%)	China	Massachusetts Gen Hosp	88
8	Stanford Univ	91 (1.50%)	United States	Seoul Natl Univ	87
9	Sun Yat Sen Univ	85 (1.40%)	China	Univ Pittsburgh	79
10	Johns Hopkins Univ	79 (1.35%)	United States	Univ British Columbia	79

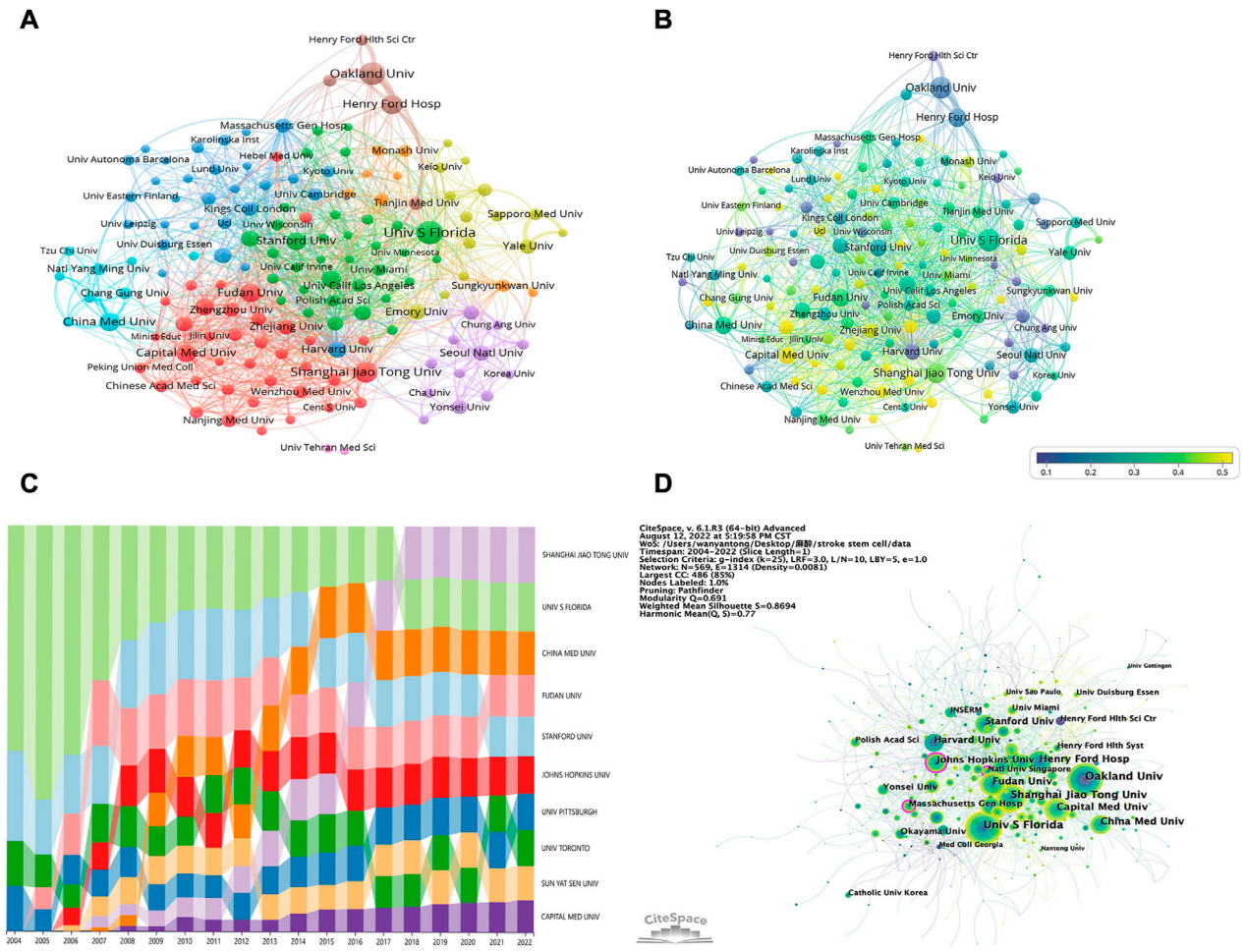


FIGURE 4
(A) Institutions clustering analysis. The node size signifies the number of publications of institutions, and the thickness of the line signifies the strength of cooperation among institutions; node colors signify different clusters. **(B)** Timeline visualization of collaboration among institutions. The blue to yellow gradient represents the number of articles published in the last 5 years as a percentage of the total number of articles published by the organization **(C)** Trends of publications in this field by different institutions over time. Different colors represent different institutions, the high and low positions represent the ranking of the institution's publication quantity, and the width represents the proportion of the institution's publication quantity among the top 10 institutions' publications of the year **(D)** Institutions related to stem cells in stroke. The larger the circle, the more articles the institution posts. The purple circle represents centrality, and the line means cooperation among institutions.

TABLE 3 Top 10 authors and co-cited authors related to stem cells in stroke.

Rank	Author	Count (%)	Total link strength	Author	Co-citations	Total link strength
1	Chopp, M.	160 (2.63%)	392	Chen, Jl	2214	36563
2	Borlongan, Cesario, V	143 (2.35%)	232	Jin, Kl	1473	29645
3	Zhang, Zheng Gang	57 (0.94%)	169	Li, Y	1367	22711
4	Yang, Guo-Yuan	52 (0.86%)	89	Zhang, Rl	1313	25956
5	Kokaia, Zaal	50 (0.82%)	77	Borlongan, Cv	1144	18474
6	Hermann, Dirk M.	45 (0.74%)	86	Arvidsson, A	1003	17582
7	Chen, Jieli	42 (0.69%)	148	Zhang, Zg	879	15015
8	Lindvall, Olle	42 (0.69%)	69	Parent, Jm	701	13781
9	Sanberg, P. R.	42 (0.69%)	64	Lindvall, O	646	10025
10	Kaneko, Yuji	40 (0.66%)	112	Savitz, Si	643	12778

more times of co-citation and greater influence. We also conducted comparative analysis of the journals' popularity, as presented in Figure 6C. Through this heat map, we can understand the change in the research direction and emphasis in this field and grasp the development trends. We found that in recent years, the popularity of *NEUROSURGERY*, *CURRENT NEUROVASCULAR RESEARCH*, and *PANS* had gradually decreased, whereas that of *CELLS*, *INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES*, and *FRONTIERS IN CELLULAR NEUROSCIENCE* had gradually increased. One of the interesting things about *STEM CELL REVIEWS AND REPORTS* is that its popularity had declined year by year, but in the last 2 years, it had returned to its level in 2004. Furthermore, on the dual-map overlay of journal publishing research (Figure 6D), we found four citation paths (colors orange, pink, and green), demonstrating that the studies published in molecular/biology/genetics journals and health/nursing/medicine journals were mainly cited by the studies published in molecular/biology/immunology, medicine/medical/clinical, and neurology/sports/ophthalmology journals.

3.6 Co-cited reference and reference bursts

The top 15 documents that were cited the most often out of the 6703 retrieved are listed in Table 5. *Mesenchymal stem cells as trophic mediators* was the most frequently cited (2,082), which is a review of studies on the applications of adult marrow-derived MCSs. It was followed by *Concise review: Mesenchymal stem cells: Their phenotype, differentiation capacity, immunological features, and potential for homing* (1,725) and then *Adult mesenchymal stem cells for tissue engineering versus regenerative medicine* (1,378).

When two or more articles are simultaneously cited in the same article, the relationship between the simultaneously cited articles is referred to as co-citation. As presented in Figure 7A, we also used the CiteSpace clustering function to create a visual map to cluster the co-citation literature, and the collected literature was separated into 11 clusters *via* cluster analysis. Each cluster was intimately connected to the others and worked together in specific areas. The weighted mean silhouette and modularity Q were 0.8694 and 0.691, respectively, demonstrating the stability, believability, and persuasiveness of the clustering structure. Figure 7A illustrates the time dimension by the color of circle changing from purple to yellow, which also shows the change in the direction and concentration of the research. While the "hippocampus" cluster had received more attention in the past, researchers recently turned their attention to "exosomes," "hydrogels," and "ischemic stroke." The name of the biggest cluster was "subventricular zone" (cluster #0), which was followed by "transplantation" (cluster #1), "ischemic stroke" (cluster #2), and "hydrogel" (cluster #3). The "hippocampus," "neurogenesis," "bone marrow stromal cells (BMSCs)," and "exosomes" clusters were other significant groups that may have represented a turning point in some sense.

As shown in Figure 7B, we created a visual map to cluster the cited literature over the last 3 years and divided them into 21 clusters through cluster analysis using CiteSpace. The weighted mean silhouette and modularity Q were 0.8729 and 0.7518, respectively, demonstrating the stability, believability, and persuasiveness of the clustering structure. The Figure 7B can help us get the latest research hotspots. The largest cluster was "cell treatment" (cluster #0), which was followed by "exosomes" (cluster #1), "biomaterials" (cluster #2), and "ischemic stroke" (cluster #3). Additional significant clusters were "adult neurogenesis," "microglia," "intracerebral hemorrhage," and "pericytes." Combined with Figure 7A, the "hippocampus" cluster has received more attention in the past, researchers have recently turned their attention to "exosomes," "hydrogels," and "ischemic stroke." In Figure 7C, the top 25 references are listed in chronological order, which have the greatest burst intensity. References that receive several citations over a period of time are known as "citation burst" references. We set the time period in CiteSpace to 2004–2022 and still kept references with a burst termination date of 2022. *Neuronal replacement from endogenous antecedents in the adult brain following stroke* by Andreas et al. was published in *Nature Medicine* in 2002, and it had the strongest burstiness (strength = 81.35), occurring from 2004 to 2007 (Arvidsson et al., 2002). The advantages and disadvantages of the top 25 articles with the strongest citation bursts are summarized in Supplementary Table S1.

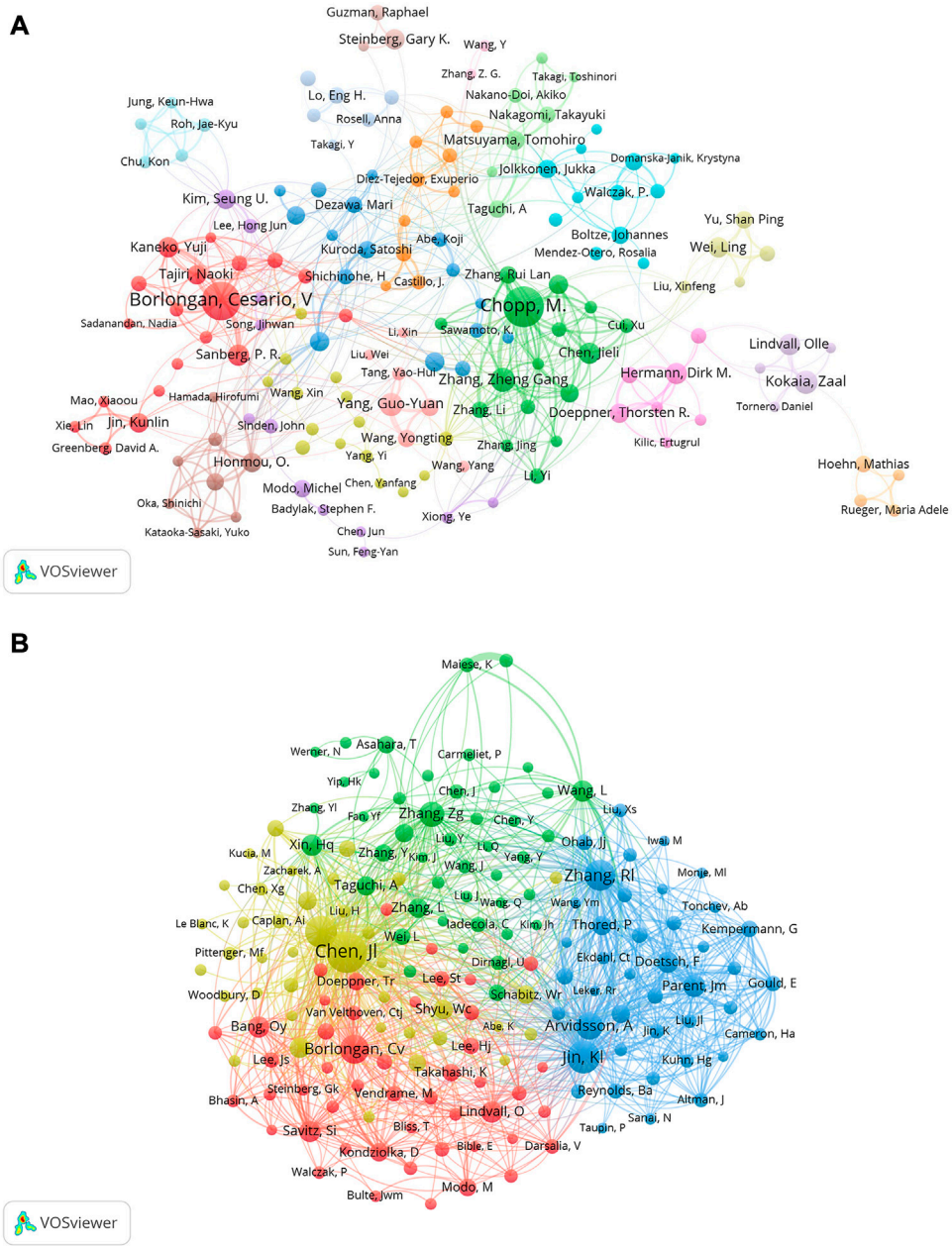


FIGURE 5
(A) Co-author related to stem cells in stroke. Each circle represents an author, the lines between the circles signify connections between the authors, and the networks of connections in different colors signify cooperative clusters among different authors **(B)** Co-cited-author clustering analysis.

3.7 Key topics of research hotspots

The use of cluster analysis to cluster the included keywords and summarize the study subjects might be helpful for relevant researchers in identifying popular topics and assisting scholars in better understanding current scientific concerns. We used VOSviewer to cluster the keywords into eight, as presented in Figure 8A: origin and behavior of stem cells (red), pathophysiological process of stroke (green), treatment of stroke and application of stem cells (purple), effects of stem cell therapy after stroke (light blue), cells that make up the central nervous system and the pathophysiological changes (orange), other diseases associated with stem cell therapy (brown), exosomes and mechanism of action (yellow), others (navy blue).

Meanwhile, we performed a series of keyword burst detections. To evaluate the development of stem cells in stroke research, researchers used a method named "keyword burst detection," which is the recognition of phrases that often occur in a certain period of time. Table 6 demonstrates that terms with a high frequency in this study, aside from "stroke" (1538), include "ischemia" (801), "neurogenesis" (660), "brain ischemia" (553), "mesenchymal stem cell" (549), and "neural stem" (541).

TABLE 4 Top 10 journals and co-cited journals related to stem cells in stroke.

Rank	Journal	Count (%)	IF(JCR 2020)	JCR Quartile	Co-cited-journal	Citations	IF(JCR 2020)	JCR Quartile
1	Cell Transplantation	172 (2.83%)	4.139	Q3	Stroke	19776	10.17	Q1
2	Brain Research	160 (2.63%)	3.61	Q3	J Neurosci	14061	6.709	Q1
3	Stroke	158 (2.60%)	10.17	Q1	P Natl Acad Sci Usa	11769	12.779	Q1
4	Plos One	147 (2.42%)	3.752	Q2	J Cerebr Blood F Met	10862	6.96	Q1
5	Journal Of Cerebral Blood Flow And Metabolism	137 (2.26%)	6.96	Q1	Brain Res	8227	3.61	Q3
6	Neural Regeneration Research	129 (2.12%)	6.058	Q2	Nature	7386	69.504	Q1
7	International Journal Of Molecular Sciences	113 (1.86%)	6.208	Q1	Plos One	7295	3.752	Q2
8	Neuroscience	103 (1.70%)	3.708	Q3	Stem Cells	6787	5.845	Q1
9	Experimental Neurology	102 (1.68%)	5.62	Q2	Exp Neurol	6630	5.62	Q2
10	Stem Cell Research and Therapy	95 (1.56%)	8.098	Q1	Science	6519	63.798	Q1

Prominent keywords were further analyzed. The top 25 keywords are listed Figure 8B in chronological order, which have the greatest burst intensity. It can be seen from the figure that the keywords that had exploded in the past 5 years mainly focused on "extracellular vesicles," "inflammation regulation," and "regulation of extracellular matrix." A timeline analysis that visually depicts the research hotspots and development paths of stem cells in stroke at various stages from a temporal perspective is presented in Supplementary Figure S2. The keywords that have received extensive attention in this field appear early, for example, cerebral ischemia, regeneration, NSC, endothelial progenitor cells, apoptosis, and cell therapy. Emerging hot words in the past decade include adhesion molecule, mesenchymal stromal cell, extracellular vesicle, pluripotent stem cell, signaling pathway, plasticity, and exosomes.

4 Discussion

4.1 Global research trends of stem cells in stroke

This study aimed to conduct a bibliometric analysis of the last 18 years' worth of research on stem cells in stroke. The citation count has been steadily increasing each year, with the 2008–2009 period showing the clearest growth pattern. Prior to 2008, an average of 150 papers per year were published in this field. From 2009 to 2022, the number of publications gradually increased, with an average of 350 papers published annually. The number of articles published in 2020 peaked at 515. These findings indicated that studies on stem cell therapy for stroke have gained increasing attention from researchers from all over the world in recent years, and the research area is currently in a steady developmental stage.

The top three authoritarian countries performing research on stem cells in stroke are the USA, China, and Japan. Four European nations, four Asian-Pacific nations, and two American nations make up the top ten nations. Furthermore, among the top 10 research institutions, five were American, and the remaining five were Chinese. We observed a close coordination between four nations, namely, Germany, Japan, China, and the United States. China also actively works with Japan, Canada, and England. There are some academic institutions that collaborate well with one another, such as Shanghai Jiao Tong University, Capital Medical University, and Harvard University. Although Oakland University ranked second in terms of paper publications, it was found to have limited collaborations with other universities. Such collaborations are detrimental for these universities in terms of long-term scholarly advancement. Consequently, it is indeed necessary that research organizations from many nations work closely together and communicate to collaboratively promote stem cell therapy for stroke.

As for the journals, those listed in Table 4 may be the core journals of the publication of research on stem cells in stroke. The most widely followed journal in this field of study is *Cell Transplantation* (IF = 4.139, Q3), with the majority of research on stem cell therapy published in this journal. *Stroke* (IF = 10.17, Q1) has the greatest IF, which also received the most citations (19,776 times). According to the Journal Citation Reports (2021 edition), five journals had IF values between 5 and 10 (*Journal of Cerebral Blood Flow And Metabolism*, *International Journal Of Molecular Sciences*, *Experimental Neurology*, *Stem Cell Research and Therapy*, and *Neural Regeneration Research*), four had an IF value between 3 and 5 (*Cell Transplantation*, *Brain Research*, *Plos One*, and *Neuroscience*), and no journal had an IF value below 3. These results indicated that most studies were published in these high-quality journals, and when researching on this topic, academics should concentrate on the content published in these journals. Most of the co-cited journals are high-impact Q1 journals, as can be seen from the list of co-cited journals. These journals support the investigation of stem cells in stroke and are undoubtedly of high quality. Furthermore, current research on stem cells in stroke is

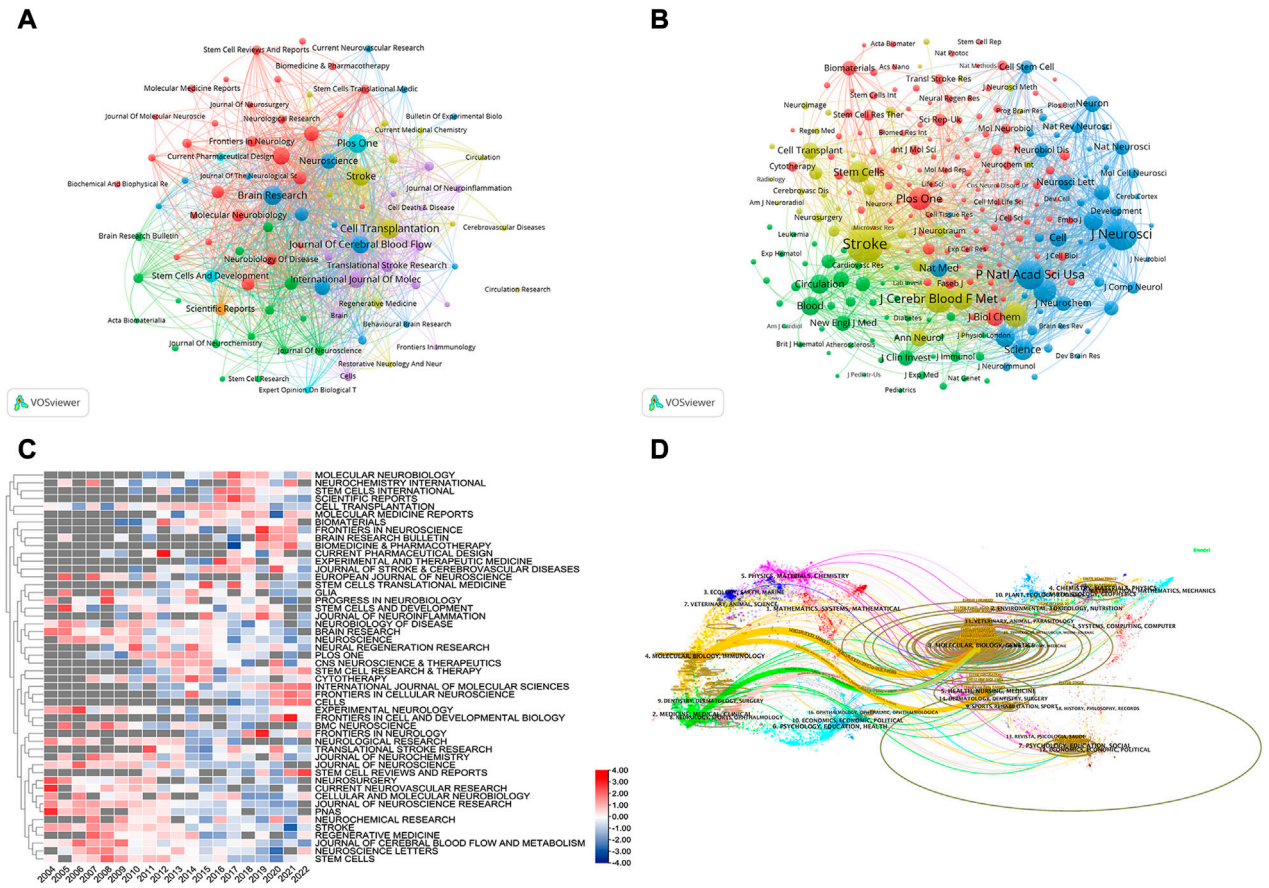


FIGURE 6
(A) Co-journal clustering analysis. Each circle represents a journal, the size of the circle depends on the strength of the connection, the number of citations, and so on. And, the color of the circle on behalf of the cluster to which it belongs, different clusters are represented by different colors. **(B)** Co-cited-journal clustering analysis **(C)** Journals heat map on stem cells in stroke. Each line was compared with each other. The value in the box is the number of articles published in the journal divided by the number of articles published in the year, verbalizing the current year's popularity of the journal, The redder the color verbalizes the hotter journal in the year, so it was called the heat map **(D)** The dual-map overlay of journal publishing research. Citing journals on the left and cited journals on the right, and the curve is the citation line, which completely shows the context of the citation, the more papers the journal publishes, the longer the vertical axis of the ellipse; the more authors they are, the longer the horizontal axis of the ellipse.

mostly published in journals pertaining to molecular biology, immunology, and medicine, followed by journals about neurology, sports, and ophthalmology. This indicates that basic research still constitutes the focus of the research and that the proportion of clinical studies is relatively low.

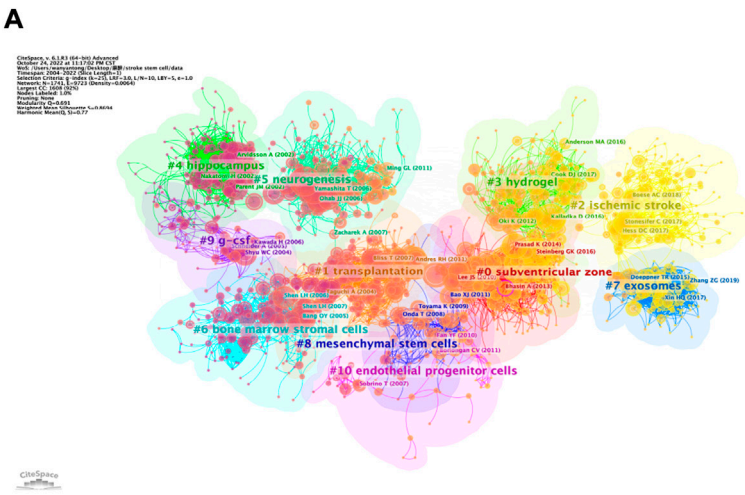
As for the authors, Chopp, M. (Oakland University, USA) is the most prolific, followed by Borlongan, Cesario, V (Stanford University, USA). They contributed to so many publications and were leaders in this field. Before 2015, Professor Chopp, M. and his team mainly focused on the role of stem cells in stroke, including BMSCs, neural progenitor cells, and human umbilical cord blood cells. In 2002, they found that after adult mouse suffered focal cerebral ischemia, endothelial progenitor cells generated from bone marrow contributed to brain neovascularization (Z. G. Zhang et al., 2002). Then in 2006, they demonstrated that intracarotid transplantation of BMSCs increased axon-myelin remodeling following stroke (L.H. Shen et al., 2006). Their experimental results in 2010 indicated that following a stroke in mice, MSC-mediated enhanced tPA activation in astrocytes encouraged neurite development (Xin et al., 2010). The next year, they published a study on how the production of astrocytic endogenous glial cell-generated neurotrophic factor was enhanced by BMSC implantation in the ischemic boundary area following stroke in adult rats (Shen et al., 2010). In the same year, they confirmed that the subventricular area has more progenitor cells dividing than normal due to the human umbilical cord tissue-derived cells (hUTCs) (Zhang et al., 2011) and had a neurorestorative effect (Ding et al., 2013). They proposed that the level of proinflammatory factors in the blood can be significantly reduced after hUTC transplantation (Bae et al., 2012). Furthermore, Chopp, M. discovered several chemicals that control stem cell migration, differentiation, and proliferation, such as specific miRNAs (X. S. Liu et al., 2013; Buller et al., 2012), atorvastatin (R. L. Zhang et al., 2012; J; Chen et al., 2008), angiopoietin 2 (X. S. Liu et al., 2009), and erythropoietin (L. Wang et al., 2008). These findings provided a solid theoretical basis for the rapid development of stem cells in stroke. Afterwards, Chopp, M. and his team shifted the attention to MSC-derived exosomes and described how they contribute to immunological reactivity, vascular remodeling, and

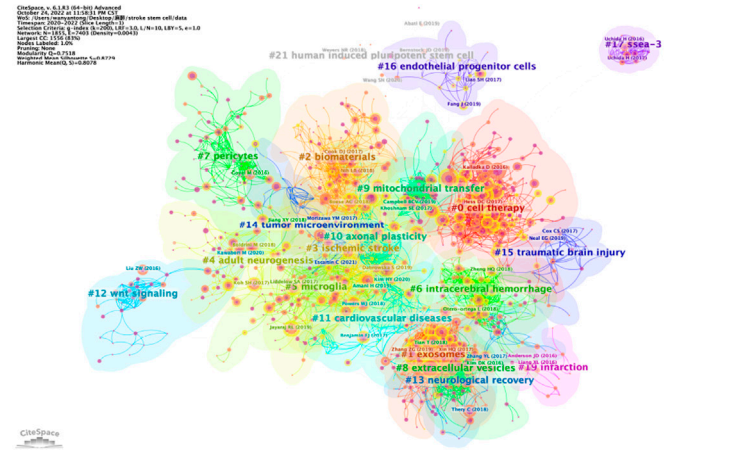
TABLE 5 Top 15 co-cited references related to stem cells in stroke.

Rank	Author	Article title	Source title	Cited	Year	DOI
1	Caplan, AI, et al.	Mesenchymal stem cells as trophic mediators	JOURNAL OF CELLULAR BIOCHEMISTRY	2082	2006	10.1002/jcb.20886
2	Chamberlain, G, et al.	Concise review: Mesenchymal stem cells: Their phenotype, differentiation capacity, immunological features, and potential for homing	STEM CELLS	1725	2007	10.1634/stemcells.2007-0197
3	Caplan, AI	Adult mesenchymal stem cells for tissue engineering *versus* regenerative medicine	JOURNAL OF CELLULAR PHYSIOLOGY	1378	2007	10.1002/jcp.21200
4	Moskowitz, MA, et al.	The Science of Stroke: Mechanisms in Search of Treatments	NEURON	1303	2010	10.1016/j.neuron.2010.07.002
5	Langhorne, P, et al.	Stroke Care 2 Stroke rehabilitation	LANCET	1290	2011	10.1016/S0140-6736 (11)60325-5
6	Schmidt-Lucke, C, et al.	Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events - Proof of concept for the clinical importance of endogenous vascular repair	CIRCULATION	915	2005	10.1161/ CIRCULATIONAHA.104.504,340
7	Imitola, J, et al.	Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1 alpha/ CXC chemokine receptor 4 pathway	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA	851	2004	10.1073/pnas.0408258102
8	Bang, OY, et al.	Autologous mesenchymal stem cell transplantation in stroke patients	ANNALS OF NEUROLOGY	838	2005	10.1002/ana.20501
9	Abrous, DN, et al.	Adult neurogenesis: From precursors to network and physiology	PHYSIOLOGICAL REVIEWS	750	2005	10.1152/physrev.00055.2003
10	Lalu, MM, et al.	Safety of Cell Therapy with Mesenchymal Stromal Cells (SafeCell): A Systematic Review and Meta-Analysis of Clinical Trials	PLOS ONE	728	2012	10.1371/journal.pone.0047559
11	Falk, E	Pathogenesis of atherosclerosis	JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY	715	2006	10.1016/j.jacc. 2005.09.068
12	Dutta, P, et al.	Myocardial infarction accelerates atherosclerosis	NATURE	688	2012	10.1038/nature11260
13	Amariglio, N, et al.	Donor-Derived Brain Tumor Following Neural Stem Cell Transplantation in an Ataxia Telangiectasia Patient	PLOS MEDICINE	671	2009	10.1371/journal.pmed.1000029
14	Ohab, JJ, et al.	A neurovascular niche for neurogenesis after stroke	JOURNAL OF NEUROSCIENCE	661	2006	10.1523/JNEUROSCI.4323-06.2006
15	Lochhead, JJ, et al.	Intranasal delivery of biologics to the central nervous system	ADVANCED DRUG DELIVERY REVIEWS	628	2012	10.1016/j.addr. 2011.11.002

brain regeneration during stroke recovery. In addition, they provided an overview of the potential and perspectives of stem cells in the fields of stroke and regenerative medicine, the links between stem cells and inflammatory factors for stroke rehabilitation, and the prospects of exosomes in the field of stroke. In the recent 2 years, Chopp et al. gradually focused on and studied the application of extracellular vesicles in neurological diseases, indicating that the extracellular vesicles may perhaps become a new research hotspot in this field.

The most commonly co-cited author is Chen, Jl. (citation = 2,214), followed by Jin, K.L. (citation = 1,473), and Li, Y. (citation = 1,367). In 2006, Chen, Jl. investigated the effects of giving human BMSCs (hBMSCs) intravenously after intracerebral hemorrhage (ICH) in rats and found that doing so dramatically improves neurological function (Seyfried et al., 2006). This paper provided the basis for the clinical investigation of BMSCs in ICH. The next year, Chen, Jl. demonstrated neurological recovery in rats intravenously injected with hBMSCs 1 month following a stroke (Shen et al., 2007b) and published the first 1-year follow-up report of BMSC therapy in stroke rats (Shen et al., 2007a). This report proved that BMSCs have an effect on scarring reduction and cell proliferation increase. In 2013, Chen, Jl. concluded that while the effect of multiple injections did not outperform single-injection therapy in terms of functional outcomes and histological





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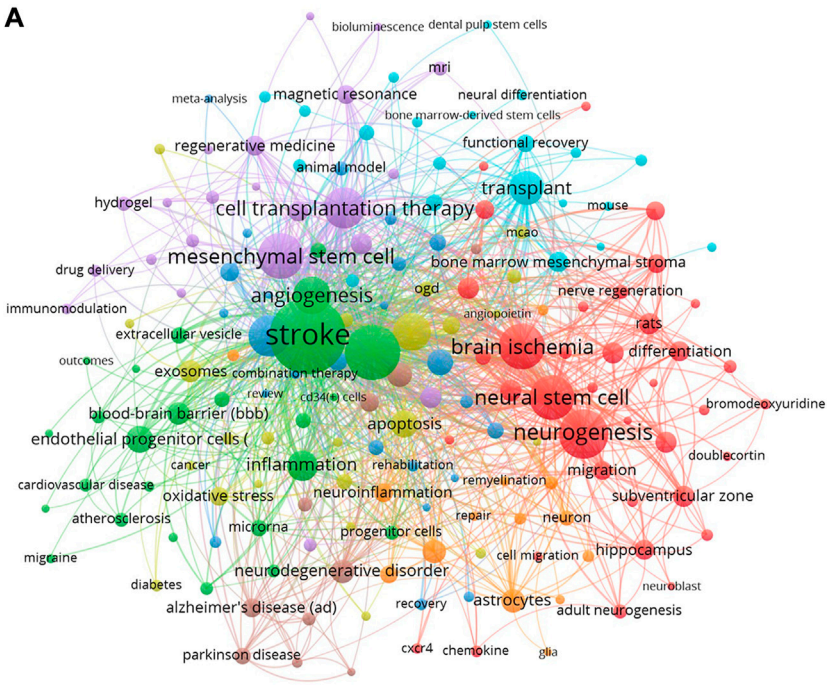
Top 25 References with the Strongest Citation Bursts

References	Year	Strength	Begin	End	2004 - 2022
Arvidsson A, 2002, NAT MED, V8, P963, DOI 10.1038/nm747, DOI	2002	81.35	2004	2007	
Parent JM, 2002, ANN NEUROL, V52, P802, DOI 10.1002/ana.10393, DOI	2002	53.74	2004	2007	
Nakatomi H, 2002, CELL, V110, P429, DOI 10.1016/S0092-8674(02)00862-0, DOI	2002	45.95	2004	2007	
Jin KL, 2001, P NATL ACAD SCI USA, V98, P4710, DOI 10.1073/pnas.081011098, DOI	2001	37.92	2004	2006	
Jin K, 2003, MOL CELL NEUROSCI, V24, P171, DOI 10.1016/S1044-7431(03)00159-3, DOI	2003	35.47	2004	2008	
Taguchi A, 2004, J CLIN INVEST, V114, P330, DOI 10.1172/jci200420622, DOI	2004	35.39	2005	2009	
Zhang RL, 2004, J CEREBR BLOOD F MET, V24, P441, DOI 10.1097/00004647-200404000-00009, DOI	2004	32.39	2005	2009	
Wang L, 2004, STROKE, V35, P1732, DOI 10.1161/01.STR.0000132196.49028.a4, DOI	2004	31.96	2005	2009	
Shyu WC, 2004, CIRCULATION, V110, P1847, DOI 10.1161/01.CIR.0000142616.07367.66, DOI	2004	31.53	2005	2009	
Bang OY, 2005, ANN NEUROL, V57, P874, DOI 10.1002/ana.20501, DOI	2005	42.8	2006	2010	
Imitola J, 2004, P NATL ACAD SCI USA, V101, P18117, DOI 10.1073/pnas.0408258102, DOI	2004	32.53	2006	2009	
Thored P, 2006, STEM CELLS, V24, P739, DOI 10.1634/stemcells.2005-0281, DOI	2006	44.03	2007	2011	
Yamashita T, 2006, J NEUROSCI, V26, P6627, DOI 10.1523/JNEUROSCI.0149-06.2006, DOI	2006	42.42	2007	2011	
Ohab JJ, 2006, J NEUROSCI, V26, P13007, DOI 10.1523/JNEUROSCI.4323-06.2006, DOI	2006	36.21	2007	2011	
Bliss T, 2007, STROKE, V38, P817, DOI 10.1161/01.STR.0000247888.25985.62, DOI	2007	32.37	2008	2012	
Zhang ZG, 2009, LANCET NEUROL, V8, P491, DOI 10.1016/S1474-4422(09)70061-4, DOI	2009	39.41	2010	2014	
Lee JS, 2010, STEM CELLS, V28, P1099, DOI 10.1002/stem.430, DOI	2010	55.4	2011	2015	
Honmou O, 2011, BRAIN, V134, P1790, DOI 10.1093/brain/awr063, DOI	2011	37.44	2012	2016	
Savitz SI, 2011, ANN NEUROL, V70, P59, DOI 10.1002/ana.22458, DOI	2011	30.96	2012	2016	
Prasad K, 2014, STROKE, V45, P3618, DOI 10.1161/STROKEAHA.114.007028, DOI	2014	42.96	2015	2019	
Doeppner TR, 2015, STEM CELL TRANSL MED, V4, P1131, DOI 10.5966/sctm.2015-0078, DOI	2015	34.4	2016	2020	
Steinberg GK, 2016, STROKE, V47, P1817, DOI 10.1161/STROKEAHA.116.012995, DOI	2016	54.4	2017	2022	
Kalladka D, 2016, LANCET, V388, P787, DOI 10.1016/S0140-6736(16)30513-X, DOI	2016	51.62	2017	2022	
Hess DC, 2017, LANCET NEUROL, V16, P360, DOI 10.1016/S1474-4422(17)30046-7, DOI	2017	43.91	2018	2022	
Xin HQ, 2017, STROKE, V48, P747, DOI 10.1161/STROKEAHA.116.015204, DOI	2017	37.72	2018	2022	

FIGURE 7
(A) Co-cited references related to stem cells in stroke. The circles represent the number of co-citations, the purple circle represents centrality, the thickness of connection indicates the cooperation degree, and the purple to yellow gradient represents the time from the past to the present. **(B)** Cluster view of references in stem cells in stroke research in recent 3 years. The larger the circle, the more times the corresponding paper has been cited in the last 3 years **(C)** CiteSpace visualization map of top 25 references with the strongest citation bursts involved in stem cells in stroke. The blue line represents the time interval. The blue line represents the time interval. The time in which a reference was found to have a burst is displayed by a red line, indicating the first year and the last year of the duration of the burst.



B **Top 25 Keywords with the Strongest Citation Bursts**

Keywords	Year	Strength	Begin	End	2004 - 2022
dentate gyrus	2004	43.57	**2004**	2009	
adult rat	2004	32.8	**2004**	2011	
subventricular zone	2004	27.9	**2004**	2009	
central nervous system	2004	23.13	**2004**	2009	
transient global ischemia	2004	16.98	**2004**	2012	
differentiate	2004	15.25	**2004**	2013	
olfactory bulb	2004	11.55	**2004**	2011	
precursor	2004	11.43	**2004**	2009	
progenitor cell	2004	11.22	**2004**	2007	
mammalian forebrain	2004	10.39	**2004**	2009	
progenitor	2004	10.36	**2005**	2013	
adult mammalian brain	2004	11.99	**2006**	2011	
marrow stromal cell	2004	10.3	**2010**	2011	
nerve regeneration	2004	14.19	**2014**	2016	
reperfusion injury	2004	10.95	**2017**	2022	
neurovascular plasticity	2004	12.32	**2018**	2022	
mesenchymal stromal cell	2004	11.69	**2018**	2022	
extracellular vesicle	2004	48.28	**2019**	2022	
exosm	2004	22.57	**2019**	2022	
protect	2004	12.82	**2019**	2022	
blood-brain barrier	2004	11.29	**2019**	2022	
neuroinflammation	2004	10.3	**2019**	2022	
ischemic stroke	2004	39.53	**2020**	2022	
oxidative stress	2004	16.26	**2020**	2022	
inflammation	2004	11.55	**2020**	2022	

FIGURE 8
(A) Clustering of co-occurrence among keywords. The circles and labels in the figure constitute a unit, and the units of different colors constitute different clusters **(B)** CiteSpace visualization map of top 25 keywords with the strongest citation bursts involved in stem cells in stroke.

TABLE 6 Top 20 keywords related to stem cells in stroke.

Rank	Keyword	Occurrences	Total link strength	Rank	Keyword	Occurrences	Total link strength
1	stroke	1538	4098	11	transplant	306	934
2	ischemia	801	2025	12	inflammation	259	804
3	neurogenesis	660	1878	13	endothelial progenitor cells (epcs)	204	436
4	brain ischemia	553	1381	14	apoptosis	191	554
5	mesenchymal stem cell	549	1307	15	stem cell	185	561
6	neural stem cell	541	1447	16	traumatic brain injury	161	490
7	stem cells	464	1351	17	neurodegenerative disorder	156	489
8	cell transplantation therapy	461	1327	18	microglia	150	444
9	angiogenesis	387	1157	19	astrocytes	144	429
10	neuronal protection	382	1087	20	blood-brain barrier (BBB)	138	391

evaluations, it significantly improved long-term functional outcomes following stroke (Shehadah et al., 2013). In 2014, in a review article published in *Progress in Neurobiology*, Chen, Jl. described the application of stem cells from various cell origins in stroke as well as the restorative mechanisms, distribution methods, and imaging methodologies, which also covered the difficulties in stem cell therapy converting to clinical applications (X. Liu et al., 2014). The aforementioned studies generally focus on the mechanisms and therapeutic benefits of stem cell treatment for stroke, indicating that the field is still in the development stage and that additional basic and clinical translational research are still required. Clearly, the achievements of Chen, Jl. have laid a theoretical and experimental basis for research on stem cells in stroke.

A reference is deemed to be co-cited if it is cited in a number of different publications, in which case the co-cited references could be viewed as the foundation of the field's study. To determine the research foundation for stem cells in stroke, we selected 15 references with the largest number of co-citations for this bibliometric analysis. Among the top 15 co-cited publications, two were written by Caplan et al., the first of which was the most often mentioned study and was published in the *Journal of Cellular Biochemistry* in 2006. This study firstly showed the trophic of the MSC-secreted bioactive molecules and summarized the application of the MSC trophic effect in injured tissues (Caplan and Dennis, 2006). The biological mechanisms of the *in vivo* functionality of MSCs during development and aging were outlined in another study (Caplan, 2007). The second most co-cited study was written by Chamberlain, G. et al., in 2011 and published in *STEM CELLS*. Their discovery that the movement of MSCs from the circulation into tissues may be facilitated by chemokine receptors and adhesion molecules attracted interest in terms of the function of stem cells in immunological regulation. Of the top 15 co-cited references (Abrous, Koehl, and Le Moal, 2005; Ohab et al., 2006), two were about the neural regeneration function of stem cells, outlining the molecular mechanisms between stem cells and neurogenesis and suggesting that stem cell therapy may be a therapeutic strategy for nervous system diseases. Three references (Schmidt-Lucke et al., 2005; Falk, 2006; Dutta et al., 2012) demonstrated that stem cells can repair blood vessels and promote neoangiogenesis. Overall, the biological function, transplantation, components, and targeted delivery of stem cells are the main subjects of discussion in the top 15 co-cited references, which represent the research foundation of stem cells in stroke.

4.2 Hotspots and frontiers

Citation bursts refer to references that have received a lot of recent citations from other scholars and highlight emerging themes within a specific research field. In accordance with the key research topics of the references with the strongest citation bursts (Figure 7C), the potential mechanism of directed migration and neurogenesis of NSCs as well as the therapeutic effects of stem cells in stroke are currently the main areas of study for research on stem cells in stroke. In addition to references with citation bursts, keywords can facilitate swift capturing of the distribution and development of hotspots in the realm of research on stem cells in stroke. Combining the citation bursts and keywords, we prepare to divide the hotspots and frontiers into two main research areas, namely, the mechanistic research hotspots and the clinical research hotspots, to discuss the distribution of their respective hot spots according to Table 6 and Figures 8A, B.

4.3 The hotspot mechanisms of stem cells in stroke

4.3.1 Stem cell and regeneration mechanism

As can be seen from Table 6, "neurogenesis," "angiogenesis," and "neural stem cells" are currently the focus of research in stem cell regeneration. Nerve and cerebrovascular regenerations are essential for recovery from stroke (Zhang et al., 2022). In 1992, Reynolds, Weiss, et al. isolated NSCs and fostered for the first time in

the presence of epidermal growth factor, leading to large cell spheres they called "neurospheres" (Reynolds and Weiss, 1992). The discovery of NSCs provides a new promising therapy for nerve and vascular regenerations in stroke. Neuronal and glial cells originate from the common immature NSC, defined as self-renewing and multipotent cells that can differentiate into neurons, astrocytes, and oligodendrocytes (Reynolds and Weiss, 1992). NSCs were found to exist not only in the developing brain but also in the mature mammalian brain. Many studies have demonstrated that NSCs can replace lost neurons and restore connectivity in neuronal circuits, contributing to improved recovery from stroke and brain injury in rats (Daadi et al., 2009; Yokobori et al., 2019; Abeysinghe et al., 2015; Hou et al., 2017; G; Wang et al., 2020). Transplanted NSCs may prevent neuronal apoptosis, exert immunomodulatory effects both inside and outside the brain, and increase endogenous neuronal regeneration and angiogenesis (G.-L. Zhang, Zhu, and Wang, 2019; Horie, Hiu, and Nagata, 2015). Numerous studies have evaluated the therapeutic efficacy and safety of transplanted exogenous NSCs in preclinical animals with cerebral ischemic stroke (Daadi et al., 2009; Horie et al., 2011; Yokobori et al., 2019). However, due to the limited regeneration capacity of NSCs, the physiological environment is complex, which limits their repair effect. The current alternative approach to the use of NSCs is the use of inducible pluripotent stem cells or MSCs. Tobin and colleagues reported that both activated and naive MSCs induced complete behavioral recovery, reduced infarct volumes, and reduced microglial activation and IL-1β, TNF-α, and IL-6 levels in treated animals compared with vehicle-treated control stroke animals (Tobin et al., 2020). The angiopoietin expression and blood vessel density in ischemic brain tissue significantly increased after MSC transplantation (Zhang et al., 2022). MSC transplantation can promote neurogenesis mainly involving enhancement of endogenous neural cell proliferation and protection of newly grown cells from the pathogenic environment. A recent study demonstrated that MSC spheroid-loaded collagen hydrogels played a therapeutic role through three upregulated signals related to cell communication and upregulated the PI3K-Akt signaling pathway, which increased the expression of proteins related to neurogenesis and neuroprotection (He et al., 2021b). As MSCs can be used to promote the differentiation of NSCs into neurons by the production of different classes of trophic factors and anti-apoptotic molecules, future studies can focus on the development of MSC cell therapies associated with NSCs to facilitate nervous system recovery.

4.3.2 Antioxidant and anti-inflammatory mechanism

Research on "oxidative stress" and "inflammation" have gradually become popular in recent years, suggesting that scholars have investigated stem cells into a deeper level. "Oxidative stress" and "inflammation" were among the top 25 keywords from 2020 to 2022 with the most citation spikes. Previous research demonstrated that oxidative stress and inflammation are two of the initial steps in the chain of events leading to cerebral ischemia injury, which disrupts various neuronal circuits (Rana and Singh, 2018; Chen H. et al., 2020; Chen S. et al., 2020). It has been commonly acknowledged that inflammation plays a major role in the development and course of the disease, as well as in recovery and wound healing following a stroke (Shekhar et al., 2018). Meanwhile, the potential of antioxidant therapies for stroke is suggested by the presence of induced oxidative modulatory pathway in the development of stroke (Pradeep et al., 2012). In recent years, it has been proven that stem cell therapy is a potentially successful treatment for inhibiting inflammation and oxidative stress following stroke (Lei et al., 2022). Researchers found that MSCs reduced the level of cellular oxidative stress and elevated the intracellular calcium and reactive oxygen species of neuronal cells when under stress from cerebral ischemia (K.-H. Chen et al., 2016; Alhazzani et al., 2018). In addition, emerging evidences have suggested that stem cells can increase the effectiveness of mitochondrial transfer to improve oxidative phosphorylation, lessen cellular oxidative stress levels and subsequently the brain damage cascade caused on by ischemic injury (Tseng et al., 2021; K; Liu et al., 2019). Based on the foregoing discussion, by lowering the degree of oxidative stress and transferring healthy mitochondria to damaged cells, stem cells engage their antioxidant ability in ischemic stroke. As for the inhibition of inflammation, recent research suggested that stem cells can regulate immune cell infiltration and polarization in ischemic brain to reduce neuroinflammation (Li et al., 2019; Yang et al., 2020). Interestingly, many studies reported no difference in or even worse efficacy of anti-inflammatory agents for stroke (Iosif et al., 2008; Tobin et al., 2014). However, some researchers used anti-inflammatory compounds to strengthen the anti-inflammatory effects of MSCs. For instance, some investigators found that MSCs from human umbilical cords that overexpress C–C motif chemokine ligand two or MSCs that have been activated with interferon-γ have a stronger anti-inflammatory ability in ischemic stroke compared with naive MSCs (Lee et al., 2020; Tobin et al., 2020). Collectively, oxidative stress and inflammation are both mechanisms of the main occurrence and development of stroke. Stem cells can fight oxidative damage, reduce inflammation, and alleviate aggravation of stroke. However, the mechanisms involves many pathophysiological processes and molecular pathways, which are needed to figure out, including the phenomenon caused by MSCs combined with anti-inflammatory agents, as described above. Therefore, in an attempt to understand the underlying mechanism, more research on mechanism is warranted.

4.3.3 Stem cells and extracellular vesicles

"Mesenchymal stem cells" are among the current study hotspots in terms of stem cell type selection. Among several stem cell types, MSCs have attracted the attention of numerous researchers. MSCs can be simply extracted from the bone marrow (51%), umbilical cord (17%), and adipose tissue (11%) (Kabat et al., 2020). MSC therapy has been proven to reduce inflammation, encourage neurogenesis, and inhibit angiogenesis and apoptosis to improve neuronal defects, neural network reconstruction, and neurological functions (Zhu et al., 2019). Cui J. et al. found that MSCs can reduce neurological impairments and enhance axonal regeneration in rats with stroke (Cui et al., 2017). Furthermore, MSCs exert angiogenic effects based on secreted angiogenic factors by significantly enhancing vitality, motility, and network formation (König et al., 2015). The fact that neuroinflammation accelerated the development of brain injury is widely accepted. The method for controlling immune response may thereby decrease brain damage.

Studies have demonstrated that stem cells can facilitate nerve repair either by boosting the protective effects of anti-inflammatory cytokines or performing immunomodulatory functions, such as neutrophil and microglia regulation (Jingli et al., 2022). For instance, following a stroke, MSCs can suppress microglial activation by upregulating growth factors and hypoxia-inducible factor-1-alpha while downregulating proinflammatory cytokines and chemokines (Yan et al., 2013). Current studies have verified that the neuroprotection of MSCs in stroke. Therefore, enhancing the therapeutic benefits and immunomodulatory capabilities of MSC may provide researchers with potential targets for their future studies.

From the stem cell derivatives used in the treatment of stroke, our data (Figure 8B) indicated that the latest research hotspots from 2019 to 2022 were "exosomes" and "extracellular vesicle." Extracellular vesicles, which can transport a variety of cargos, including lipids, nucleic acids, and proteins, and are released from the cell surface into body fluid, help cells communicate with one another (Allan et al., 2020). One of the most currently appealing subcategories of extracellular vesicles is exosome (Lawson et al., 2016). In recent years, studies have suggested restrictions and potential hazards with stem cell therapy, such as tumorigenicity and the inability to successfully penetrate the blood-brain barrier (BBB) (Lukomska et al., 2019). Researchers also found that the use of exosomes generated from stem cells could be an alternative strategy to stem cell (Venkat, Chopp, and Chen, 2018; Cai et al., 2020). The nanoscale characteristics of exosomes allow themselves to efficiently disperse throughout the body and pass across the BBB. Furthermore, it can imitate the ability of stem cells and other supporting cells to regenerate. Stem cells release exosomes to communicate with other cells, when they detect alterations in the microenvironment, such as "inflammation" or "oxidative stress." Exosomes have been demonstrated to be useful for the regulation of post-stroke inflammation, neurovascular remodeling, angiogenesis, neurogenesis, synaptic plasticity, and apoptosis and autophagy control (Seyedaghamiri et al., 2022). According to some authors, exosomes can be used as natural biomarkers to gage the seriousness of clinical manifestations of neurological diseases (Hong et al., 2019).

4.3.4 Clinical application prospect of stem cells in stroke

Basic science and animal models have laid the groundwork for advancing stem cell therapy for stroke in clinical setting. NSC transplantation has been performed mainly as a treatment for chronic phase of post-stroke. In a PISCES one clinical trial (Kalladka et al., 2016), the NSC transplantation to the patients within 6 months to 5 years after stroke showed that the embedded delivery of the NSC line CTX0E03 was safe and suggested improved neurological function. However, the study was conducted on only 11 men; thus, further study is needed that includes female patients and larger patient populations. In another PISCES two study that included adults aged over 40 years with significant upper-limb motor deficit 2–13 months following ischemic stroke, 23 patients underwent CTX cell transplantation, and their upper-limb functions improved at 3, 6, and 12 months (Muir et al., 2020). Subsequently, the PISCES-3 study recruited approximately 130 patients with moderate to severe functional disability from 6 to 24 months after the stroke. The primary outcome was an improvement in the modified Rankin scale (mRS) score at 6 months following surgery (Wechsler et al., 2018). However, due to the ethical issues related to the harvesting of fetal NSCs, as well as the limited number of donors, very few trials used NSCs. In addition, the clinical use of NSCs has several disadvantages, such as immunogenicity and the possibility of rejection of allogeneic human NSCs, which limits its application.

A large number of preclinical data have proven the feasibility of MSCs in the treatment of stroke, and clinical administration of stem cell therapy is expected. Most of the clinical trials have evaluated the efficacy and safety of MSCs for the treatment of stroke. MSCs, especially bone marrow-derived ones, are most widely used in clinical trials. In a randomized study of 30 patients with severe stroke, Bang et al. reported that MSCs improved the mRS and Barthel index scores within 1 year following stroke and exerted no adverse cell-related, serological, or imaging-defined effects (Bang et al., 2005). Honmou et al. reported that there were no major side events after the intravenous infusion of autologous BMSCs expanded in human serum into 12 participants 36–133 days post-stroke (Honmou et al., 2011). Overwhelming evidence supports the safety of the approach, although data on its efficacy are scarce or indicate only a transient improvement (Nistor-Cseppentö et al., 2022). Majority of the adverse events in these clinical trials were minor (Kvistad et al., 2022). For example, two minor side effects in a clinical trial (n = 57) using MSCs to treat stroke may be linked to venous internal position stimulation and urinary tract infection (Levy et al., 2019). However, some animal studies have demonstrated that MSCs may increase the risk of autoimmune disease and the onset of tumors (Djouad et al., 2003). This prompted the researchers to look for some countermeasures. Furthermore, most of the relevant clinical trials included only a few patients (n < 100), and large multicenter randomized controlled trials are absent; thus, further research is warranted to determine the efficacy and security of MSCs for stroke treatment and also to identify the optimal cell concentration, time, patient selection criteria (age, stroke subtype, and damage area), and combination therapy for routine clinical uses.

Most of the studies on exosomes have focused on animal and *in vitro* experiments. According to available information, a few clinical studies have used exosomes for stroke treatment. As of August 2022, only three clinical trials involving stroke patients were available on the public clinical trial database (https://clinicaltrials.gov/). One study evaluated improvement in patients suffering from acute ischemic stroke who were administered with allogenic MSC-derived exosomes. In a different study, the diagnostic value of blood extracellular vesicles was investigated in stroke patients undergoing rehabilitation. The final study aimed to determine how acupuncture-induced exosomes may help treat post-stroke dementia. In addition, a pilot randomized clinical trial suggested that local injection of exosomes produced by allogenic placenta MSCs is safe after a malignant middle cerebral artery infarction (Dehghani et al., 2022). These data suggest that exosome therapy is a novel promising strategy for stroke in clinical translational application. In conclusion, although the therapeutic effect, biosafety, kinetics, and biodistribution of exosomes still need to

be thoroughly investigated, their capacity for regeneration and repair provides new possibilities for the treatment of stroke.

4.3 Advantages and limitations

This study has several advantages. First, based on research published from 2004 to 2022, this bibliometric analysis is the first investigation of patterns and contentious topics relating to stem cells in stroke. Second, we used three bibliometric methods simultaneously for the survey and analysis in this study, which significantly increased the likelihood that our data analysis process is impartial; VOSviewer and CiteSpace were extensively used in this study (C. Chen et al., 2012). This study also involved a thorough analysis of the number and growth tendency of annual publications; relationships among journals, authors, nations, and institutions; and various references, citations, and keywords.

This study also has limitations that need to be acknowledged. First, because this study only included articles in English, non-English writings may have been underrepresented. Second, only data obtained from the WoSCC database were used in this study; therefore, some pertinent studies from other databases may have been missed. Furthermore, insufficient data prevented full inclusion of articles in 2022.

5 Conclusion

This analysis may help researchers in identifying new trends and research hotspots for stem cells in stroke in the period of 2004–2022. The steadily increasing number of publications indicates that research on stem cells in stroke is becoming more and more important to academics worldwide. The top 3 countries with the most number of publications were China, the USA, and Japan. The journal, organization, and author with the most influence on were *Cell Transplantation*, Florida State University, and Chopp, M. respectively. The keywords that highlight recent hot topics about research on stem cells in stroke were "neurogenesis," "angiogenesis," "mesenchymal stem cells," "oxidative stress," "inflammation," "exosomes," and "extracellular vesicles," which will probably become promising in the future. Notably, stem cells have numerous positive effects, such as neuroprotection, enhanced angiogenesis and neurogenesis, and diminished inflammatory and immunological responses; however, the main mechanisms for mitigating the damage caused by stroke are still unknown. Clinical challenges may include complicating factors, such as the effect of age, stroke subtype, and stroke severity, all of which can affect the efficacy of stem cell therapy. In the future, to successfully create a therapeutic scheme, all of complicating factors must be carefully taken into account.

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 authors.

Author contributions

QZe, GH, and YW: conception and design the logic, revising the article. QZh and YZ: study conduct and editing the article. SZ, JZ, and XyZ: data acquisition, analysis and interpretation. QZh, YZ, SZ, LC, HL, HC, and XfZ: writing the manuscript. QZh, YZ, and SZ contributed equally to this work and should be considered co-first authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

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A retrospect and outlook on the neuroprotective effects of anesthetics in the era of endovascular therapy

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Studies on the neuroprotective effects of anesthetics were carried out more than half a century ago. Subsequently, many cell and animal experiments attempted to verify the findings. However, in clinical trials, the neuroprotective effects of anesthetics were not observed. These contradictory results suggest a mismatch between basic research and clinical trials. The Stroke Therapy Academic Industry Roundtable X (STAIR) proposed that the emergence of endovascular thrombectomy (EVT) would provide a proper platform to verify the neuroprotective effects of anesthetics because the haemodynamics of patients undergoing EVT is very close to the ischaemia–reperfusion model in basic research. With the widespread use of EVT, it is necessary for us to re-examine the neuroprotective effects of anesthetics to guide the use of anesthetics during EVT because the choice of anesthesia is still based on team experience without definite guidelines. In this paper, we describe the research status of anesthesia in EVT and summarize the neuroprotective mechanisms of some anesthetics. Then, we focus on the contradictory results between clinical trials and basic research and discuss the causes. Finally, we provide an outlook on the neuroprotective effects of anesthetics in the era of endovascular therapy.

KEYWORDS

neuroprotection, anesthetics, ischaemic stroke, endovascular procedures, therapy

Introduction

Stroke is the leading cause of disability and death worldwide. It can be classified into haemorrhagic stroke and ischaemic stroke, the latter of which is characterized by the sudden loss of blood flow to an area of the brain due to thrombosis or thromboembolism (Campbell et al., 2019). A nationwide community-based study showed that the incidence of acute ischaemic stroke (AIS) in all incident stroke cases was as high as 70%, and the high incidence and disability rates of AIS have seriously increased the socioeconomic and healthcare burdens (Gorelick, 2019; Wu S. et al., 2019).

Nevertheless, only limited options for treatment are available at present.

Intravenous recombinant tissue plasminogen activator (IV-rtPA) was the only pharmacologic treatment approved by the United States Food and Drug Administration (FDA) until endovascular thrombectomy (EVT) emerged. IV-rtPA has played an integral role in treating AIS in recent decades. However, the multiple contraindications and narrow therapeutic window restrict the application of IV-rtPA (Patel et al., 2020). In addition, rtPA has a low recanalization rate (13–50%) in patients suffering from large vessel occlusion (LVO) because of the unresponsiveness of large thrombi to the enzyme (Saqqur et al., 2007).

Advances in interventional neuroradiology promoted the development of EVT. The publication of five clinical trials of EVT in 2015 with positive findings launched a new era in AIS treatment. EVT is beneficial to most patients with AIS caused by the occlusion of the proximal anterior circulation (Goyal et al., 2016). Compared with IV-rtPA, EVT has a broader application time window and can be used in patients with contraindications to thrombolysis or intracranial LVO.

Inevitably, EVT must be performed under anesthesia. Thus, anesthetics are more widely available to patients with AIS than ever before. The choice of anesthesia, however, is still based on team experience without definite guidelines. Recently, the option of general anesthesia (GA) and conscious sedation (CS) during EVT was discussed in many multicentre randomized controlled trials (RCTs) (Schonenberger et al., 2016; Simonsen et al., 2018; Goldhoorn et al., 2020; Maurice et al., 2022), which indicated that anesthetics may affect the outcomes of patients with EVT. Moreover, a retrospective study preliminarily showed that propofol anesthesia was related to improved functional independence compared with inhalational GA [odds ratio (OR) = 2.65; 95% confidence interval (CI), 1.14–6.22; $p < 0.05$] (Diprose et al., 2021). These effects may be attributed to the haemodynamic effects of anesthetic drugs or the neuroprotective properties of anesthetics (Simonsen et al., 2022). Whether anesthetics have neuroprotective effects will directly affect the selection of anesthesia for EVT treatment. However, different results on the neuroprotective effects of anesthetics have been shown in clinical trials and basic research.

In this paper, we describe the research status of anesthesia in EVT in Part 1 and summarize the mechanisms related to the neuroprotective effects of commonly used anesthetics in Part 2. Then, we focus on the contradictory results between clinical trials and basic research and discuss the causes of the heterogeneity in Part 3. Finally, we provide a brief outlook on the neuroprotective effects of anesthetics in the era of endovascular therapy.

Anesthetics may affect the outcomes of EVT

With advances in stroke treatment, highly effective thrombectomy devices are being used more widely for patients with LVO (Wasselius et al., 2022). As a result, anesthetic drugs are more widely available to stroke patients than ever before. However, it remains unclear which type of anesthesia and what kind of anesthetic drug used in EVT are better for reducing postoperative complications and improving the prognosis.

General anesthesia or conscious sedation

Thus far, the best anesthetic strategy during EVT is still a matter of debate. GA and CS are the two main anesthetic methods used in EVT. While allowing for immobility and airway control, GA can delay endovascular treatments and may be associated with hemodynamic instability. On the other hand, CS is faster and allows for neurologic assessment during a procedure, but thrombectomy can be less safe due to patient movement. As for which type of an anesthesia is better for the prognosis of patients, the views are constantly changing with the deepening of research. More than 10 years ago, a non-randomized retrospective study performed in 12 stroke centers in the United States demonstrated that GA was related to poorer neurological outcomes after 3 months (OR = 2.33; 95% CI, 1.63–3.44; $P < 0.0001$) (Abou-Chebl et al., 2010). In the same year, another study compared the safety and clinical outcomes between GA with intubation and CS in a non-intubated state (NIS). This study reported that a NIS was associated with lower infarct volume (OR = 0.25, $P = 0.004$) and better clinical outcomes (OR = 3.06, $P = 0.042$) (Jumaa et al., 2010). Although the same conclusion was drawn in the subsequent meta-analysis, the authors noted that patients receiving GA had higher average National Institute of Health Stroke Scale (NIHSS) scores in the 6 studies included (Brinjikji et al., 2015). This finding means that non-randomized retrospective studies have some methodological limitations (Talke et al., 2014; van den Berg et al., 2015). The stroke severity at baseline in the GA group and the CS group was inevitably imbalanced because the anesthetic protocol was decided by teams rather than by randomization (Albers et al., 2017). As a result, the severity of stroke in the GA group would be more severe than that in the CS group due to selection bias, which may have prevented drawing correct conclusions (Brinjikji et al., 2015; van den Berg et al., 2015).

Recently, a series of large-scale multicentre RCT studies on this topic have been carried out (Goyal et al., 2016), and different conclusions from previous retrospective studies have been drawn. The authors found that the functional outcomes of patients undergoing EVT after 3 months were similar in patients receiving GA and those receiving CS (relative risk, 0.91; 95% CI, 0.69–1.19), and even better recanalization was observed in the GA group (Goyal et al., 2016). In a meta-analysis including 3 RCTs [SIESTA (Schonenberger et al., 2016), ANSTROKE (Lowhagen et al., 2017), and GOLIATH (Simonsen et al., 2018)] and 368 patients with AIS in the anterior circulation, the application of GA during EVT was significantly associated with less disability on the 90th day (OR = 1.58; 95% CI, 1.09–2.29; $P = 0.02$) than the application of procedural sedation (Schonenberger et al., 2019). This may be because GA provides a more comfortable environment for the surgeon during EVT that will be safer and easier with a motionless patient (Chen et al., 2009). Recently, Simonsen et al. (2022) performed a mediator analysis to explore whether the better outcome in patients receiving GA was mediated by better recanalization and a higher reperfusion rate. Their meta-analysis also included 3 RCTs and 368 patients [SIESTA (Schonenberger et al., 2016), ANSTROKE (Lowhagen et al., 2017), and GOLIATH (Simonsen et al., 2018)]. The mediator analysis demonstrated that the indirect effect (i.e., better reperfusion) on outcome was small

[risk difference (RD) = 0.03], and the direct effect of GA itself on outcome was much more significant (RD = 0.12). Moreover, they observed that even for non-reperfused patients, GA resulted in a better outcome than CS (Simonsen et al., 2022). This finding suggested the direct effects of GA, such as neuroprotection, as the source of a better outcome. An RCT in patients undergoing EVT where GA is induced by different anesthetic drugs could be valuable.

Before a definite conclusion is drawn, either GA or CS seems reasonable because GA and CS have their own advantages in EVT (summarized in **Table 1**). The American Heart Association and American Stroke Association guidelines advised selecting an anesthesia technique during EVT according to clinical characteristics, patient risk factors, and the technical performance of the procedure rather than a fixed anesthesia technique (Xie et al., 2016).

Different influences on haemodynamics

Improving collateral blood flow is a potential approach to protect the penumbra before recanalization (ENOS Trial Investigators, 2015; Savitz et al., 2019). Anesthetic agents can directly affect vessels and endogenous regulatory mechanisms (Nowak et al., 1984). Blood pressure reduction during EVT could impair collateral perfusion (Froehler et al., 2012). At present, there are no studies that have directly evaluated the haemodynamic effects of different anesthetic drugs on patients undergoing EVT. However, we can make some inferences from past research. Therefore, here, we summarize the findings of some past studies focusing on the effects of anesthetic drugs on cerebral haemodynamics.

(1) It has been debated for many years whether ketamine can be used as an anesthetic for neurologically compromised patients (Gregers et al., 2020). Early studies in the 1970s and 1980s reported that ketamine increased intracranial pressure (ICP), leading to a reduction in cerebral blood flow (CBF) and oxygen supply (Evans et al., 1971; List et al., 1972; Wyte et al., 1972; Nelson et al., 1980). However, subsequent studies found that when combined with propofol, ketamine (1.5, 3, and 5 $mg.kg^{-1}$) could decrease ICP in patients with traumatic brain injury (Albanese et al., 1997). Subanaesthetic doses of ketamine increased regional cerebral blood flow (rCBF) in the frontal cortex (25.4% increase from baseline, $P < 0.001$) but did not change the regional metabolic rate of oxygen ($rCMRO_2$) (Langsjo et al., 2003). A recent meta-analysis including 11 studies with a total of 334 patients showed that there was no evidence indicating that the application of ketamine worsened the cerebral condition (Gregers et al., 2020). It is currently thought that ketamine administration does not result in increased ICP when used as a part of a typical modern anesthesia protocol, and ketamine can be used safely in neurologically impaired patients (Himmelseher and Durieux, 2005; Slupe and Kirsch, 2018). However, no relevant studies have evaluated the safety of ketamine in EVT.

(2) Hypotension is a common side effect of propofol. As a result, the application of propofol in EVT necessitates higher requirements for blood pressure control since a drop of more than 40% in mean arterial blood pressure during EVT in GA is an independent risk factor for poor neurological outcomes (Lowhagen et al., 2015). Blood pressure is one of the determinants of CBF. In a study where positron emission tomography (PET) was used to quantify the effect of propofol on CBF and $rCMRO_2$, propofol reduced rCBF and $rCMRO_2$ to approximately 60% of the baseline at a concentration producing a bispectral index value of 40 (Slupe and Kirsch, 2018). Another similar study also showed a roughly equal reduction in $rCMRO_2$ and rCBF (Himmelseher and Durieux, 2005), indicating that propofol could preserve the regional ratio between rCBF and $rCMRO_2$. Thus, propofol has become an anesthetic in neurosurgical procedures (Gregers et al., 2020), but the haemodynamics of propofol in EVT should be further studied because haemodynamics do not change equally across the whole brain during EVT. Previous study findings may not apply to EVT.

(3) Volatile anesthetics such as sevoflurane and isoflurane have an intrinsic cerebral vasodilatory effect (Matta et al., 1999) that is related to the activation of adenosine triphosphate-sensitive K^+ channels (Iida et al., 1998). Unlike propofol, sevoflurane and isoflurane at 1 minimum alveolar concentration (MAC) can increase CBF but decrease $CMRO_2$ (Oshima et al., 2003), and this property may contribute partly to preventing postoperative ischaemic stroke. A retrospective cohort study that included 314,932 patients undergoing GA showed that volatile anesthesia was related to lower odds of postoperative ischaemic stroke compared with total intravenous anesthesia by propofol (Raub et al., 2021). However, in regard to application in EVT, the lesion and CBF autoregulation caused by volatile anesthetics should be considered. Autoregulation is a vasodilator reflex that maintains CBF within the physiological range under normal circumstances and helps build collateral blood supply around the ischaemic core after stroke (Hoffmann et al., 2016). It was reported that volatile anesthetics can impair autoregulation in rats and dogs (Archer et al., 2017; Esposito et al., 2020) and have similar effects in humans (Strebel et al., 1995; Goettel et al., 2016). It is necessary to carry out further research on the effects of volatile anesthetics.

TABLE 1 Comparison of the advantages of general anesthesia and conscious sedation in EVT.

General anesthesia	Conscious sedation
Improve procedural conditions (Maurice et al., 2022)	Less haemodynamic instability (Davis et al., 2012)
Facilitate airway management	A shorter delay from arrival at the neurointerventional suite to groin puncture (Schonenberger et al., 2016)
Less pain, anxiety, and agitation and low aspiration risk (Emiru et al., 2014)	Fewer ventilation-associated complications (Takahashi et al., 2014)

Neuroprotection in EVT

During AIS, a sudden decrease in blood flow to the brain area supplied by the blocked artery occurs, which is not uniform across the whole ischaemic area. The ischaemic core is the area in which < 20% of basal blood flow remains, and the penumbra is defined as the area where approximately 40% of the basal blood

flow is maintained by collateral circulation (Zhao et al., 1997). The concept of neuroprotection involves preventing extraneuronal cell death by protecting the salvageable penumbral region around the ischaemic core after an ischaemic insult (Ginsberg, 2008). Although decades of failures have been experienced in clinical trials on neuroprotection, and none of the neuroprotective drugs have been approved for treatment, numerous studies are still ongoing.

Endovascular thrombectomy within 24 h of symptom onset could benefit patients with LVO (Berkhemer et al., 2015; Bracard et al., 2016; Nogueira et al., 2018). However, nearly 50% of patients may still undergo "futile recanalization" (Xu et al., 2020), which means that the recanalization of the occluded vessel fails to improve the neurological outcome (Nie et al., 2018). The no-reflow phenomenon after EVT may be one of the causes of futile recanalization. This phenomenon is defined as severe tissue hypoperfusion despite timely recanalization of an occluded artery, which may be due to abnormalities at the level of the microvasculature. Microvascular obstruction from endothelial cell swelling, pericyte contraction, luminal clogging with leukocytes and microthrombi can impede the reperfusion after EVT because EVT only clears blockages in large arteries (Nie et al., 2023). In clinical studies, the incidence of the no-reflow phenomenon after EVT has ranged from 25 to 38% (Ng et al., 2018; Rubiera et al., 2020; Ter Schiphorst et al., 2021). Another important cause of futile recanalization is cerebral ischaemia–reperfusion injury (Stoll and Nieswandt, 2019). During reperfusion, reactive oxygen species (ROS) are produced by the xanthine (XO) system, the NADPH oxidase (NOX) system, and the mitochondrial enzymatic system (Granger and Kvietys, 2015), leading to direct cellular damage and indirect damage, such as inflammation. Moreover, ROS can result in apoptosis and necrosis through lipid peroxidation and DNA/RNA damage (Mizuma et al., 2018). A more detailed mechanism is shown in **Figure 1**. Experimental studies showed that transient middle cerebral artery occlusion (3-hour occlusion and 3-hour reperfusion) in rats caused a larger infarct volume and blood–brain barrier disruption than permanent middle cerebral artery occlusion (6 h) (Yang and Betz, 1994). In clinical research, a similar ischaemia–reperfusion injury was indirectly observed in magnetic resonance imaging through a hyperintense acute reperfusion marker (Warach and Latour, 2004), suggesting that ischaemia–reperfusion injury also exists in humans. Therefore, neuroprotective drugs are particularly needed in EVT.

According to the Stroke Therapy Academic Industry Roundtable X (STAIR), in the current era of EVT, neuroprotective agents need to work synergistically with endovascular therapy to reduce ischaemia–reperfusion injury rather than work as monotherapies (Savitz et al., 2019). Perhaps the treatment of stroke is similar to precision surgery, which requires much cooperation. Neuroprotection in the new era should be verified on the basis of endovascular therapy. Therefore, many neuroprotective drugs that failed in clinical trials are currently being revisited (Yang et al., 2019). However, before that, anesthetics should be examined first in EVT, since anesthetics will be confounding factors in the validation of other drugs. For example, the neuroprotective effects of a tested drug might be masked if anesthetics also act on the same pathway.

The neuroprotective properties of some anesthetic drugs in basic research

Over the decades, accumulating evidence has displayed the neuroprotective effects of anesthetic drugs involving multiple mechanisms and pathways. Here, we have selected several anesthetic drugs commonly used in clinical practice that have the neuroprotective potential for a brief discussion. We focus more on differences in the properties of different anesthetics and some studies with contradictory findings that may explain why these medicines "lose" their neuroprotective effects when used clinically.

Ketamine

Ketamine is a phenyl cyclohexylamine derivative that consists of two optical enantiomers, (S)- and (R)- ketamine. The anesthetic properties of ketamine are mainly attributed to the direct inhibition of the N-methyl-D-aspartate receptors (NMDARs). Other lower-affinity pharmacological targets of ketamine include γ-aminobutyric acid (GABA) receptors, dopamine receptors, serotonin opioid receptors, cholinergic receptors, hyperpolarization-activated cyclic nucleotide-gated channels, and so on (Paoletti et al., 2013). The mechanisms of brain injury after stroke include excessive activation of NMDARs, an imbalance in intracellular and extracellular calcium concentrations, neuroinflammation, NO production, ROS production, apoptosis, and so on (Campbell et al., 2019). Blocking one of these mechanisms alone has only a limited effect. Studies on ketamine have found that its neuroprotective mechanism also involves multiple pathways and mechanisms.

N-methyl-D-aspartate receptors (NMDARs), ionotropic glutamatergic receptors, are permeable to calcium ions (Ca^{2+}). These channels are blocked by magnesium at resting membrane potentials. However, when they are depolarized, the magnesium will be removed, and NMDAR conduction will be substantially higher (Nowak et al., 1984). In pathological conditions such as stroke, NMDAR overstimulation causes a series of Ca^{2+}-dependent cascades of events (shown in **Figure 2**), which ultimately lead to neuronal demise. This process is excitotoxicity (Granzotto et al., 2022). Ketamine is a non-competitive inhibitor of NMDARs, and it can act on NMDARs in two ways. One is to block the open channel directly; the other is to act on the binding site outside the channel and indirectly affect NMDARs through an allosteric effect, reducing the number and frequency of NMDAR openings (Orser et al., 1997). In addition to the effects on NMDARs, ketamine has also been reported to affect glutamate release. A recent study showed that ketamine could reduce neuronal glutamate release by stimulating presynaptic adenosine A1 receptors (Lazarevic et al., 2021). However, other studies have demonstrated that ketamine application increases synaptic glutamate release (Abdallah et al., 2018; Lisek et al., 2017). This may be the result of differences in experimental design as well as in measurement methods.

Spreading depolarization (SD) is a kind of pathological wave that contributes to secondary lesions after stroke. The cumulative effect of many SDs is the same as a single persistent depolarization, leading to cell death and delayed lesions (Hartings et al., 2017).

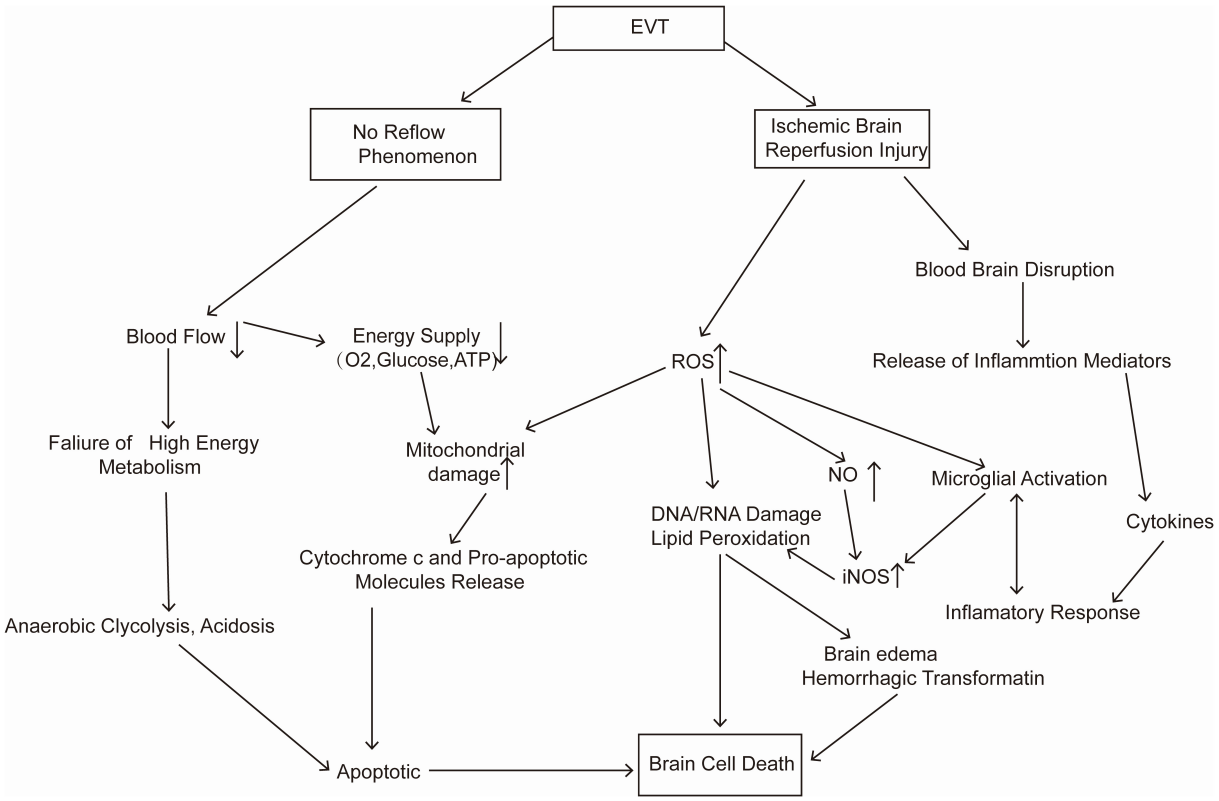

FIGURE 1
Pathological changes after endovascular thrombectomy (EVT).

It has been proven that ketamine can suppress SD in acute brain injury (Carlson et al., 2018). In a retrospective international multicentre analysis, the administration of ketamine was associated with a reduction in spreading depolarizations (OR = 0.38; 95% CI, 0.18–0.79; p = 0.01) (Hertle et al., 2012). Moreover, Reinhart and Shuttleworth (2018) found that applying a lower concentration of ketamine (30 μM, brain slice) does not completely prevent SD but prevents its damaging consequences and retains the potential protective effect of SD. This finding is consistent with the study by Shu et al. (2012) in which they found that low-dose ketamine (25 mg.kg^{-1}, intraperitoneal injection in rats) has a smaller infarct volume than high-dose ketamine (50 or 100 mg.kg^{-1}, intraperitoneal injection in rats) in the treatment of stroke. However, there are also studies drawing contradictory conclusions. Some studies have shown that higher doses (60 and 90 mg.kg^{-1}, intraperitoneal injection in rats) of ketamine improve neurological outcomes, but low doses do not (Reeker et al., 2000; Proescholdt et al., 2001). This difference may be associated with the different properties of R-ketamine and S-ketamine. Studies on S-ketamine tended to use high doses (Reeker et al., 2000; Proescholdt et al., 2001), whereas R-ketamine showed neuroprotective effects at low doses (Xiong et al., 2020). In an ongoing study in our laboratory, S-ketamine also initially showed a dose-dependent effect. The specific mechanism is being further studied.

Neuroinflammation and apoptosis are not only the result of the loss of ion homeostasis caused by NMDAR overactivation but also the cause of neuronal cell death. Ketamine has been proven to inhibit neuroinflammation (Tanaka et al., 2013; Liu et al., 2016; Wang et al., 2021) and apoptosis (Engelhard et al., 2003; Shu et al., 2012; Qi et al., 2020). Inflammatory factors and apoptosis-related molecules are dynamically changed in stroke patients. They not only change with time but also change drastically after recanalization in EVT. The timing and method of ketamine administration can significantly impact the outcome. In mice, applying ketamine by intraperitoneal injection immediately after ischaemia onset could not remarkably induce a significant change in infarct volume. However, injection immediately after the onset of ischaemia–reperfusion significantly reduced infarct volume (Xiao et al., 2012). Similarly, a preclinical study has shown that ketamine dramatically reduced infarct volume when combined with IV-rtPA. However, ketamine alone could not achieve this effect (Gakuba et al., 2011), which might be related to the upregulation of NMDARs after ischaemia–reperfusion (Sutcu et al., 2005). Many studies have confirmed that NMDARs are related to ischaemia–reperfusion injury, and antagonizing NMDARs can reduce ischaemia–reperfusion injury (Kaur et al., 2016; Xie et al., 2016; Singh et al., 2017). Using ketamine in combination with IV-rtPA may be a promising way to extend the time window of IV-rtPA. However, routine treatment with rtPA does not require the use of ketamine. In regard to EVT, anesthetic drugs are routinely used. If relevant studies could confirm that ketamine can reduce ischaemia–reperfusion injury and prolong the time window of EVT application, it will change the current situation where anesthesia during EVT is based on the experience and habits of anesthesiologists and lead to a better prognosis for patients.

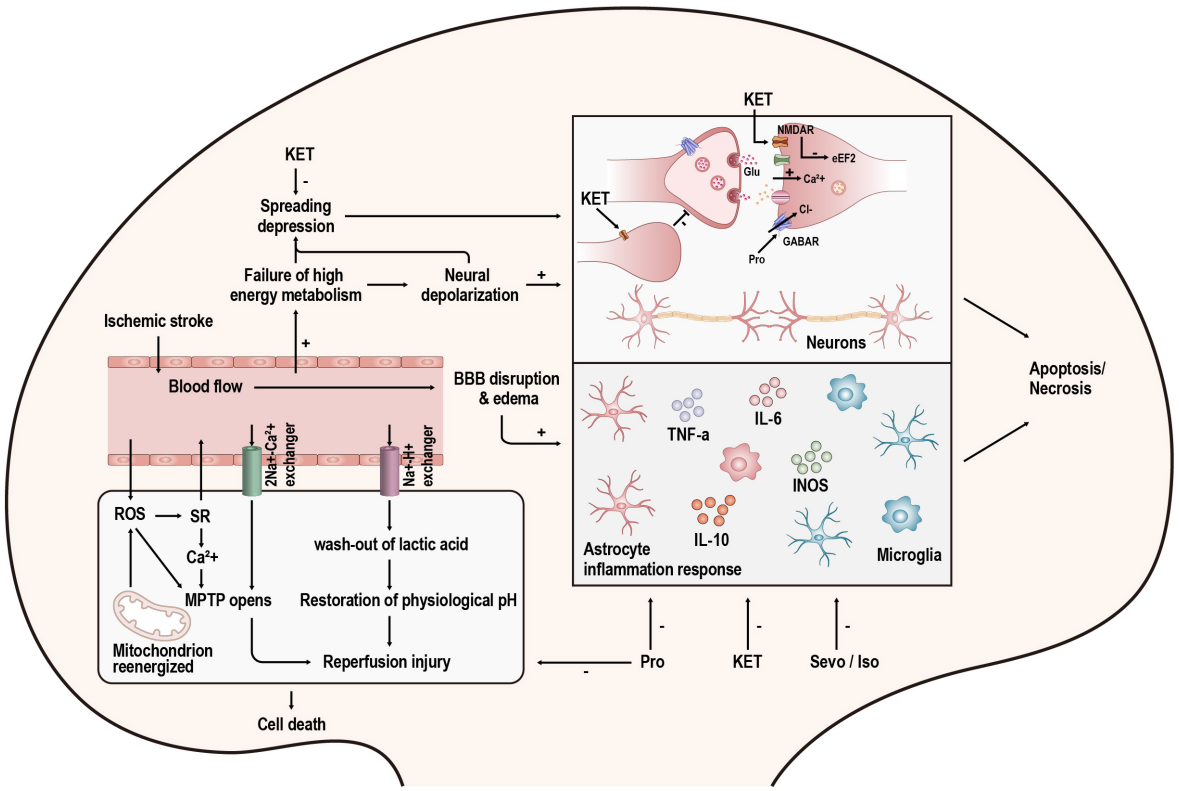

FIGURE 2
Overview of the mechanisms of injury after stroke and the targets of anesthetics. KET, ketamine; Pro, propofol; Sevo, sevoflurane; Iso, isoflurane.

Propofol

Propofol is a widely used intravenous agent. Experimental studies have shown that propofol might protect the brain from ischaemic stroke (Bayona et al., 2004; Ulbrich et al., 2016; Wang et al., 2016). When propofol is used as an anesthetic drug for the induction and maintenance of anesthesia, it mainly acts by activating γ-aminobutyric acid ($GABA_A$) receptors (Walsh, 2018). However, the function of $GABA_A$ receptors in neuroprotection is complicated.

γ-aminobutyric acid (GABA) signaling has two forms. Tonic GABA signaling is a form of extrasynaptic GABA receptor-mediated inhibition. Reducing excessive GABA-mediated tonic inhibition promoted the recovery of motor function after stroke (Clarkson et al., 2010), indicating that excessive tonic inhibition is detrimental to the recovery of function. One of the interpretations was that the cortical hypometabolism caused by excessive astrocytic GABA would prevent functional recovery (Nam et al., 2020). At clinically relevant concentrations, propofol can affect extrasynaptic GABA receptors, although the effect is small (Wakita et al., 2013). Thus, propofol mainly affects the GABA receptors at the synapse rather than the extrasynaptic GABA receptors, which mediate a classic form of inhibition called phasic GABAergic inhibition. In the acute phase of stroke, enhancing phasic GABAergic inhibition can reduce excitotoxic neuron death (Lyden and Hedges, 1992; Green et al., 2000). Similarly, motor function can be improved when a GABA-positive allosteric modulator is used to enhance phasic GABAergic signaling during the repair phase (Hiu et al., 2016). However, due to the lack of direct evidence, further research on whether propofol exerts neuroprotective effects by enhancing phasic GABAergic signaling is needed.

Many studies have demonstrated the anti-apoptosis and anti-inflammation characteristics of propofol (Kotani et al., 2008; Fan et al., 2015; Peng et al., 2020; Qi et al., 2020). In addition to these classic effects, propofol has several other properties. It has a similar chemical structure to antioxidant substances such as vitamin E. It was reported that propofol could scavenge ROS, inhibit the generation of free radicals, and reduce lipid peroxidation to protect the brain from oxidative injury (Cheng et al., 2002; Kobayashi et al., 2008). Moreover, cell ferroptosis is one of the cell death processes correlated with overwhelming lipid peroxidation and cellular ROS. Recently, it was revealed that propofol may help attenuate ferroptosis in HT-22 cells treated with a ferroptosis activator (Erastin) (Xuan et al., 2022), providing a new therapeutic method to treat cerebral ischaemia. However, when used in cancer therapy, propofol appeared to enhance ferroptosis (Zhao and Chen, 2021; Zhao et al., 2022). Further study of the two opposing effects of propofol on ferroptosis is needed. Parthanatos is another form of programmed cell death induced by ROS. Zhong et al. (2018) found that propofol could inhibit parthanatos by impeding calcium release from the endoplasmic reticulum, ROS overproduction, and mitochondrial swelling.

As mentioned earlier, the excitotoxicity caused by glutamate and NMDARs has an essential impact on cerebral ischaemic injury. Propofol could inhibit NMDARs in some studies (Wu et al., 2018; Zhou et al., 2018), but the doses of propofol they used

in their experiments exceeded clinically relevant concentrations. Another study on the effects of propofol on NMDAR-mediated calcium increase in neurons revealed that the overall effects of propofol were minor when the propofol concentration was at clinically relevant concentrations (Grasshoff and Gillessen, 2005). Therefore, the neuroprotective effect of propofol may be partly through the inhibition of NMDARs, but that is not the primary mechanism. Moreover, propofol may prevent excitotoxicity in other ways. Numerous studies have demonstrated that propofol can reduce glutamate concentrations during cerebral ischaemia by decreasing glutamate release (Ratnakumari and Hemmings, 1997; Lingamaneni et al., 2001) and increasing glutamate uptake (Cai et al., 2011; Gong et al., 2016). However, the glutamate concentration may not necessarily play a decisive role in the neuroprotective effect of propofol. Yano et al. (2000) found that propofol and Intralipid (a vehicle for propofol) could similarly reduce glutamate increase in CA1. In contrast, propofol, but not Intralipid, alleviated delayed CA1 neuron death when administered intracerebroventricularly in a transient global forebrain ischaemic model (Yano et al., 2000).

Hypothermia has been demonstrated to be an effective way to alleviate the damage caused by stroke (Gonzalez-Ibarra et al., 2011). When the ischaemic cascade is activated, therapeutic hypothermia can alleviate central nervous

TABLE 2 Clinical studies on the neuroprotective effects of propofol, ketamine, sevoflurane and isoflurane.

References	Research type	Comparison of drug treatment	Experimental subjects	Outcomes
Bhutta et al., 2012	RCT (n = 24)	Ketamine (2 mg.kg^{-1}) vs. placebo (saline)	Infants undergoing cardiopulmonary surgery	No evidence for neuroprotection or neurotoxicity.
Loo et al., 2012	RCT (n = 46)	Ketamine (0.5 mg.kg^{-1}) or placebo (saline)	Patients undergoing electroconvulsive therapy	Slight improvement in the first week of treatment.
Nagels et al., 2004	RCT (n = 106)	S (+)-ketamine (2.5 mg.kg^{-1}) vs. remifentanil	Patients undergoing open-heart surgery	No greater neuroprotective effects than with remifentanil.
Hudetz et al., 2009	RCT (n = 26)	Ketamine (0.5 mg.kg^{-1}) vs. placebo (saline)	Patients undergoing open-heart surgery	Ketamine attenuated POCD 1 week after cardiac surgery.
Guo et al., 2019	RCT (n = 60)	Propofol (1.2 μg.ml^{-1}, TCI, plasma target concentration) vs. 0.5–2% sevoflurane	Patients undergoing aneurysm clipping	Propofol may protect the brain from oxidative stress injury up to 7 days.
Tanguy et al., 2012	RCT (n = 59)	Propofol (depending on the procedure requirements) vs. midazolam (depending on the procedure requirements)	Patients with severe traumatic brain injury	Results did not support a difference between propofol and midazolam for sedation in traumatic brain injury.
Kanbak et al., 2004	RCT (n = 20)	Isoflurane (1 to 1.5% until CPB and 0.5 to 1% during CPB) vs. propofol (6 mg.kg^{-1}.h^{-1} until CPB and 3 mg.kg^{-1}.h^{-1} during CPB)	Patients undergoing coronary artery bypass grafting	Propofol appeared to offer no advantage over isoflurane for cerebral protection during cardiopulmonary bypass.
Schoen et al., 2011	RCT (n = 128)	Propofol (3–5 mg.kg^{-1}.h^{-1}) vs. sevoflurane (0.6-1MAC)	Patients undergoing on-pump cardiac surgery	Sevoflurane-based anesthesia was associated with better short-term postoperative cognitive performance than propofol.
Mahajan et al., 2014	RCT (n = 66)	Propofol (attain a burst suppression ratio of 75 ± 5% in bispectral index monitoring) vs. placebo (saline)	Patients undergoing temporary clipping during intracranial aneurysm surgery	Propofol did not offer any neuroprotective effects on improving postoperative cognition.
Roach et al., 1999	RCT (n = 225)	Propofol (computer-assisted continuous infusion titrated to achieve EEG burst suppression) and sufentanil (5 μg.kg^{-1}) vs. sufentanil (5 μg.kg^{-1})	Patients undergoing cardiac valve replacement	Propofol did not significantly reduce the incidence or severity of neurologic or neuropsychologic dysfunction.
Wu B. et al., 2019	RCT (n = 80)	Propofol (depending on the procedure requirements) vs. dexmedetomidine (depending on the procedure requirements)	Patients undergoing endovascular therapy	This study did not show any difference between propofol and dexmedetomidine in good outcomes or in-hospital mortality.
Yoon et al., 2020	RCT (n = 152)	Sevoflurane vs. no intervention	Patients with moyamoya disease undergoing revascularization surgery	Sevoflurane postconditioning did not reduce the incidence of SCH after revascularization surgery in patients with moyamoya disease.
Dabrowski et al., 2012	Observational study (n = 128)	Sevoflurane vs. isoflurane vs. control	Patients undergoing coronary artery bypass graft surgery	Isoflurane and sevoflurane reduced brain injury markers such as plasma matrix metalloproteinase-9 and glial fibrillary acidic protein.

POCD, postoperative cognitive dysfunction; CPB, cardiopulmonary bypass.

system hyperexcitability by reducing extracellular levels of excitatory neurotransmitters such as dopamine and glutamate. Hypothermia also protects the brain from ischaemic injury by reducing cerebral blood flow, oxygen and glucose consumption, and metabolic rate. The decrease in cerebral metabolic demands results in slower enzyme activity, allowing Adenosine triphosphate (ATP) stores to be preserved (Otto, 2015). When used in GA, propofol can induce heat redistribution from the core to the periphery by impairing thermoregulatory vasoconstriction and preventing shivering (Noguchi et al., 2002). However, it is difficult to quantify the extent to which the decrease in body temperature caused by propofol plays a role in its neuroprotective effect. This is because in basic experiments, we often use holding devices to ensure that the body temperature of the animals is constant and to prevent the neuroprotective effects of hypothermia from interfering with the experiment. In the context of temperature control, there are still basic research studies that confirm the neuroprotective effect of propofol (Fan et al., 2022).

The neuroprotective effect of propofol involves multiple mechanisms, but whether propofol can improve the long-term prognosis of stroke is uncertain. A study found that using propofol to treat cerebral ischaemia can significantly enhance the infarct volume and motor function on the third day after treatment. However, there was no difference in infarct volume on the 21st day in the propofol group compared with the control group (Bayona et al., 2004). In addition, in a preclinical trial of propofol combined with IV-rtPA, propofol failed to reduce infarct size after thrombolysis (Gakuba et al., 2011). Some clinical studies did not support the neuroprotective effect of propofol (as shown in **Table 1**). The reasons for this difference will be discussed in detail in the second part.

Sevoflurane and isoflurane

Sevoflurane and isoflurane are both commonly used volatile anesthetics for the induction and maintenance of GA. The targets of these inhaled anesthetics include but are not limited to GABARs, NMDARs, and TWIK-related K^+ channels (TREK-1) (Orser et al., 2019). As mentioned before, NMDARs play a vital role in excitotoxicity. Although volatile anesthetics can protect against excitotoxicity partly by inhibiting NMDARs, the efficiency of volatile anesthetics is less than selective NMDAR antagonism (Kudo et al., 2001). Thus, the neuroprotective effect of volatile anesthetics partly contributes to NMDAR inhibition, but this is not the main mechanism.

Some existing studies have indicated that sevoflurane and isoflurane can reduce ischaemia and ischaemia–reperfusion injury by affecting inflammatory and apoptotic processes (Bedirli et al., 2012; Hwang et al., 2017; Zhang and Zhang, 2018; Yang et al., 2022). A recent review of the neuroprotective mechanisms of sevoflurane and isoflurane specifically summarized how they affect classic inflammatory and apoptotic pathways (Neag et al., 2020). However, not all reports about inhaled anesthetics are positive (Zhang et al., 2016; Wu et al., 2020). Orset et al. (2007) developed a mouse model of thromboembolic stroke that is closer to the physiological situation than traditional stroke models. Then, Gakuba et al. (2011) used this model to assess the different effects of the combination of anesthetics and IV-rtPA on the infarct volume. Unexpectedly, isoflurane and propofol failed to enhance the benefits brought by rtPA-induced thrombolysis (Gakuba et al., 2011). Moreover, sevoflurane applied in different models can even have the opposite effect. When used in rats that were subjected to brain hypoxia-ischaemia, sevoflurane could protect the brain by inhibiting apoptosis (Ren et al., 2014). However, sevoflurane showed neurotoxicity and tended to exacerbate apoptosis when rat pups were exposed to it for as long as 4 h (Shan et al., 2018).

Therefore, it seems that simply evaluating whether a drug is neuroprotective is unscientific. The protective effect is based on a specific environment, and the application of the same medication to different subjects at different doses can even produce opposite effects. For example, the effects of anesthetic drugs on NMDARs, GABARs, or some other receptors may be detrimental in some patients but may reduce excitotoxicity in patients experiencing cerebral ischaemia. The narrow concept of neuroprotection is based on the condition of ischaemia, and it is a process that reduces brain injury after the onset of stroke (Ginsberg, 2008). Therefore, our clinical research on the neuroprotective effects of anesthetics should be precisely linked to stroke. However, many clinical studies in the past have used other diseases and surgeries to study the neuroprotective effects of anesthetics (summarized in **Table 2**). Past studies may not accurately evaluate the neuroprotective effect of anesthetic drugs.

The considerable gap between clinical trials and basic research

Patients undergoing endovascular therapy need GA or CS to undergo the procedure. However, there are few guidelines to help in the selection of anesthetic drugs. Although the findings from many basic research studies support the neuroprotective effects of anesthetics (Sanders et al., 2005), the results are ambiguous when evaluating anesthetic neuroprotective effects in clinical trials. Here, two authors independently searched PubMed and Medline for randomized controlled trials published between 1 January 1995 and 1 September 2021, using the permutation and combination of the keyword terms "neuroprotective," "neuroprotection," "ischaemia," "ketamine," "propofol," "sevoflurane," and "isoflurane"; excluded the studies that were not relevant to the theme of this paper after discussion; and finally summarized the results in **Table 2**. We can see in **Table 2** that the conclusions of these clinical trials are not unified, and some are even contradictory. Here, we discuss why there is a considerable gap between clinical trials and basic research.

Defects in basic research

According to the STAIR criteria (Fisher et al., 2009), a large number of studies on neuroprotection seem to exhibit low methodological quality. Here, we summarize some common defects in research on the neuroprotective effects of anesthetics.

(1) Some basic research focuses more on infarct volume (Shu et al., 2012; Xiong et al., 2020) than on subsequent outcome several months later, which is commonly evaluated through the modified

Rankin Scale (mRS) in clinical trials (Saver et al., 2021). The latest STAIR trial advised that the main endpoints should include not only infarct volume but also behavioral outcomes, gray versus white matter protection, and the potential negative effects of the agent tested (Savitz et al., 2019).

(2) Another problem is the incompatibility between the doses of medicine used in basic research and those used in clinical practice. Due to receptor affinity, some drugs that show significant neuroprotective effects at concentrations higher than clinically applied often fail to improve patient prognosis after entering clinical studies (Muir, 2006; Morgan et al., 2012), and their clinical application value is limited.

(3) Basic research studies pay more attention to whether an anesthetic drug has a neuroprotective effect, so they tend to determine the timing when the phenomenon is most obvious through preliminary experiments and then proceed from there (Zhou et al., 2013; Yang et al., 2018). However, clinical practice has more demand for the time window of drug application since patients suffering from stroke have a variable duration of ischaemia. If the time window of a drug is very narrow or the time of administration and the method of administration is unrealistic (Saver et al., 2021), its clinical significance is still limited even if a positive result is obtained.

(4) Transient middle cerebral occlusion (tMCAO) is the most widely used model of stroke and has advantages in the study of reperfusion injury. With the continuous development of endovascular therapy, it is increasingly important to research how to reduce ischaemia/reperfusion injury and promote prognosis. However, there is still a large number of patients without vessel recanalization (Yoshimura et al., 2014), which is closer to permanent middle cerebral occlusion (pMCAO). When we evaluate the neuroprotective effects of drugs, pMCAO should also be taken into consideration (McBride and Zhang, 2017).

(5) Sex and age have long been neglected factors. A meta-analysis including 80 publications compared the neuroprotective effects of anesthetics in animals of different sexes and aged animals. It showed neuroprotective effects in female and aged animals (Archer et al., 2017). Although it was based on a *post hoc* analysis and a small number of studies, this meta-analysis raised a thought-provoking question: Are normal male animals appropriate animals in which to simulate human stroke?

(6) Clinical trials mostly test neuroprotectants in active, awake patients. In five large clinical trials of neuroprotectants involving 9,560 patients, only 664 had suffered night-time strokes (Esposito et al., 2020). However, rodent tests are always performed during the day, when they are inactive. The opposite circadian rhythm of rodents to that of humans impacts the effectiveness of neuroprotectants, which may be one reason for translational failure (Esposito et al., 2020; Boltze et al., 2021). Some moderate-quality studies have shown that anesthetic drugs affect circadian rhythms (Orts-Sebastian et al., 2019; Imai et al., 2020; Wang et al., 2020). Therefore, the influence of circadian rhythm must be considered for translational studies on anesthetic neuroprotection.

The transient effects of anesthetic drugs

Common anesthetic drugs such as propofol, ketamine, and volatile anesthetics all have a short half-life in humans (Freiermuth et al., 2016; Peltoniemi et al., 2016; Sahinovic et al., 2018), which is an advantage in fast recovery after anesthesia. However, in regard to neuroprotection, the transient effects of anesthetic drugs may become a disadvantage because some injurious factors can last for a long time. For example, the elevation of excitatory amino acid (EAA) concentrations in MCAO lasts only 1–2 h (Takagi et al., 1993; Baker et al., 1995); however, in humans with AIS, glutamine increase may persist for 24 h or longer (Bullock et al., 1995; Davalos et al., 1997). Moreover, microglial cells, a type of immune cell in the brain, peak in activity 2–3 days after injury (Barthels and Das, 2020), and they can release variable inflammatory factors that lead to secondary injury around the ischaemic core (Yenari et al., 2010). As a result, the injurious factors are still in effect after the neuroprotective effects of anesthetics have passed. In addition, the compensatory function of patients is established within several months after suffering a stroke. This may be the reason why the neuroprotective effect of anesthetic drugs is not significant when we evaluate the recovery of neurological function of patients after several months in clinical studies. In the future, we may be able to introduce some therapies suitable for long-term use to restrict damage-causing factors. Electroacupuncture may be an option. Electroacupuncture, an extension of traditional acupuncture, is used as a complementary treatment with minimal side effects (Wei et al., 2016). Studies have shown that electroacupuncture can attenuate inflammation after ischaemic stroke by inhibiting the activation of microglia (Liu et al., 2020), improving cerebral blood flow, and alleviating neurological deficits (Zheng et al., 2016). As anesthetic drugs are not suitable for prolonged use after EVT, electroacupuncture can be used as an adjunctive technique to help reduce ischaemia–reperfusion injury after recanalization and to promote functional recovery. However, the reporting quality of randomized controlled trials on electroacupuncture for stroke is generally moderate, and further improvement is needed (Wei et al., 2016).

Heterogeneity in experimental subjects

In MCAO, a filament is sent into the middle cerebral artery from the internal or external carotid arteries to mimic stroke, and it allows reperfusion through the withdrawal of the filament (Smith et al., 2015). This kind of reperfusion is different from the pathophysiology of thrombolysis in human stroke because the blood flow is restored promptly. Compared with thrombolysis, MCAO more closely simulates the clinical situation of mechanical thrombectomy (Sommer, 2017). However, past clinical studies on the neuroprotective effects of anesthetic agents were based neither on patients undergoing thrombolysis nor on those undergoing mechanical thrombectomy. As shown in **Table 2**, a large part of the past clinical research is based on other operations or diseases that may cause cerebral ischaemia, such as heart surgery and intracranial aneurysm surgery. These studies are not sufficiently convincing to evaluate whether anesthetic drugs have neuroprotective effects because most of these surgeries cause only transient ischaemia and postoperative cognitive dysfunction, which cannot cause large areas of brain tissue necroptosis such as stroke.

In addition, stroke is a heterogeneous disease with diverse additive risk factors (Caprio and Sorond, 2019). Although strict

inclusion and exclusion criteria and grouping can reduce the effect of patient heterogeneity, the heterogeneity of patients in clinical studies is still more significant than that in animal models. The emergence of EVT provides a good translational platform for drugs that exhibit neuroprotective effects in the MCAO model. Among patients undergoing EVT, we were able to screen out patients with similar proximal intracranial artery occlusion by Computed Tomography (CT) and angiography, in which the heterogeneity of haemodynamics will be smaller and the haemodynamic changes will be much closer to those of MCAO.

There are already large multicentre, double-blind, randomized controlled trials of neuroprotective drugs in EVT patients (Hill et al., 2020). In the future, more rigorous basic research and clinical trials based on EVT will more rationally evaluate the neuroprotective effects of anesthetics. Regardless of the outcome, this research will provide more conclusive answers to decades-old questions on the neuroprotective effects of anesthetics.

Conclusion

Anesthetics have great potential in neuroprotection, involving various mechanisms such as excitotoxicity, SD, inflammation, apoptosis, and ischaemia–reperfusion injury, but this has not been clearly observed in previous clinical trials due to the mismatch between basic research and clinical trials. The emergence of EVT has brought new hope to the study of the neuroprotective effects of anesthetics that once had been shelved. EVT might become a bridge connecting basic and clinical research. Anesthetics have long been confounding factors in translational stroke research. With an increasing number of neuroprotective techniques coming into clinical trials (Baker et al., 1995; Davalos et al., 1997; Sahinovic et al., 2018), it is necessary to determine the effects of anesthetics during EVT, and anesthetists also need a definitive study to guide clinical anesthetic administration.

Author contributions

TZ, DD, SH, and DF contributed substantially to the article concept and manuscript writing. TZ, SH, FX, LM, YW, YD, and KW retrieved the literature and reviewed the manuscript. XC and TW revised and approved the final version before submission. All authors have participated actively in the study and have read and approved the final manuscript.

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Conflict of interest

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

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Ginkgo biloba extract EGb 761® improves cognition and overall condition after ischemic stroke: Results from a pilot randomized trial

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Background: Patients who experienced an ischemic stroke are at risk for cognitive impairment. Quantified *Ginkgo biloba* extract EGb 761® has been used to treat cognitive dysfunction, functional impairment and neuropsychiatric symptoms in mild cognitive impairment and dementia.

Objectives: To assess the cognitive-related effects of EGb 761® treatment in patients after acute ischemic stroke, as well as the feasibility of patient selection and outcome measures.

Methods: We conducted a randomized, multicentric, open-label trial at 7 centers in China. Patients scoring 20 or lower on the National Institutes of Health Stroke Scale were enrolled between 7 and 14 days after stroke onset and randomly assigned to receive 240 mg per day of EGb 761® or no additional therapy for 24 weeks in a 1:1 ratio. Both groups received standard treatments for the prevention of recurrent stroke during the trial. General cognitive function and a battery of cognitive tests for sub-domains were evaluated at 24 weeks. All patients were monitored for adverse events.

Results: 201 patients ≥50 years old were included, with 100 assigned to the EGb 761® group and 101 to the reference group. The mean change from baseline on the global cognitive function as assessed by the Montreal Cognitive Assessment score was 2.92 in the EGb 761® group and 1.33 in the reference group (between-group difference: 1.59 points; 95% confidence interval [CI], 0.51 to 2.67; $p < 0.005$). For cognitive domains, EGb 761® showed greater effects on the Hopkins Verbal Learning Test Total Recall (EGb 761® change 1.40 vs. reference −0.49) and Form 1 of the Shape Trail Test (EGb 761® change −38.2 vs. reference −15.6). Potentially EGb 761®-related adverse events occurred in no more than 3% of patients.

Conclusion: Over the 24-week period, EGb 761® treatment improved overall cognitive performance among patients with mild to moderate ischemic stroke.

Our findings provide valuable recommendations for the design of future trials, including the criteria for patient selection.

Clinical Trial Registration: www.isrctn.com, identifier ISRCTN11815543.

KEYWORDS

Ginkgo biloba extract, ischemic stroke, cognitive function, post-stroke recovery, randomized trial

1 Introduction

Patients surviving stroke are experiencing cognitive impairment faster than those of stroke-free controls and several large population-based studies have reported significant cognitive decline in long-term follow-up (Wang et al., 2012; Levine et al., 2015; Zheng et al., 2019; Lo et al., 2022). To date, the mechanisms, magnitude and predictors of cognitive decline after stroke is still incompletely understood. Clinical characteristics are a key determinant of the variability in dementia incidence among post-stroke patients. The presence of lesion burden, multiple acute infarcts and total infarct volume, all have been shown to predict post-stroke cognitive impairment independently from demographic and vascular risk factors (Pendlebury and Rothwell, 2009; Pendlebury and Rothwell, 2019). There is also a stepwise association between the manifestation of severe small vessel diseases (SVD) or multiple lacunes and delayed-onset dementia after stroke (Mok et al., 2016). Moreover, vascular factors contribute to dementia through cerebral infarcts and white matter changes (Pendlebury and Rothwell, 2019). In particular, Foster *et al.* have provided clinicopathological evidence that pyramidal neuron atrophy in the dorsolateral prefrontal cortex, rather than loss of neuronal numbers, was associated with distinct cognitive deterioration in post-stroke dementia and vascular dementia (Foster et al., 2014). Similarly, neuropsychiatric symptoms (NPS) such as depression, irritability, agitation, apathy and anxiety have also been found in significant proportions (23%–33%) of stroke patients (Angelelli et al., 2004; Zhang et al., 2013; Karakus et al., 2017; Salinas et al., 2017; Villa et al., 2018). Acute stroke treatment is typically followed by secondary prevention in accordance with current guidelines (Wang et al., 2017). This mainly consists of antithrombotic treatments (anti-platelet drugs, anticoagulants), and management of risk factors (such as hypertension, hyperlipidemia, metabolic syndrome or diabetes mellitus). There are limited pharmacological therapies to address subsequent cognitive impairment aside from brain stimulation techniques and physical exercise (Bordet et al., 2017), and little attention has been paid to cholinesterase inhibitors and memantine. Only one randomized controlled trial (RCT) showed the effectiveness of Actovegin on cognitive outcomes in patients with mild-to-moderate ischemic stroke (Guekht et al., 2017), highlighting the need for more evidence from large prospective cohorts. Some patients with post-stroke depression responded to selective serotonin inhibitors, whereas treatments of other NPS appears to be less straightforward (FOCUS Trial Collaboration, 2019; Hankey, 2020; Lundström, 2020).

Ginkgo biloba extract EGb 761® is a standardized product prepared to a ratio of 35–67:1 from dry extract from Ginkgo biloba leaves to final extract, extraction solvent: acetone 60% (weight/weight) (DeFeudis, 2003). It contains 22.0%–27.0% ginkgo flavonoids calculated as ginkgo flavone glycosides and 5.0%–7.0% terpene lactones consisting of 2.8%–3.4% ginkgolides A, B, C and 2.6%–3.2% bilobalide, 7% proanthocyanidins, certain low-molecular-weight organic acids, and less than 5 ppm ginkgolic acids (Müller et al., 2012). It is commercially available as tablets and drops and freshly dissolved in growth medium (EGb 761® is a registered trade mark of Dr. Willmar Schwabe GmbH & Co. KG, Karlsruhe, Germany) (Koltermann et al., 2009). EGb 761® has been proven to in clinical studies enhance perfusion and decrease blood viscosity (Müller et al., 2012). Randomized, placebo-controlled clinical trials have demonstrated that EGb 761® improved cognitive performance and neuropsychiatric symptoms in patients with age-associated mild impairment in cognitive function, vascular dementia and Alzheimer's disease (Gavrilova et al., 2014; Li et al., 2018). Meanwhile, an injectable form of the extract reduced the infarct size, neurological deficits, and further restored motor function with mitochondrial dynamics in a rat model of stroke (Li et al., 2018). These findings suggest that EGb 761® may have a role in the prevention and treatment of cognitive impairment following stroke.

Since there is still a lack of effective drugs that can reliably prevent or treat cognitive decline in this population, we designed this pilot trial to assess whether EGb 761® 240 mg/day for 24 weeks would confer cognitive benefits after an acute stroke; we also planned to evaluate the effect of EGb 761@ in various cognitive domains and on NPS, as well as obtain data which can inform the process of designing a randomized controlled trial with oral EGb 761® treatment in post-stroke cognitive impairment.

2 Methods

2.1 Study design and setting

We conducted a parallel-group, randomized, multicentric, open-label pilot trial at 7 centers of China in accordance with international (International Conference on Harmonization, ICH) and national guidelines for Good Clinical Practice and applicable laws of the People's Republic of China. Details around the centers are described in the supplementary material (Supplementary Table S1). It was approved by the ethics committees of all participating clinical sites. Two amendments to the protocol were also approved by all ethics committees. Informed consent was obtained from all patients before

enrolment in the trial. Clinical trials registration: study ID ISRCTN11815543 on the ISRCTN registry.

2.2 Inclusion and exclusion criteria

Patients of both sexes, at least 50 years old, who had given informed consent and scored no higher than 20 on the National Institutes of Health Stroke Scale (NIHSS) were enrolled during 7–14 days after an acute stroke (Lyden, 2017). Their MRI scans had to indicate acute ischemic cerebral infarction and rule out signs of hemorrhage, tumor, normal pressure hydrocephalus or other serious cerebral disorder. Lacunes, white matter hyperintensities or mild atrophy, or combinations thereof consistent with pre-dementia Alzheimer's disease or cerebrovascular disease were acceptable. Patients had to be able to understand and respond to interview questions, complete questionnaires and take part in neuropsychological testing with the necessary language skills. Each patient needed a regular contact person (e.g., partner, close relative, friend) who was willing to accompany him/her to provide information about the patient's cognitive problems, functional abilities and neuropsychiatric symptoms during the hospital visits.

Patients were excluded from the study if they had any type of dementia or other major neurological disorder (e.g., Parkinson's, Huntington's, Pick's or Creutzfeldt-Jakob disease, seizure disorder), psychiatric disorder (e.g., *major* depression, generalized anxiety disorder), alcohol or substance abuse/addiction, severe and uncontrolled cardiovascular disease, severe renal or hepatic dysfunction, insufficiently controlled diabetes mellitus, clinically significant thyroid dysfunction, vitamin B12 or folic acid deficiency, HIV or syphilitic infection, active malignant disease or any gastrointestinal disease with impaired absorption of orally applied drugs. Moreover, other criteria for exclusion were long-term hospitalization, aphasia, dysarthria, paresis of the dominant upper extremity, severe and insufficiently corrected loss of vision or hearing, severe language difficulties and any other disability that could have prevented valid cognitive testing. Female patients of childbearing potential and patients with known sensitivity to Ginkgo biloba extract were also excluded. In addition, patients who were taking psychoactive drugs (e.g., antidepressants, neuroleptics), anticholinergic drugs, anti-epileptics, anti-coagulants, anti-dementia drugs, cognition enhancers or perfusion-enhancing agents were not allowed to take part in the study.

2.3 Randomization and treatment

Randomization by means of a validated computer program (SAS macro RANSCH) was performed by a member of the biometrics department who was not otherwise involved in conducting of the trial. Eligible patients were identified by the clinician conducting the assessments. Randomization numbers were allocated to the patients in the order of inclusion. To minimize allocation bias, treatment information (EGb 761® or reference group) was contained in sealed envelopes matched to the randomization numbers.

All patients received standard treatment in accordance with current guidelines for the prevention of stroke recurrence, including general supportive care, antiplatelet drugs and treatment for acute complications (Liu et al., 2020); nootropic agents were not allowed for use in this trial. Patients randomized to EGb 761® treatment took 2 tablets at 40 mg EGb 761® three times a day, for a daily dose of 240 mg in addition to standard treatment for a period of 24 weeks. Those randomized to the reference group received standard treatment only.

2.4 Outcome measures

Cognitive assessments: the main interest of this study was the change in cognitive status from baseline assessed by the validated Beijing version of the Montreal Cognitive Assessment (MoCA) at 24 weeks. The MoCA assesses a number of sub-domains of cognition (visuospatial/executive, naming, memory, attention, language, abstraction, delayed recall, orientation) (Julayanont and Nasreddine, 2017). Scores range from 0–30, with lower scores indicating more severe impairment. One point is added for patients who have less than 12 years of education. Cognitive impairment was defined as MoCA <26 (Nasreddine et al., 2005). The cognitive domains evaluated also included the changes in Hopkins Verbal Learning Test–Revised (HVLT-R), a scale that measures verbal learning and memory with lower scores indicating worse functioning (Shi et al., 2012); the Shape Trail Test (STT), checking the set shifting of executive function, with higher scores predicting worse ability (Zhao et al., 2013); a category version of the Verbal Fluency Test (VFT), which assesses the role of frontal lobe function by generating exemplars in the given category within 1 min (Mok et al., 2004); the Digit Symbol Substitution Test (DSST) of the Wechsler Adult Intelligence Scale—Revised (WAIS-R), a test for associative learning by correctly matching symbols to numbers within limited time (Jaeger, 2018).

Clinical status was assessed using the following instruments: the Neuropsychiatric Inventory (NPI), a multidimensional scale for the assessment of neuropsychiatric symptoms, the total composite score ranging from 0 to 144, where higher scores indicates greater abnormalities (Wang et al., 2012); the Hospital Anxiety and Depression scale (HADS), with higher scores indicating more psychological distress (Leung et al., 1999); the Clinical Global Impression of Change (CGI-C), targeting overall change in the patients' mental health within the context of clinical experience (Busner and Targum, 2007); National Institutes of Health Stroke Scale (NIHSS), a measure of patients' neurological status as well as stroke severity (Chalos et al., 2020); and incidence of recurrent stroke. Safety outcomes included vital signs, physical examination, 12-lead electrocardiograms, laboratory tests and recording of adverse events. All efficacy and safety outcomes were documented at baseline, at week 12 and 24. The members of an independent clinical-event adjudication committee confirmed all the assessments since they were unaware of the trial assignments.

2.5 Statistical analysis

Taking into account the exploratory nature of the trial, a sample size of 200 patients (100 per treatment group, 1:1 randomization, assumed drop-out rate of 10%) was chosen to achieve a statistical power of 80%

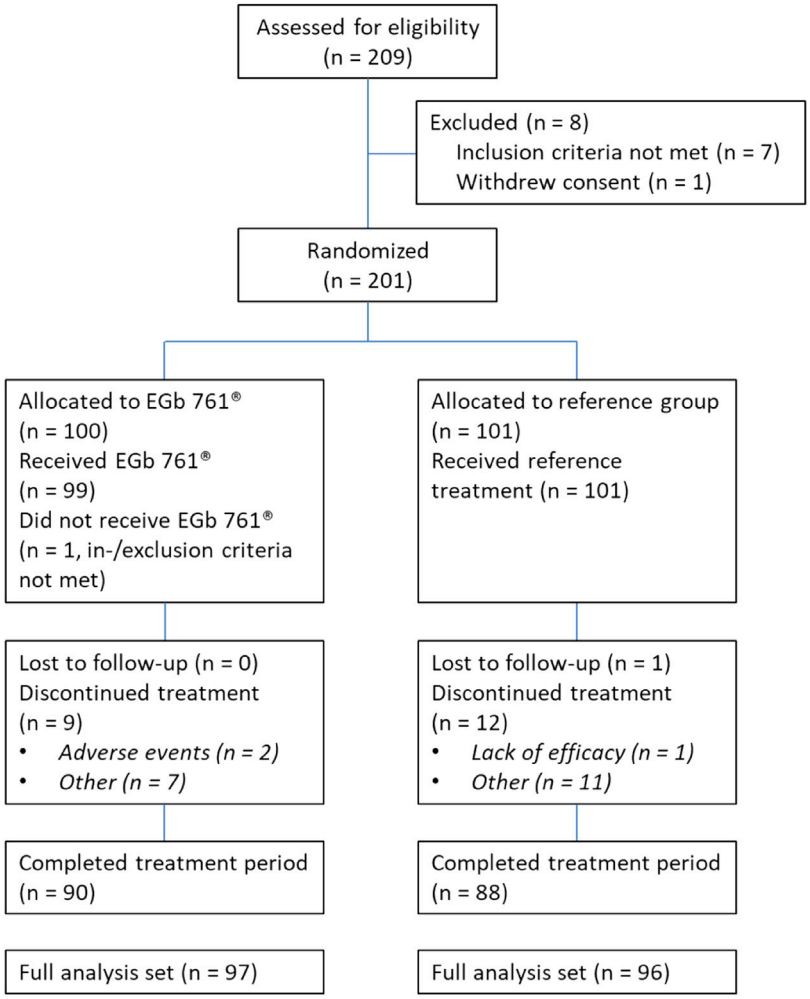

FIGURE 1
Patients flow diagram.

to detect a minimum standardized difference of 0.5 within a two-group multivariate repeated measures design for a two-sided test, three time points, 3 variables at a descriptive significance of $\alpha = 0.05$.

The outcome analyses were predefined using data from the full analysis set (FAS) population. For comparisons within and between treatment groups, two-sided *p*-values were calculated, applying the *t*-test to quantitative variables (paired *t*-test for within-group comparisons) and Fisher's exact test to qualitative variables. Missing values were replaced by the last observation carried forward method. Statistical analysis was performed using SAS software, version 9.2 and higher (SAS Institute Inc., Cary, NC, United States) and SPSS version 24.0 (SPSS Inc., Chicago, IL, United States, 2001).

3 Results

3.1 Characteristics of the study population

Between January 2014 and May 2016, 209 patients were screened for the study. Two hundred one were enrolled and underwent randomization along with standard treatment, with 100 to the EGb 761® group and 101 to the reference group (no additional treatment). Figure 1 shows the patient flow chart. Overall, 23 patients discontinued the trial. The full analysis set (FAS) for the evaluation of treatment effects (EGb 761® group, n = 97; reference group, n = 96) comprised all randomized patients who had at least one cognitive test result or one NPS rating after baseline and all patients randomized to receive EGb 761® who terminated the trial early due to lack of efficacy or an adverse event for which a causal relationship with the study drug could not be ruled out. All patients of the reference group (n = 101) and all patients of the EGb 761® group who took at least one dose of the study drug (n = 99) were included in the safety analysis (safety population, SAF).

Demographic characteristics and scales at baseline were similar between two groups except for the Neuropsychiatric Inventory (NPI) total score (Table 1). The numerically higher mean score and the high standard deviation in the reference group were due to a small number of outliers with high scores. The mild mean National Institutes of Health Stroke Scale (NIHSS) score indicated minimal

TABLE 1 Characteristics of patients at baseline; n (%) or mean (SD); two-sided *p*-values of *t*-test (continuous variables) or Fisher's exact test (categorical variables).

	EGb 761® (n = 97)	Standard treatment (n = 96)	*p*-value
Female [n, %]	26 (26.8%)	20 (20.8%)	0.3988
Male [n, %]	71 (73.2%)	76 (79.2%)	
Age (years) [mean, SD]	62.6 ± 8.3	64.1 ± 8.3	0.2026
NIHSS total score [mean, SD]	1.98 ± 2.22	2.18 ± 2.23	0.5384
MoCA total score [mean, SD]	23.02 ± 4.68	23.47 ± 4.26	0.4876
HVLT total recall [mean, SD]	16.53 ± 4.53	17.20 ± 5.64	0.3628
STT trail 1 [mean, SD]	121.5 ± 109.5	109.7 ± 67.28	0.3669
STT trail 2 [mean, SD]	234.8 ± 176.6	239.6 ± 196.0	0.8588
VFT [mean, SD]	12.91 ± 5.00	12.44 ± 4.22	0.4814
WAIS-R DSST [mean, SD]	25.58 ± 11.97	25.58 ± 12.97	0.9973
NPI total score [mean, SD]	4,90 ± 13,04	8,88 ± 54,83	0.4906
HADS-Anxiety [mean, SD]	4.79 ± 3.99	5.27 ± 3.98	0.4066
HADS-Depression [mean, SD]	4.92 ± 4.01	5.08 ± 4.01	0.7742

Abbreviations: NIHSS, national institutes of health stroke scale; MoCA, montreal cognitive assessment; HVLT, hopkins verbal learning test; STT, shape trail test; VFT, verbal fluency test; WAIS-R, Wechsler Adult Intelligence Scale-Revised; DSST, digit symbol substitution test; NPI, neuropsychiatric inventory; HADS, hospital anxiety and depression scale; SD, standard deviation.

TABLE 2 Efficacy outcomes. Changes from baseline to 24 week in cognitive tests and neuropsychiatric rating scales. Data are n (%) and mean (SD).

	EGb 761® (n = 97) [mean ± SD]	Standard treatment (n = 96) [mean ± SD]	*p*-value
MoCA total score	2.92 ± 3.90	1.33 ± 3.75	<0.005
MoCA delayed recall	0.88 ± 1.42	0.17 ± 1.39	<0.001
MoCA orientation	0.28 ± 0.75	−0.11 ± 0.75	<0.001
MoCA language	0.45 ± 1.05	0.09 ± 1.08	<0.05
HVLT total recall score	1.40 ± 5.47	−0.49 ± 5.20	<0.05
Shape trail test—trail 1	−38.2 ± 93.8	−15.6 ± 55.4	<0.05
Shape trail test—trail 2	−68.8 ± 174.5	−62.7 ± 174.9	n. s
Verbal fluency test	1.21 ± 6.14	−0.16 ± 4.29	n. s
WAIS-R DSST	7.22 ± 14.49	5.60 ± 14.36	n. s
HADS anxiety	−1.79 ± 3.72	−1.45 ± 4.05	n. s
HADS depression	−1.25 ± 4.74	−0.57 ± 4.17	n. s
NPI total score	−3.98 ± 13.86	−6.59 ± 55.68	n. s

neurological deficits and those slight differences between groups did not reach statistical significance.

3.2 Efficacy

A significant difference in total MoCA scores favoring EGb 761® was observed at 24 weeks. The mean change from the baseline MoCA rating at week 24 was 2.92 in the EGb 761® group and 1.33 in the reference group (drug-reference control difference: 1.59 points; 95% confidence interval [CI], 0.51 to 2.67; $p < 0.001$; Table 2; Figure 2). For three sub-domains of the MoCA, scores of delayed recall, orientation and language in the EGb 761® group at 24 weeks showed significantly larger improvements (delayed recall: EGb 761® change 0.88 vs. reference 0.17, 95% CI, 0.31 to 1.10, $p < 0.001$; orientation: EGb 761® change 0.28 vs. reference −0.11, 95% CI, 0.18 to 0.60, $p < 0.001$; language: EGb 761® change 0.45 vs. reference 0.09, 95% CI, 0.06 to 0.66, $p < 0.05$).

Results involving the cognitive domains are presented in Table 2. The change score of the Hopkins Verbal Learning Test (HVLT) and

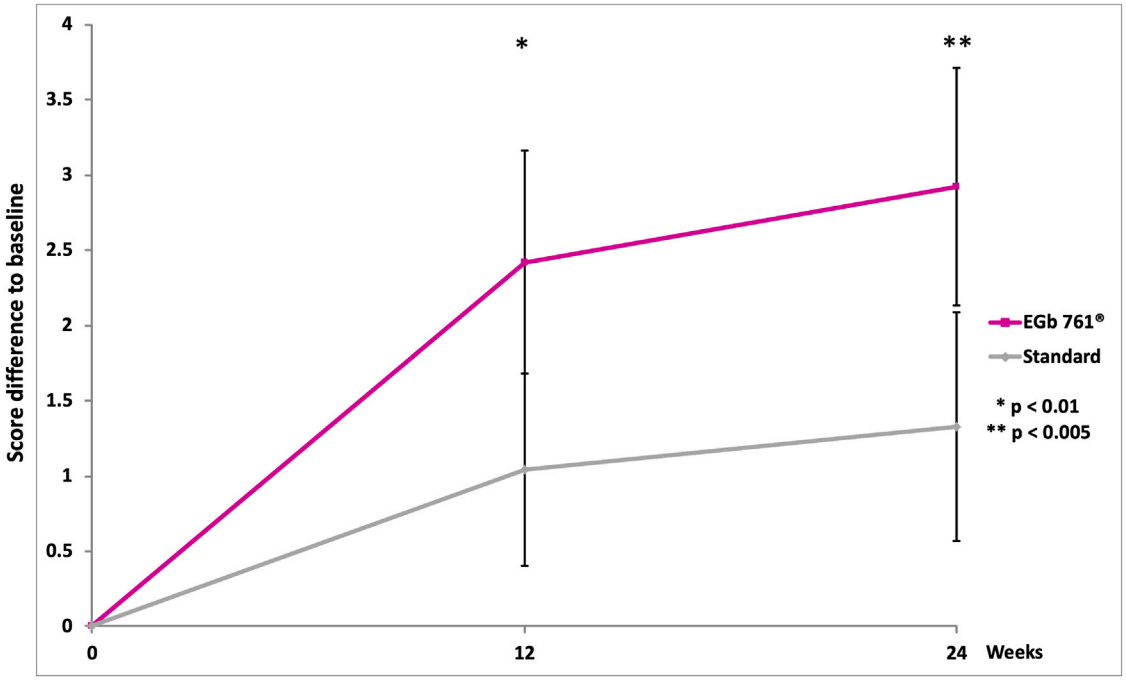

FIGURE 2
Change in Montreal Cognitive Assessment (MoCA) scores over the course of the study (means, 95% confidence intervals, two-sided *p*-values of *t*-test for between-group differences).

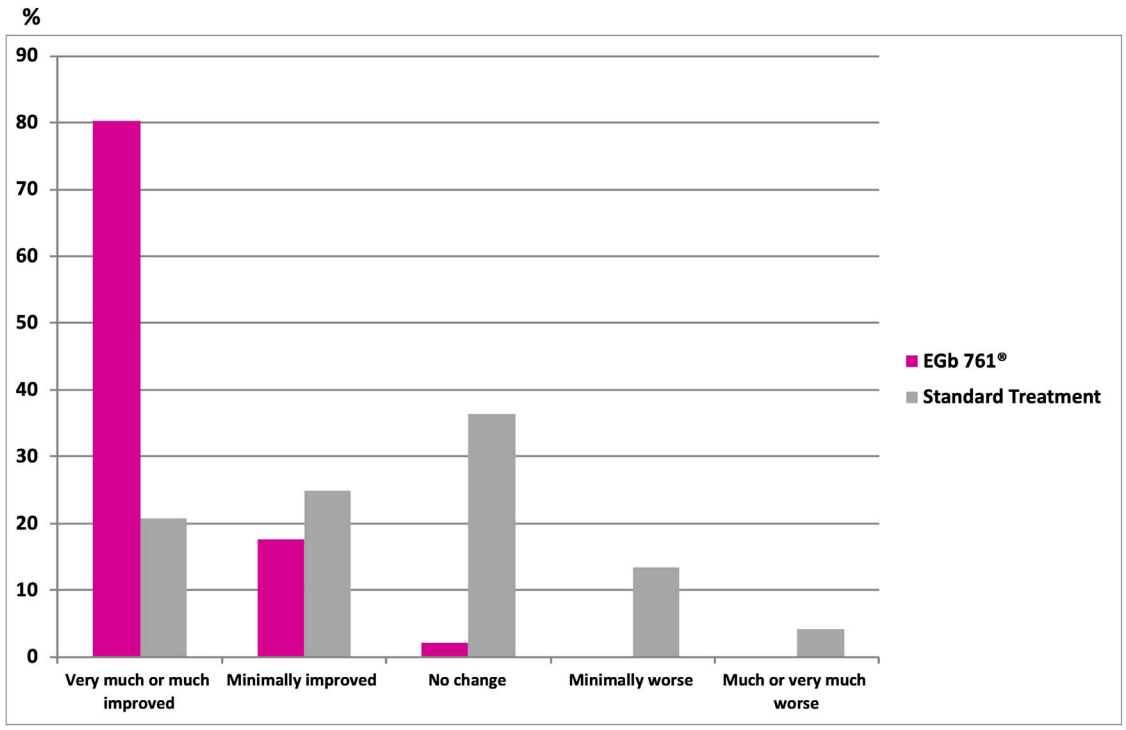

FIGURE 3
Clinical Global Impression of Change (CGI-C) at 24 weeks; $p < 0.001$ vs. standard (Fisher's exact test two-sided).

the Shape Trail Test—Trail 1 at week 24 revealed that the drug-reference difference were 1.89 points (95% CI, 0.38 to 3.40; $p < 0.05$; Table 2) and −22.6 points (95% CI, −44.31 to −0.89; $p < 0.05$; Table 2). According to the clinicians' global ratings (CGI-C), 80.2% of the patients treated with EGb 761® improved much or very much compared to their baseline condition (Figure 3) versus only 20.8% of those who received standard treatment alone. The rate of recurrent strokes was low; only one recurrent stroke was observed in one patient of the EGb 761® group, and three recurrent strokes were reported for two patients in the reference group. The analysis did not find significant difference for the Shape Trail Test Trail 2 (STT-2), the Verbal Fluency Test (VFT), the Digit Symbol Substitution Test (DSST) of the Wechsler Adult Intelligence Scale-Revised (WAIS-R), the Hospital Anxiety and Depression scale (HADS) and NPI in both groups at week 24.

3.3 Safety

Overall, 10.5% of patients experienced at least one adverse event during the 24-week randomized treatment period. The incidence of adverse events was similar between the two groups.16 adverse events were observed in 11 patients (11.1%) of the EGb 761® group, and 14 adverse events were reported for 10 patients (9.9%) of the reference group. Most adverse events were mild and unlikely to be related to EGb 761®. The most frequently reported adverse event was nasopharyngitis, which was experienced by 3 patients (3%) in each treatment group. Other adverse events occurred in no more than 1% of patients in either group. Altogether, 5 serious adverse events were reported: 2 events (cerebral infarction, chronic kidney disease, one patient each) in the EGb 761® group and 3 events (2 cerebral infarctions in one patient, lacunar infarction in one patient) in the reference group. A causal relationship could not be ruled out for three events: rash, dizziness (both possibly related) and chronic kidney disease (relationship unlikely). There were no clinically significant changes from baseline observed in vital signs, biochemical markers or electrocardiography results in either group.

4 Discussion

Considering the high incidence of stroke and the cognitive decline experienced by survivors in the Chinese population, there is a pressing need for novel interventions during the early post-stroke stage (Rajan et al., 2014); however, only few drugs have been dedicated to the topic. Our prospective randomized, multicenter, open-label trial focused on the effects of *Ginkgo biloba* extract EGb 761® vis-à-vis cognitive function in patients after a recent mild-to-moderate ischemic stroke. The results provide valuable insight into the optimal selection of patients, efficacy measures and determining treatment duration for this disorder. Our study had an exploratory design. Evidence of previous trials has supported the use of EGb 761® in the treatment of cognitive impairment (Gauthier and Schlaefke, 2014; Gavrilova et al., 2014). Thus we applied a cost-effective attempt to repurpose EGb 761® at the same dose regimen to treat cognitive change after stroke. We adopted a 6-month treatment period because previous large-scale drug trials demonstrated that this interval was sufficient to observe symptomatic benefits in patients with vascular cognitive impairment (Ritter and Pillai, 2015).

We demonstrated that EGb 761® exhibited slight improvements of cognitive performance, mainly documented by a larger increase in MoCA scores in the treatment group compared with the reference group. MoCA has been found sensitive when it comes to identifying changes in cognitive function, especially in executive deficits that add to the outcome prediction of post-stroke cognitive impairment and post-stroke dementia (Shopin et al., 2013; Dong et al., 2014; Burton and Tyson, 2015; Ritter and Pillai, 2015; Tan et al., 2017). In our study, we only enrolled patients with mild neurological deficits because they had to be able to participate in functional cognition evaluation: a ceiling effect can therefore not be ruled out while explaining the similar high response in both groups after 24 weeks. Nevertheless, the difference between drug and reference treatment, although minor, stayed in line with the typical results of other vascular cognitive impairment trials (Guekht et al., 2017; Nijsse et al., 2017; Chabriat et al., 2020), providing further evidence that the MoCA is a useful instrument to assess cognitive outcomes after mild stroke. It is noteworthy that both our trial and the findings from the previous study (Li et al., 2017) have shown that Ginkgo biloba extract as an add-on to the standard treatment promoted MoCA improvement at 6 months. Comparatively, our current research used lower EGb 761® dosages (240 mg daily) compared to Li et al. (450 mg daily). While several studies have indicated that EGb 761® may increase the risk of bleeding by inhibiting platelet aggregation and platelet activating factor function (Kudolo et al., 2002; Bent et al., 2005), our results supported that the use of lower dosage of EGb 761® (240 mg/d) did show clinical benefits in cognitive performance within a 24-week period and alleviated the concern of bleeding in future study designs. A long-term study lasting for 1–3 years would help to fully explore the efficacy of EGb 761® in preventing post-stroke dementia. Strengths of our study also included the exclusion of patients who take antidementia drugs, psychoactive drugs and so forth regularly to avoid possible confounding in the result of the trial on the cognitive function.

In particular, the cognitive function of patients in the reference group did improve to some extent at 24 weeks, confirming the benefits of secondary prevention, including risk-factor management. It has been postulated that risk-factor management could restore cognitive performance and NPS. Therefore, we recommend that future analyses also include medical history, concomitant disease, as well as significant laboratory values such as blood pressure, blood glucose, and blood lipid during treatment. A trend towards a progress in learning and memory along with executive functions was also detected with the EGb 761® group versus control. Furthermore, there is a marked and statistically significant difference in favor of EGb 761® treatment in terms of the clinicians' global judgment of change on the patients' overall condition. This may be somewhat overestimated: if there is any doubt in an open-label study, it is entirely possible that the better of two possible ratings was chosen if a patient had more intensive treatments. Addressing additional

aspects of quality of life or instrumental activities in further studies may provide more insight. Other secondary outcomes assessed did not achieve clinical significance and our analysis demonstrated that EGb 761® did not improve psychological outcomes by concomitant treatment in our pilot period. The threefold higher number of recurrent strokes in the standard treatment group might be a hint towards a preventive effect of EGb 761® treatment. However, due to the very low rate of recurrence, this data point has to be interpreted with caution. In terms of safety, the application of EGb 761® was well tolerated, which was in line with an established safety profile in the treatment of dementia (Gauthier and Schlaefke, 2014; Gavrilova et al., 2014) as well as a low incidence of adverse events in this study population.

The findings above were not representative of patients with more severe stroke sequelae due to our restrictive inclusion criteria. Indeed, patients tend to develop focal neurologic symptoms like aphasia or paralysis after a stroke. Despite a higher risk of post-stroke cognitive deterioration, these patients are not likely to be enrolled due to several reasons: they may not able to understand and complete the cognitive assessments; they could also be hospitalized in a long-term care, which may not allow sufficient time for them to be identified and enrolled; they are also likely to comorbid other serious medical conditions that are inappropriate for clinical trials. These factors limit the generalizability of study population, making it challenging to gain valid evidence towards the effects of EGb 761® on patients with severe cognitive impairment. As expected, most patients enrolled in our groups had only mild neurological deficits as well as mild cognitive decline. Considering the slow nature of cognition-associated decline after stroke, the cognitive state of those patients might stay normal or stable during a 24-week follow up (an interval which might only represent the early warning stage of post-stroke cognitive impairment). In general, pharmacological intervention at the early stage of associated diseases is more effective in preserving cognitive function than delayed treatment (Lissek and Suchan, 2021; Marcolini et al., 2022). Ginkgo biloba extract contains several compounds that have been shown to improve blood flow to the brain, reduce oxidative stress and enhance the activity of neurotransmitters such as acetylcholine, which is important for learning and memory (Ahlemeyer and Krieglstein, 2003). Therefore, the neuroprotective effects of EGb 761® could produce a modest yet consistent benefit in slowing or halting the progression of cognitive decline, rather than reversing it once it has already developed into dementia.

Moreover, it is noteworthy that most adopted cognitive tests in current vascular dementia trials have much in common with the assessment tools in patients with Alzheimer's disease (AD). These traditional screening measures seem general but presumably lack sensitivity in detecting the early subtle changes in cognition after stroke. Indeed, those insufficient evaluations of AD-related abnormalities might also confound our results, since vascular and degenerative factors always interact and exacerbate cognitive deterioration together in the long term (Korczyn, 2002). Thus more attention is needed to develop more specific cognitive tests for assessments after stroke, taking into account the fact that cognitive injuries vary, depending on different ischemic types including stroke location, volume, number of incidents and severity (Kalaria et al., 2016).

Additional limitations of our study include the open-label design and the lack of placebo, which has the potential of bias and would have made the study not feasible, especially in clinical event reporting and ascertainment. However, given the highly objective nature of cognitive tests, it is unlikely that the performance of our outcome measures of main interest were adversely affected. Another limitation might be the absence of biomarker evaluation related to cognitive status, which could help the assessment of cognitive function. In addition, we acknowledge the lack of diet regulation among the participants and this variability could have influenced the post-stroke recovery as well as the pharmacokinetics of the drug, potentially impacting the study outcomes. Moreover, the duration of our study—only 24 weeks—may have been too short to reveal the potential of EGb 761® in attenuating the decline in cognitive function, since cognitive deficits in patients after stroke tend to develop slowly. Larger samples and longer follow-up for 1–3 years might be required in future studies for major post-stroke cognitive impairment to manifest. In conclusion, evidence from our trial suggests that EGb 761® 240 mg/d showed clinical benefits in cognitive functioning of patients compared with standard care after a mild-to-moderate ischemic stroke. Future randomized controlled trials with a larger sample size and higher statistical power may help further establish the inclusion criteria and treatment duration; they can also further validate the effects of EGb 761® in patients with a broader range of severity of cognitive impairment.

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.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Huashan Hospital, Fudan University, Shanghai, China. The patients/participants provided their written informed consent to participate in this study.

Author contributions

QD proposed the project notion. MC, TY, YZ, RL, YG, JL, XL, XW, and QD conducted the research. MC and TY analyzed data and wrote the manuscript. All authors contributed to manuscript revision and approved the final manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1147860/full#supplementary-material

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Sanhua decoction: Current understanding of a traditional herbal recipe for stroke

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Both thrombolytic and endovascular therapies are optimal treatment options for patients with acute ischemic stroke, but only less than half of these patients can benefit from these treatments. Traditional Chinese medicine has a long history of successfully managing ischemic stroke using both herbal and physical therapeutics. Among herbal recipes, Sanhua decoction (SHD) is one of the classical prescriptions for ischemic stroke. The present review aimed to summarize evidence from both clinical and basic research to demonstrate its efficacy in managing ischemic stroke and the potential mechanisms underlying its therapeutic effects, which will provide evidence on the therapeutic effect of this herbal recipe and guide future studies on this recipe. SHD is composed of four herbs, *Rheum palmatum* L. [Polygonaceae], *Magnolia officinalis* Rehder & E.H.Wilson [Magnoliaceae], Citrus × aurantium L. [Rutaceae], Hansenia weberbaueriana (Fedde ex H.Wolff) Pimenov & Kljuykov [Apiaceae]. We found that the majority of clinical studies on SHD are case reports and they showed positive therapeutic effect of SHD on both acute and chronic ischemic stroke. There are over 40 bioactive compounds identified in SHD, but few experimental studies have examined their individual molecular mechanisms. As an extract of SHD, it improves neurological functions through suppressing inflammation, protecting the blood brain barrier from degradation, restoring the number of neural stem cells, inhibiting apoptosis and brain edema, scavenging oxygen free radicals, and regulating the brain-gut axis. These will lay the theoretical foundation for future studies on this prescription and its clinical application. Future research may need to confirm its clinical efficacy in large-scale clinical trials and to disentangle its bioactive compounds and their potential mechanisms.

KEYWORDS

Sanhua decoction, ischemic stroke, composition analysis, pharmacological analysis, clinical efficacy

Introduction

Ischemic stroke is the most common cerebrovascular disease in clinical practice. Its incidence is steadily increasing with the improvement of modern living standards and the acceleration of the pace of life (Wang et al., 2019), and its onset of age is getting younger (Hankey, 2017). Hemiplegia, slurred speech, loss of consciousness, are the major manifestations (Zhang and Wu, 2017). Stroke remains one of the leading causes of death worldwide, with a high incidence, a high morbidity rate, and a high recurrence rate (Wang et al., 2019), especially in China where stroke is the leading cause of adult disability and death (Gbd 2016 Headache Collaborators, 2018). It poses heavy medical and financial burden to patients and their families, as well as the society (Wang W. et al., 2017; Pandian et al., 2018).

Studies have shown that stroke has complex etiologies and pathogenic mechanisms. Western medicine remains the first choice of treatments. Tissue derived plasminogen activator (tPA) is the only drug approved by FDA. It has an ideal time window of 4.5 h after symptom onset before starting intravenous infusion (Rangaraju et al., 2015). Emerging studies also support the use of endovascular thrombectomy. A few randomized clinical trials have extended the time window of endovascular thrombectomy from 6 to 24 h with the guidance of imaging results (Fransen et al., 2014; Pirson et al., 2021). However, the reality is that a decent proportion of ischemic stroke patients do not reach the hospital within the time window and others do not meet the criteria for thrombolysis or thrombectomy though they arrive at the hospital within the time window. These patients will be managed with secondary prevention measures leaving them with disabilities of various extents. Even in China where Traditional Chinese Medicine (TCM) was developed, few people received TCM therapies in the emergency room. One of the hurdles for the wide application of TCM therapies is that the therapeutic mechanisms of these herbal recipes or physical therapies are unclear though they have been empirically used for thousands of years. However, a large proportion of patients prefer to receive TCM therapies at the sequelae stage when western medicines have limited effects on their conditions.

Traditional Chinese Medicine has a long history of managing stroke with herbs or physical therapies, either to treat ischemic or hemorrhagic stroke (Li et al., 2016; Seto et al., 2016). Many literatures show that TCM treatment can prevent the exacerbation of this condition, significantly improve clinical outcomes of patients by promoting their functional recovery (Li et al., 2016; Seto et al., 2016; Bi, 2022). Recent studies have found that the addition of TCM recipes to conventional medicine at the acute stage of ischemic stroke displays superiority to pure conventional medicine, and the incidence of adverse events is low (Liu and Xiong, 2013). More importantly, many patients may have missed the time window for intravenous thrombolysis or endovascular thrombectomy, and they have no better options to choose but secondary prevention measures. In this particular respect, TCM treatments have displayed advantages over conventional medicines. Therefore, there is a surge of both clinical and basic research on the therapeutic effect and their underlying mechanisms of TCM recipes. Findings from these studies will demonstrate both clinical and mechanistic evidence of these recipes on ischemic stroke, and promote their use both in China and other countries (Zhang, 2019).

TCM understanding of ischemic stroke

According to the TCM theory, ischemic stroke is closely related to accumulation of internal injury, deficiency of Qi (a form of energy which waxes and wanes in the body depending on health) and blood, excessive fatigue and lack of rest, emotional disturbance, unhealthy dieting habit, and obesity (Wang Y. et al., 2017). Pathogenic mechanisms mainly include external wind, extra heat, phlegm, blood stasis, and deficiency of Qi and blood (Si et al., 2019), which are closely related to the climate, emotional response, pressure, and other factors in daily life (Duan, 2017). These pathological factors contribute to the imbalance of Yin and Yang, dysfunction of Zang (solid organs) and Fu (hollow organs like guts, stomach), altered homeostasis of Qi and blood. As a result, ischemic stroke occurs (Li et al., 2019). Treatment of ischemic stroke should also be tailored based on the etiologies and pathogenic mechanisms of patients. Commonly used treatments mainly include nourishing Yin to expel the wind, clearing extra heat to protect the liver, invigorating Qi, improving blood circulation, cleansing blood stasis, dissipating extra heat and phlegm in internal organs, and restoring the consciousness (Li, 2018; Zhao and Zhou, 2021).

Composition and application of Sanhua decoction

Sanhua decoction (SHD) first appeared in the book named "Su Wen Bing Ji Qi Yi Bao Ming Ji" (literally translated into Su Wen—collection of experience on how to live longer by understanding the pathogenic mechanisms and Qi) written by Liu (2007). He pointed out that ischemic stroke can be the result of dysfunction of a variety of organs. If it is due to the dysfunction of Fu (hollow organs), it is very likely to manifest with symptoms of both Fu and Zang (solid organs), like stasis of Qi and loss of consciousness. Treatments should aim to recanalize Fu (hollow organs) or to restore the consciousness (Chen et al., 2021). SHD is a representative prescription for stroke that recanalizes Fu. It has the effect of harmonizing Qi, blood, and body fluid, cleansing stasis in Fu through which the environment and the internal organs are connected (Zhao and Jie, 2016; Wang Y. et al., 2020).

Sanhua decoction is composed of four herbs, *Rheum palmatum* L. [Polygonaceae], *Citrus* × *aurantium* L. [Rutaceae], *Magnolia officinalis* Rehder & E.H.Wilson [Magnoliaceae], and *Hansenia weberbaueriana* (Fedde ex H.Wolff) Pimenov & Kljuykov [Apiaceae]. It is used for treating apoplexy of six meridians (Dong and Fang, 2005; Gong, 2007). These four herbs have been recorded in ancient books to effectively treat stroke (Song et al., 2016). "Yi Xue Wen Dui" (literally translated to Questions and Answers in Medicine) recorded that SHD is composed of Xiao Chengqi decoction and Qiang Huo [*Hansenia weberbaueriana* (Fedde ex H.Wolff) Pimenov & Kljuykov [Apiaceae]] (Gao, 1959). It is interpreted that *Rheum palmatum* L. [Polygonaceae] is the king herb, responsible for purging heat and the bowel; *Citrus* × *aurantium* L. [Rutaceae] is the minister herb, removing Qi stasis and retention of food in the bowel in addition to dissolving phlegm. These two herbs, when used together, can

effectively eliminate extra heat, remove Qi stasis, and expel the retained food in the bowel. As a result, the gastrointestinal system is well-restored. *Magnolia officinalis* Rehder & E.H.Wilson [Magnoliaceae] is the assistant herb, specialized in removing Qi stasis and distention, and assists the other two herbs to recanalize the gastrointestinal system. These three herbs from Xiao Chengqi decoction are used for conditions manifesting with regurgitation of the gastrointestinal system. *Hansenia weberbaueriana* (Fedde ex H.Wolff) Pimenov & Kljuykov [Apiaceae] is the envoy herb, responsible for dispelling cold and wind, eliminating extra water, and ameliorating pain (Song et al., 2016). The combined use of these four herbs recanalizes meridians, purges Fu (hollow organs), tonifies Qi and blood, removes stasis, and restores the function of the brain and other organs (Fan et al., 2012b; Wang and Xie, 2013).

To search for studies on SHD, 4 English databases including PubMed, Web of Science, EMBASE, Cochrane Central Register of Controlled Trials, four Chinese databases including the Chinese National Knowledge Infrastructure, Wanfang Database, Chongqing VIP Database, and the Chinese Biomedical Database, and two clinical trial registration websites-the International Clinical Trials Registry Platform and the Chinese Clinical Trial Registry were searched up to March 30, 2022. Studies including randomized controlled trials, case control studies, reviews, or systematic reviews were included for analysis. Discrete searching strategies were used, including: "Sanhua decoction" or "Sanhua Tang" and "acute ischemic stroke" or "acute cerebral infarction" or "apoplexy" or "stroke" or "ischemic attack." No language restriction was used. We found 5 articles from Pubmed, 7 from Web of Science, 0 from Cochrane database, 7 from EMBASE, 29 from CNKI, 34 from Wanfang Database, 10 from VIP Database, and 25 from Chinese Biomedical Database. Two out of seven articles written in English are published in Chinese but their titles are translated into English. Articles from four Chinese databases are overlapping with each other. In total, the number of articles directly related to SHD is 34, and partially related to SHD is 37. The latter includes studies on one herb or single or multiple bioactive compounds. Among these 34 articles, 12 are clinical studies.

Clinical studies on Sanhua decoction

Sanhua decoction has a wide range of clinical applications. Many clinical studies have found that it has significant therapeutic effects on stroke without apparent adverse reactions.

Though there is a lack of randomized controlled trials on this herbal recipe, a number of Chinese studies did report its efficacy in managing ischemic stroke. It was reported that the efficacy of this recipe alone ranged from 83 to 95% for acute stroke patients compared with conventional western medicines excluding tPA and endovascular thrombectomy (Li and Dou, 2008; Yang et al., 2009; Liu, 2011; Wang C. et al., 2017). When SHD was used along with conventional western medicines, the therapeutic effect was even more significant (88.46 vs. 61.54%) (Wang, 2015). Among the patients, not only their NIHSS was improved, but also their Barthel indices (Yang et al., 2009; Liu, 2011). Apart from this, SHD significantly improved rheological parameters, including the decreased viscosity of the blood, decreased levels of fibrinogen, hematocrit (Yang et al., 2009), and TXB2 (Wang C. et al., 2017) when used in combination with conventional western medicines. In the meantime, plasma AT-III and 6-keto-PGF1α were increased after SHD treatment (Wang C. et al., 2017). In the largest cohort of 120 stroke patients, 60 received modified SHD. It was shown that neurological functions of 28 patients were completely restored, 20 significantly improved, 9 improved, and 3 unchanged. In comparison, the conventional western medicine group had 23 completely restored, 19 significantly improved, 8 improved, 10 unchanged. The difference between these two groups was statistically significant (Li and Dou, 2008), demonstrating the effectiveness of SHD in managing ischemic stroke.

In addition, SHD was found to be effective for chronic ischemic stroke patients with sequelae. Zhu and Guo (2000) reported that it improved neurological functions of ischemic stroke patients and prevented the recurrence of stroke after optimizing the dosage of the four herbs of SHD.

Apart from clinical studies using the standard SHD, a couple of studies modified this recipe based on patients' conditions and also found that the modified recipes were also effective for ischemic stroke patients (Duan, 2005; Meng and Liu, 2007). For example, when *Conioselinum anthriscoides* 'Chuanxiong', *Angelica sinensis* (Oliv.) Diels [Apiaceae], *Acorus calamus* var. angustatus Besser [Acoraceae], and *Asarum sieboldii* Miq. [Aristolochiaceae] were added to the recipe, over 90% of patients with ischemic stroke showed improvement in their neurological functions (Meng and Liu, 2007). Similarly, another study added *Neolitsea cassia* (L.) Kosterm, *Pueraria montana* var. lobata (Willd.) Maesen & S.M.Almeida ex Sanjappa & Predeep [Fabaceae], *Mitragyna inermis* (Willd.) Kuntze [Rubiaceae], *Lycopus virginicus* L. [Lamiaceae], and *Tragia involucrata* L. [Euphorbiaceae] to the recipe, and 84.7% of the patients showed improvement in their neurological functions (Duan, 2005). There are other reports on the clinical use of SHD, but the number of patients was so small that they were not stated in this review. One example is the case report by Zhang and Ren (2002), where intractable hiccup due to stroke was alleviated by SHD.

Formula analysis of SHD

Rheum palmatum L. [Polygonaceae]

This herb is a commonly used one in TCM. It tastes bitter and has a cooling effect, specifically entering the spleen, stomach, large intestine, liver, and the pericardium meridians (Chen et al., 2019). It is known to remove bowel stagnation, clear extra water in the body, purge extra heat, cool the blood, clear blood stasis, and to detoxify toxins (Chinese Pharmacopoeia Commission, 2015). To be specific, this herb has a variety of pharmacological effects. It can regulate the function of the gastrointestinal system through anti-inflammatory and cholagogic effects, and increasing pancreatic secretion. It can also protect the cardiovascular system through regulating the metabolism of blood lipids, inhibiting the pathogenesis of atherosclerosis (Chen et al., 2019; Liu et al., 2020), scavenging free radicals (Heo et al., 2010; Song, 2019), and regulating the hemopoietic system. It improves the renal function, presenting with anti-inflammatory, antibacterial, antiviral, and anti-tumor effects

(Du et al., 2018; Jin et al., 2020). In the central nervous system, it inhibits the production of nitric oxide and nitrosation or oxidation of proteins (Zhao et al., 2018), as well as the aggregation of platelets (Nemmar et al., 2015). As a result, it is empirically used to treat ischemic stroke.

Magnolia officinalis Rehder & E.H.Wilson [Magnoliaceae]

This herb has been used in China for over 2,000 years. It is warm and bitter in nature, spicy in taste, and enters the spleen, stomach, lung, and the intestine meridians (Chinese Pharmacopoeia Commission, 2015). It mainly removes Qi stasis and stagnation, clears extra water and dispels distention, suppresses regurgitation, and pacifies asthma (Wei et al., 2019).

It is known for a number of therapeutic effects on the digestive, nervous, cardiovascular, and respiratory systems. It inhibits leukocyte infiltration to the brain under ischemia, production of free radicals (Liou et al., 2003) and TXB2 (Yu et al., 2016b), suppresses brain edema (Nemmar et al., 2015), and increases the content of dopamine, serotonin, 5-HIAA in the brain (Yang et al., 2017). Consequently, the cerebral blood flow is increased (Yu et al., 2016a), brain edema attenuated, and oxidative stress relieved. In addition, it has anti-inflammatory, analgesic, anti-bacterial, anti-tumor effects (Tan et al., 2020).

Citrus × *aurantium* L. [Rutaceae]

This herb has a long history of clinical use. It first appeared in "Shen Nong Ben Cao Jing." It tastes bitter, spicy, sour, and is slightly cold in nature. It enters the spleen and the stomach meridians. It is known to remove Qi stasis and bowel stagnation, to dissolve phlegm, and to ameliorate paralysis of internal organs (Heo et al., 2010). It is mainly used to treat bowel stagnation, distention and pain, diarrhea with tenesmus, constipation, phlegm stagnation, Qi stasis, and other conditions (Zhang et al., 2015).

Pharmacologically, this herb improves the function of the gastrointestinal tract, and exerts anti-tumor, anti-oxidation, anti-bacterial, and anti-inflammatory effects (Qu et al., 2017). In the central nervous system, it can ameliorate neuronal apoptosis and oxidative stress induced by the ischemia and reperfusion injury (Wang et al., 2017a), attenuate mitochondrial dysfunction (Wang et al., 2017b). It also suppresses levels of nucleotide-binding oligomerization domain 2 (NOD2), receptor-interacting serine/threonine kinase (RIP2), nuclear transcription factor-kappa B (NF-kB), matrix metalloproteinase-9 (MMP-9) and up-regulates claudin-5, and minimizes the infarct volume and edema. Consequently, neurological functions are improved (Bai et al., 2014; Wang K. et al., 2020).

Hansenia weberbaueriana (Fedde ex H.Wolff) Pimenov & Kljuykov [Apiaceae]

This herb was recorded in "Lei Gong Pao Zhi Yao Xing Jie" (literally translated to analysis of herb processing and pharmacological effects) (Li, 1998). It has a Yang nature with a light smell and spicy and bitter taste, and can tonify and dissipate Qi, expel the cold through the skin. It enters the bladder and the kidney meridians, dispelling the cold and the wind from the skin, removing extra water in the body, recanalizing the merians, and exerting an analgesic effect (Shi and Shi, 2017).

Pharmacological studies have shown that this herb possesses anti-inflammatory, antipyretic, antioxidant, antibacterial, analgesic (Guo et al., 2019), and anti-hypoxia effects (Li et al., 2015). In the central nervous system, it has been shown to reduce viscosity of the plasma, and to inhibit platelet aggregation and thrombosis (Lv et al., 1981; Zhang et al., 1996). Therefore, it is widely used to treat cardiovascular, cerebrovascular, gynecological, and gastrointestinal diseases (Yang et al., 2022b).

Dosage of Sanhua decoction and possible bioactive compounds

The dose of each herb in SHD was clearly recorded in the book named "Su Wen Bing Ji Qi Yi Bao Ming Ji" by Liu (2007). Converted to current units, the dose of each herb equals to 30.5 g. They are boiled in 2,831.4 ml water and only half of the water is retained after being boiled twice. This water extract will be drunk within a day. Changes of the patients' conditions will be closely monitored and drinking of SHD is ceased if diarrhea occurs (Zhang X. et al., 2022). Though this herbal recipe has been empirically used in clinical practice for centuries, no human pharmacokinetic research has been conducted. Little information is available to characterize this herbal recipe in this respect.

Possible bioactive compounds

With the assistance of the rat middle cerebral artery occlusion (MCAO) model and the network pharmacology technique, correlations between active compounds, compound targets and signaling pathways were described. These targets or compounds were tested *in vivo* for verification, aiming to reveal the therapeutic mechanisms of SHD in managing ischemic stroke. Forty active compounds and 47 direct target genes were identified, indicating that this herbal recipe plays a pharmacological role in the treatment of ischemic stroke through multiple targets. Among the purified compounds, emodin anthrone, isopropamidine, and scopoletin were identified as key bioactive compounds. Numerous targets, including interleukin-6 (IL-6), amyloid precursor protein (APP), protein kinase B (AKT1), and vascular endothelial growth factor A (VEGFA) were considered to be major targets (Yang et al., 2022b). Multiple signaling pathways including endocrine resistance, estrogen, tumor necrosis factor (TNF), advanced glycation endproducts/receptor for advanced glycation endproducts (AGEs/RAGE), and microRNAs were regulated by SHD. As a result, ischemic injury and inflammatory reactions were attenuated (YingHuang et al., 2022).

In another study, 78 shared targets by ischemic stroke and SHD were identified through the network pharmacology technique. Based on these targets, 9 compounds targeting over 10 target genes were identified and they might be the key bioactive molecules of SHD. These included apigenin, luteolin, nobiletin, naringenin,

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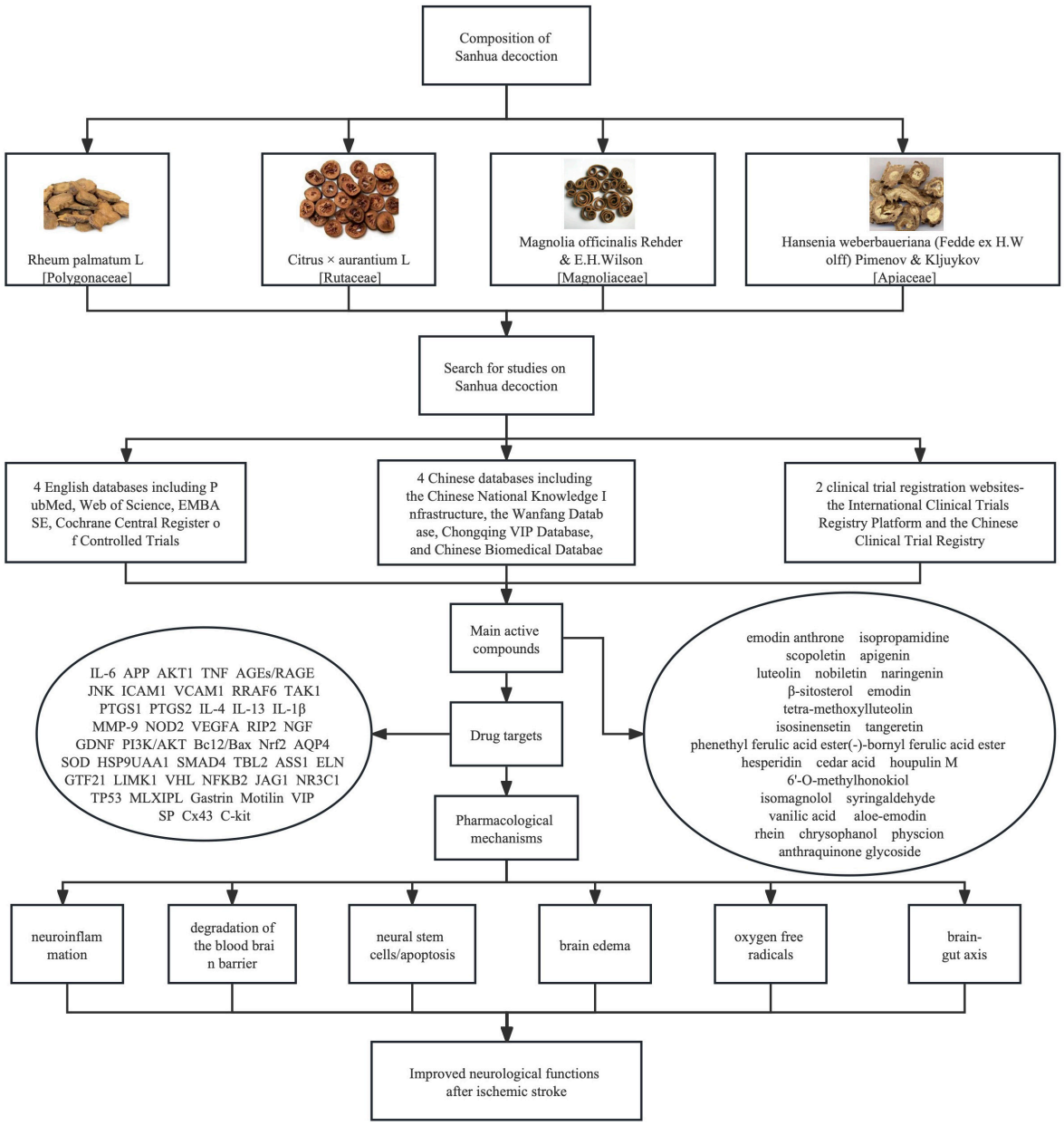

FIGURE 1
Pictures of four herbs constituting Sanhua decoction (SHD), searching strategies for articles on SHD and bioactive compounds identified from SHD, molecular mechanisms underlying their therapeutic effect on ischemic stroke. Both English and Chinese databases were searched for publications on SHD. Bioactive compounds were summarized from network pharmacology studies. These compounds can improve neurological functions of ischemic stroke patients through suppressing neuroinflammation, ameliorating, or preventing the degradation of the blood brain barrier, protecting neural stem cells and reducing apoptosis, preventing or mitigating brain edema, removing oxygen free radicals, and regulating brain-gut axis.

β-sitosterol, emodin, tetra-methoxylluteolin, isosinensetin, and tangeretin (Zhang W. et al., 2022). In another study, phenethyl ferulic acid ester and (-)-bornyl ferulic acid ester were found to be the active compounds which can inhibit platelet aggregation (Zhang and Shen, 2008).

A similar study found that SHD had 24 key bioactive compounds for stroke and some of them showed potential to become medicines, including hesperidin, cedar acid, houpulin M, 6′-*O*-methylhonokiol, isomagnolol, syringaldehyde, and vanillic acid. These compounds mainly interact with 19 targets including APP, heat shock protein 90 alpha (HSP90AA1), recombinant mothers against decapentaplegic homolog 4 (SMAD4), argininosuccinate synthase 1 (ASS1), elastin (ELN), general transcription factor II-I (GTF2I), LIM domain kinase 1 (LIMK1), transducin (beta)-like 2 (TBL2), Von Hippel-Lindau (VHL), nuclear transcription factor-kappa B 2 (NFKB2), Jagged 1 (JAG1), Nuclear Receptor Subfamily 3 Group C Member 1 (NR3C1), tumor protein P53 (TP53), and MLX interacting protein-like (MLXIPL), etc. Seven core signaling pathways were identified and they were dominantly related to the anti-inflammatory effect of SHD, such as the interleukin-4 (IL-4) and interleukin-13 (IL-13) mediated signaling pathways (Yang et al., 2022a).

Aloe-emodin, rhein, emodin, chrysophanol, and physcion are in the same category of rhubarb aglycone. They were shown to improve neurological functions of MCAO rats through attenuating neuronal apoptosis, scavenging free radicals, suppressing nitric oxide mediated cytotoxicity and neuroinflammation, as well as preventing platelet aggregation (Li et al., 2003, 2005; Guan et al., 2014).

Pharmacological mechanisms of SHD in treating ischemic stroke

Studies have found that SHD is an effective therapy for acute ischemic stroke without apparent adverse reactions (Yang et al., 2009; Liu, 2011). However, mechanisms underlying the therapeutic effect of SHD are still unclear. A summary of the known mechanisms was listed below.

The anti-inflammatory effect of SHD

After cerebral infarction, a large amount of proinflammatory cytokines are released, among which IL-6 and TNF-α are the major players. They are used as biomarkers for the recurrence of vascular events (Boehme et al., 2016). IL-6 induces brain injury and hippocampal neuronal necrosis through activating the *N*-methyl-D-aspartate (NMDA) receptor and up-regulating the c-Jun N-terminal kinase (JNK) (Armstead et al., 2019). SHD can significantly attenuate swelling, degeneration, and necrosis of neural cell, as well as infiltration of inflammatory cells caused by cerebral ischemia, and can significantly reduce the expression of interleukin-1β (IL-1β) and intercellular adhesion molecule 1 (ICAM-1) (Wu et al., 2009; Fan, 2010; Fan et al., 2011). Emodin is likely to be the bioactive compound for this effect as it was shown to downregulate NF-κB and ICAM1 in MCAO rats (Wu et al., 2009). In network pharmacology studies stated above, IL-6, TNF-α, NF-κB, AGEs/RAGE, and other targets were identified, suggesting that SHD may target these genes or their proteins to take the therapeutic effect (Yang et al., 2022b; Zhang W. et al., 2022; Zhang X. et al., 2022). In addition, another study reported seven major signaling pathways targeted by SHD, including the NF-κB mediated signaling pathway where TNF receptor associated factor 6 (TRAF6) is involved, the NF-κB signaling pathway activated through TGF-beta activated kinase 1 (TAK1) phosphorylation and the Ikappa B kinases (IKKS), IL-4, and IL-13 mediated signaling pathways (Yang et al., 2022a). In another network pharmacology study, the targets of emodin were estimated, which included caspase 3, prostaglandin-endoperoxide synthase 1 (PTGS1), TNF, matrix metalloproteinase 9 (MMP9), protein kinase C epsilon (PRKCE), prostaglandin-endoperoxide synthase 2 (PTGS2), tumor protein 53 (TP53). Among them, caspase 3, PTGS1, TNF, and MMP9 are associated with inflammation (Jia et al., 2021). *In vivo* experiments also confirmed the decreased expression of IL-6 and TNF-α in one of these studies (Zhang W. et al., 2022). Fu et al. (2020) reported that SHD significantly improved neurological functions and reduced the expression of p-tau. The latter is a pathogenic protein involved in neurodegeneration, and triggers neuroinflammation in Alzheimer's disease models. In another study on dogs with global ischemia-reperfusion injury, honokiol has been shown to attenuate the increased level of thromboxane B2 (TXB2), but it has no impact on the level of endothelin and NO. Surprisingly, whether neurological functions were improved by this compound was not reported in this study (Yu et al., 2016b). Another study used model rats of the ischemia-reperfusion injury and rats with spontaneous hypertension which are prone to develop stroke. They found that honokiol significantly increased the production and release of NO by endothelial cells, which mediates the dilation of blood vessels (Liu et al., 2016). Whether other bioactive compounds have the same effect is unknown.

Inhibition of degradation of the blood brain barrier

Destruction of the blood-brain barrier (BBB) plays an important role in the occurrence and development of neurological dysfunction in ischemic stroke (Yang et al., 2019; Li et al., 2022). Fan (2010) showed that SHD could significantly reduce cerebral edema, increase the expression of zonula occludin-1 (ZO-1) in rats with the cerebral ischemia-reperfusion injury, and significantly reduce the content of S100β protein in the serum of these rats. When BBB is open or broken, S100β protein can reach the peripheral blood. This protein protects BBB in rats with the cerebral ischemia-reperfusion injury, which may be achieved by up-regulating the expression of ZO-1 protein, a linker protein between transmembrane proteins and cytoskeleton proteins (Li et al., 2022).

Matrix metalloproteinases (MMPs) are involved in neuronal injury after cerebral ischemia (Cuadrado et al., 2009; Fan et al., 2012a). Fan (2010) showed that SHD could significantly ameliorate neurological deficits of rats with the cerebral ischemia-reperfusion injury and attenuate the swelling, degeneration, and necrosis of neural cells. The level of MMP-9 mRNA and MMP-9 protein was also significantly reduced. Wang et al. not only found the decreased expression of MMP-9 in MCAO rats, but also decreased expression of nucleotide oligomerization domain-like receptors 2 (NOD2), receptor-interacting protein 2 (RIP2), and NF-κB, which are all regulators of the expression of proinflammatory genes, after pretreatment with naringenin. In addition, naringenin upregulated the expression of claudin-5 and consequently decreased the permeability of the BBB (Bai et al., 2014). Another study showed that SHD can upregulate the expression of krüppel-like factor 2 (KLF2), suppress the expression of thrombomodulin and eNOS, sustaining the integrity of the endothelial cells and maintaining their physiological functions (Wang et al., 2022).

Whether other compounds identified in SHD can protect the integrity of the BBB still needs investigation.

Protection of neural stem cells and inhibition of apoptosis

Two studies reported the therapeutic effect of SHD in the rat model of the ischemia-reperfusion injury and found that this herbal recipe restored the number of endogenous neural stem cells. Not only BrdU positive and doublecortin positive cells, but

also BrdU positive and GFAP positive cells were increased. Stem cell migration and differentiation was promoted by SHD, which was closely related to the improvement of neurological functions (Li, 2015; Fu et al., 2020). However, which bioactive compounds impart this effect is unknown. Phytochemicals are recognized by their multi-target characters (Khan et al., 2020). One herb contains thousands of chemicals and a number of them can protect or augment neurogenesis in the brain, like Egb761 (a chemical present in ginkgo) and resveratrol (present in grape products) (Jahan et al., 2018). Another study on bone marrow derived stem cells found that rhubarb aglycone, a bioactive compound from Rhubarb, increased the level of nerve growth factor (NGF) and glial derived neurotrophic factor (GDNF) after transplanting the stem cells to the brain of MCAO model rats (Li et al., 2008). This suggests that rhubarb aglycone might have positive effect on neural stem cells. The PI3K-Akt pathway is an important one in the development of ischemic brain injury (Samakova et al., 2019). It regulates many cellular functions, such as cell survival, autophagy, protein synthesis, and glycolysis (Xie et al., 2019). Phosphorylation of Akt increases the level of an anti-apoptotic protein B-cell lymphoma 2 (Bcl2). Recent studies reported that Akt phosphorylation enhanced nuclear translocation of the nuclear factor E2-related factor 2 (Nrf2), and phosphorylation of the cyclic adenosine monophosphate response element binding protein (CREB), a survival regulatory protein, and protected against cerebral ischemia (Zhang et al., 2018, Zhang X. et al., 2022). A study on the rhubarb extract showed that it significantly attenuated the increase in apoptosis, caspase-3, BCL2-associated X (Bax) in the MCAO model and increased the level of Bcl-2, suggesting that this extract might take effect through inhibiting the apoptosis pathway (Tang et al., 2020). In a study by Wang et al. (2017a), naringenin was found to prevent neuronal apoptosis and to suppress translocation of Nrf2 from the cytoplasm to the nucleus in MCAO model rats (Wang et al., 2017a). Whether other compounds are able to protect neurons from apoptosis still needs further investigation.

Inhibition of brain edema

Aquaporin 4 (AQP4) is a functional regulator of astrocytes and a common water-conducting membrane integrin channel in the brain. Through regulating the inflow and clearance of cerebral water, AQP4 is involved in the development of cerebral edema and pathogenesis of various neurological conditions (Suzuki et al., 2020). In the study by Lu et al. (2015) SHD significantly alleviated neurological deficits after the cerebral ischemia/reperfusion injury and reduced the expression of AQP4. Through injecting lentivirus-mediated AQP4-siRNA into the ventricle of rats before inducing MCAO, decreased expression of AQP4 in the ipsilateral hippocampus and attenuated cerebral edema was found after modeling MCAO. Therefore, SHD can reduce the water content of the brain and effectively ameliorate the permeability of BBB.

Sodium channels are key to control the transmission of electrical signals in the nervous system, and its abnormality contributes to the development of cerebral infarction. In a study by Dai et al. (2011), they found that SHD significantly reduced the volume of cerebral infarction in rats and increased the expression of Nav1.1 mRNA. The latter resulted in reduce sodium influx and protection of neurons. Neuroinflammation is often accompanied by infiltration of inflammatory cells from the blood and increased levels of proinflammatory cytokines. The latter can injure the endothelial cells and activate microglia as well as astrocytes through multiple signaling pathways. One of the consequences is brain edema. Therefore, bioactive compounds present in SHD that attenuate inflammation may alleviate brain edema as well (Wu et al., 2009; Fan, 2010; Bai et al., 2014; Yu et al., 2016b).

Scavenging oxygen free radicals

Reactive oxygen species (ROS) are important players in the development of ischemic stroke, especially during the ischemia-reperfusion injury. Therefore, scavenging or neutralizing ROS is one of the potential therapies for ischemic stroke. In the body, the superoxide dismuatase (SOD) is a cytoplasmic antioxidant enzyme that catalyzes the reaction with free radicals and scavenges them. SHD was reported to increase the content of SOD in rats with the cerebral ischemia-reperfusion injury when administered intragastrically. In the meantime, the content of malonaldehyde (MDA), a product of lipid peroxidation, was significantly decreased (Tang et al., 2008), indicating that SHD takes its effect partially through suppressing lipid peroxidation and accelerating the scavenging of ROS. In another study, honokiol, a compound present in Magnolia officinalis, was found to protect the brain tissue during the ischemia-perfusion injury through suppressing neutrophil infiltration and lipid peroxidation evidenced by the reduced level of MDA (Liou et al., 2003). Naringenin significantly decreased the production of ROS through activating the Nrf2/antioxidant response element signaling pathway (Wang et al., 2017b). Whether other compounds have this effect is still unknown.

Potential involvement of the microbiota-brain-gut axis

The microbiota-brain-gut axis has been widely acknowledged to be involved in a large variety of disorders. Emerging studies have shown that the brain regulates the digestive system through not only the sympathetic, parasympathetic nervous system, but also through the hypothalamus-pituitary-adrenal gland axis as well as the endocrine system. Vice versa, the gut impacts the brain not only through neural transmission *via* the vagus nerve to upper brain regions, but also through diverse signals, including intestinal peptides like cholecystokinin and vasoactive intestinal peptide, small molecular weight compounds like dopamine, serotonin, and metabolites of bacteria in the gut. Both the nervous, endocrine, and immune systems are involved in this regulation. The detailed molecular mechanisms of these neural pathways, compounds, and immune cells as well as mediators in the pathogenesis of neurological conditions have been comprehensively reviewed by

many researchers (Cryan et al., 2019; Agirman et al., 2021; Socała et al., 2021; Barrio et al., 2022).

In TCM, 6 Yang meridians are traveling through the brain, indicating their involvement in the pathogenesis of brain diseases. Among these six meridians, the colon meridian is responsible for the expelling of the remaining substance after absorption in the digestive system. The normal function of the colon meridian ensures that the brain receives supply of nutrients and essential molecules from the digestive system. When stroke occurs, the colon will be impacted more or less. One of the eight therapeutic strategies in TCM is catharsis. SHD, a recipe containing rheum palmatum, can recanalize the gut through purging the digestive tract. This not only removes the food residue retained in the gut, but also attenuates inflammation, and improves neural functions (Liu et al., 2005). It is reasonable to expect that the purge of the digestive tract will significantly alter the composition of the microbiota in it and subsequently the metabolites of bacteria, the interaction between bacteria and the gut epithelial cells. Eventually, neurological functions will be altered. However, few studies have examined this type of molecular mechanisms underlying the therapeutic effect of SHD. In the ischemia rat model, Fan et al. found that intragastric administration of SHD in aged rats increased the activities of Na^+-K^+- and Ca^{2+}-ATPases (Fan et al., 2009), protecting the gastrointestinal tissue from injury caused by acute stroke. In another study, anthraquinone glycoside, a compound identified in rheum palmatum, was found to alleviate the ischemia-reperfusion injury and to increase the activity of SOD. In the meantime, Escherichia coli and enterococci were suppressed, lactobacillus and bifidobacterial were increased (Yu et al., 2019). In the thesis of Li (2012), it was found that citrus aurantium significantly attenuated inflammation in the mucosa of the stomach due to acute ischemic stroke, which might be related to reduced levels of gastrin, motilin, and vasoactive intestinal peptide. In the same model, suspension of citrus aurantium was shown to ameliorate inflammation in both the stomach and the small intestine, with even better results when nimodipine was used together. The expression of substance P (SP), connexin 43 (Cx43), C-kit in the stomach and the intestine was decreased along with a reduced number of interstitial Cajal cells, suspension of citrus aurantium plus nimodipine significantly reversed these changes, indicating that citrus aurantium is able to protect the mucosa of the digestive tract and restore its functions after ischemic stroke (Qiu, 2014). It is possible that SHD might take its therapeutic effect partially through regulating the brain-gut axis.

Conclusion and outlook

The present review summarized evidence supporting the therapeutic effect of SHD in managing ischemic stroke and its potential therapeutic mechanisms (**Figure 1**). There are a few possibilities to explain the potential advantages of SHD over western medicines in managing ischemic stroke patients. Firstly, SHD can be used beyond the time window for intravenous thrombolysis or endovascular thrombectomy, even at the chronic stage of ischemic stroke. This might be related to the anti-inflammatory, anti-apoptosis and other therapeutic mechanisms listed above. It is widely acknowledged that free radical scavengers like edaravone can effectively attenuate neurological deficits. The bioactive compounds isolated from SHD like naringenin and honokiol might have similar effects at the acute stage of ischemic stroke. Secondly, SHD contains multiple bioactive compounds which are targeting multiple pathways that are responsible for pathological changes after ischemic stroke. The synergistic effect of these compounds may augment their individual therapeutic effect. For example, the anti-inflammatory and free radical scavenging effects of SHD may more significantly alleviate brain edema than suppressing neuroinflammation alone or scavenging free radicals alone. This might be confirmed by applying different combinations of bioactive compounds to ischemic stroke models and even through randomized clinical trials. Thirdly, no specific medications have been developed so far to treat ischemic stroke and this is due to the lack of insight into the pathogenesis or mechanisms of ischemic stroke. There might be potential mechanisms that we have not discovered yet. SHD or other herbal recipes might have compounds that can target these mechanisms.

It is found from clinical practice that the therapeutic effect of TCM treatment based on syndrome differentiation is often better than that of Western medicine, and the incidence of adverse reactions is lower. The combination of SHD with conventional Western medicine in the treatment of stroke can effectively improve clinical efficacy, and is of great significance to ensure the long-term quality of life of patients. However, strict attention should be paid to drug interactions when Chinese and western drugs are used together. At present, large-scale, multi-center randomized, double-blind, controlled clinical trials on SHD are still lacking. In the future, it is necessary to reinforce the training of research design and clinical research capabilities of traditional Chinese medicine researchers, constantly explore opportunities to integrate Chinese and western medicine in managing a variety of medical conditions. The aim is to accelerate the recovery of these patients and to improve their quality of life.

Author contributions

JY and HL conceived the study and revised the manuscript. LZ and LM searched the literatures and prepared the draft of this manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Postoperative cerebral hemorrhage death in a patient with secondary hyperparathyroidism: a report of one case and literature review

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Secondary Hyperparathyroidism (SHPT) is a common complication of end-stage renal disease (ESRD), and parathyroid surgery (PTX) is an effective way to treat patients with severe SHPT. ESRD has multiple associations with cerebrovascular diseases. For example, the incidence of stroke in patients with ESRD is 10 times higher than that in the general population, the risk of death after acute stroke is three times higher, and the risk of hemorrhagic stroke is significantly higher. High/low serum calcium, high PTH, low serum sodium, high white blood cell count, previous occurrences of cerebrovascular events, polycystic kidney disease (as a primary disease), and the use of anticoagulants are independent risk factors for hemorrhagic stroke in hemodialysis patients with uremia. The risk of stroke in patients who undergo PTX decreases significantly in the second year of follow-up and persist thereafter. However, studies on the risk of perioperative stroke in SHPT patients are limited. After undergoing PTX, the PTH levels in SHPT patients drop suddenly, they undergo physiological changes, bone mineralization increases, and calcium in the blood gets redistributed, often accompanied by severe hypocalcemia. Serum calcium might influence the occurrence and development of hemorrhagic stroke at various stages. To prevent bleeding from the operated area, the use of anticoagulants after surgery is reduced in some cases, which often decreases the frequency of dialysis and increases the quantity of fluid in the body. An increase in the variation in blood pressure, instability of cerebral perfusion, and extensive intracranial calcification during dialysis promote hemorrhagic stroke, but these clinical problems have not received enough attention. In this study, we reported the death of an SHPT patient who suffered a perioperative intracerebral hemorrhage. Based on this case, we discussed the high-risk factors for perioperative hemorrhagic stroke in patients who undergo PTX. Our findings might help in the identification and early prevention of the risk of profuse bleeding in patients and provide reference for the safe performance of such operations.

KEYWORDS

secondary hyperparathyroidism, end-stage renal disease, hemorrhagic stroke, parathyroid surgery, perioperative management

1. Introduction

Stroke is the main cause of disability and death in hemodialysis (HD) patients. The chances of stroke are several times higher in HD patients than that in the general population, especially because cerebral hemorrhage has a huge impact on patient outcomes (Kelly et al., 2021). Secondary hyperparathyroidism (SHPT) is a common and serious complication in patients with chronic renal failure, and parathyroidectomy is the main way of treating SHPT patients who are refractory to medical treatment (Lau et al., 2018; Choi et al., 2021). Various dialysis methods, comorbidities, and drugs can affect platelet aggregation and/or coagulation cascade and, thus, affect the homeostasis of bleeding and hemostasis. The level of serum calcium, high PTH, low serum sodium, high leukocyte count, and a history of cerebrovascular events are important factors that influence hemorrhagic stroke in patients with hemodialysis. Several clinical studies have investigated the pathogenesis of CKD-MBD stroke, along with its prevention and treatment (Xiao et al., 2017; Yang et al., 2022). After undergoing PTX due to PTH, SHPT patients experience many physiological changes. The perioperative usually strictly control fluid intake because the changes in calcium homeostasis, dialysis, and the effect of multiple factors, such as antihypertensive drugs, blood pressure fluctuations, vascular tone, blood-brain barrier changes, and the risk of hemorrhagic stroke, might increase (Wakasugi et al., 2015). Few studies have investigated the prevention and treatment of perioperative stroke in patients after PTX. In this study, we reported the death of a patient due to cerebral hemorrhage after she underwent PTX. We analyzed the cause to provide a reference for increasing the safety of such surgeries. We present the following case following the CARE reporting checklist.

2. Case description

In 2020, a patient with SHPT expired due to cerebral hemorrhage after parathyroid surgery in our unit. The details regarding this case are as follows and Figure 1 is showcasing a timeline with relevant data from the episode of care. The patient was a 51-year-old female who was admitted to the hospital with uremic dialysis for 9 years and elevated parathyroid hormone for more than 5 years. She was suffering from hypertension for more than 10 years, epilepsy for more than 9 years, and cerebral infarction for more than 2 years. She was taking various oral medications. On admission, the patient suffered from bone pain in extremities with limited mobility and pruritus all over the skin, and no other uncomfortable physical symptoms. During admission, she was diagnosed with secondary hyperparathyroidism, the uremic phase of chronic renal failure, renal anemia, renal hypertension, and sequelae of cerebral infarction. After admission, a thyroid ultrasound examination showed posterior nodules in both lobes of the thyroid gland, which were considered to be of parathyroid origin. Re-examination showed the following: PTH: 2,405 pg/mL; Ca: 2.672 mmol/L; P: 1.6 mmol/L; WBC: 4.07 x 10^9/L; HB: 132 g/L. Total parathyroidectomy was performed on July 7, 2020, and postoperative pathology showed (upper right, lower right, upper left, and lower left) parathyroid nodular hyperplasia. PTH decreased to 565.6 pg/L on intraoperative examination, and blood calcium and phosphorus decreased to the normal range on the first day after surgery. The clinical symptoms of the patients, such as bone pain with limited mobility and skin pruritus, improved significantly after surgery. On the morning of July 12, 2020, the patient suddenly became unconscious (deep coma with a GCS score of 3). She was unresponsive to calls and vomited a large quantity of watery material. Her pupillary reflex to light was poor, and emergency cranial CT showed left basal ganglia hemorrhage with a break in the ventricular system; multiple lacunar cerebral infarcts, and ischemic focus. The neurosurgeon evaluated the intracranial hemorrhage by CT imaging at about 75 ml. The patient was transferred to the intensive care unit, where she was treated for dehydration and intracranial pressure and was administered anti-infectives and continuous renal replacement therapy (CRRT), followed by intracranial hematoma drainage and lateral ventriculotomy (hematoma drainage 50 ml). Cranial CT reexamination showed the area of high density in basal ganglia was reduced. Re-examination showed the following: WBC: 13.99 x 10^9/L; PLT: 124 x 10^9/L; D-dimer: 1.00 mg/L; Ca: 1.87 mmol/L; P: 0.60 mmol/L. The patient was resuscitated twice during hospitalization (continuous infusion of norepinephrine and vasopressin by intravenous pump) and was in a deep coma, mechanically ventilated (respirator: SIMV mode, VT 480 ml, PEEP 4 cmH2O), and without spontaneous respiration. The patient's family refused to continue using the ventilator and stopped further treatment.

3. Discussion

Secondary hyperparathyroidism is a common complication of end-stage renal disease (ESRD). Such patients mostly have multiple comorbidities. The overall mortality rate of patients with renal diseases, such as uremia, is 15–20% per year. Cerebral hemorrhage is ∼3.8 times more common in ESRD patients than in the general population (Thome et al., 2019). According to the US Renal Data System, 3.1% of dialysis patients died of a stroke in 2011 (Collins et al., 2012). With the advancements in hemodialysis technology, the survival time of uremic patients has increased substantially. However, the incidence of SHPT is increasing, and more patients are opting for parathyroidectomy. According to the latest edition of the AAES guidelines (2022), PTX can reduce cardiovascular and all-cause mortality in patients with SHPT. The benefits of surgery significantly exceed the drawbacks in patients who do not respond to medication and have significant symptoms (Dream et al., 2022). To prevent perioperative cerebral hemorrhage or reduce its severity, a comprehensive understanding of the risk factors for cerebral hemorrhage in HD patients with modifiable factors is necessary.

3.1. Risk factors for concurrent cerebral hemorrhage

The risk of perioperative cerebral hemorrhage increases due to the application of anticoagulants such as heparin during dialysis, making this treatment method life-threatening. Hyperlipidemia, hypertension, diabetes mellitus, age on dialysis, and mean arterial

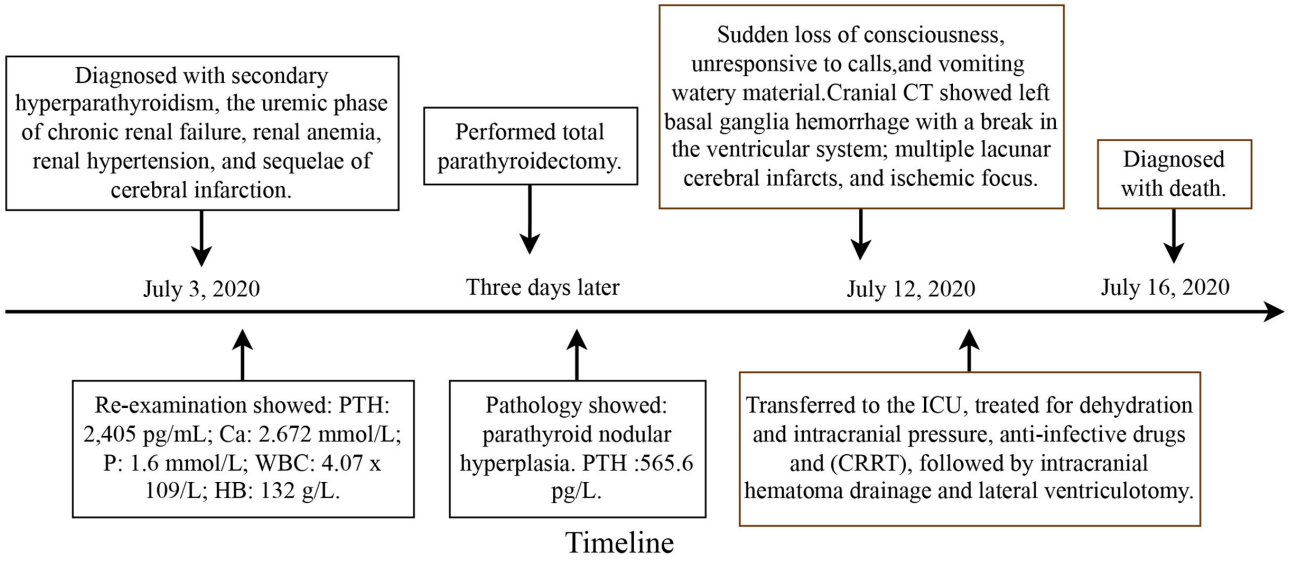

FIGURE 1
The timeline of case details.

pressure are high-risk factors for the development of complications of cerebral hemorrhage (Lau et al., 2018). Patients with a history of combined hyperlipidemia, hypertension, and diabetes mellitus may have atherosclerosis of intracranial vessels, which in turn might increase the fragility of intracranial vessel walls. Patients with advanced uremia may have increased blood volume and extracellular fluid and increased renin-angiotensin activity, resulting in water and sodium retention. This can cause an increase in mean arterial pressure, progressive aggravation of atherosclerosis, and an increase in vascular fragility. Sharp blood pressure fluctuations might cause dysregulation of the autonomic nervous system, which can lead to arterial and microaneurysm rupture and affect prognosis (Xiao et al., 2017; Yang et al., 2022). Several studies have investigated blood pressure control after CH; however, data on blood pressure before CH are limited, particularly on the increase in blood pressure before CH. Age on dialysis is strongly associated with the development of cerebral hemorrhage; the longer the age on dialysis, the higher the frequency of dialysis, which in turn gradually decreases the functions of the patient's organs (Kitamura et al., 2020). Administering exogenous anticoagulants causes fibrinogen to bind to platelets in the body, which can stimulate the production of platelet antibodies, induce thrombocytopenia, and cause bleeding. The greater the cerebral hemorrhage, the worse the prognosis. Brain tissue edema is more pronounced, and intracranial pressure is higher in patients on maintenance dialysis for uremia, and the prognosis is extremely poor if the volume of cerebral hemorrhage is >30 mL (Kitamura et al., 2019). Additionally, a decrease in hemoglobin and albumin levels increases hemorrhage and affects prognosis. The changes in hemoglobin levels after cerebral hemorrhage might be related to the higher incidence of hematoma enlargement in the early stages of a cerebral hemorrhage. Conversely, hemoglobin levels might reflect the severity and prognosis of a cerebral hemorrhage. A decrease in albumin levels can lower the resistance of the body, increase the risk of infection, and thus, aggravate the disease. Hypoproteinemia can decrease the plasma colloid osmotic pressure, promote the retention of a large quantity of fluid in the interstitial space, decrease the effective circulating blood volume, and decrease the perfusion of vital tissues and organs surrounding the site of intracranial hemorrhage, leading to a poor prognosis (Xiao et al., 2017; Kitamura et al., 2019; Yang et al., 2022). Additionally, high serum calcium, low serum sodium, high leukocyte count, a history of cerebrovascular diseases, primary polycystic kidney disease, and daily application of warfarin are independent risk factors for cerebral hemorrhage in uremic patients on regular hemodialysis.

Serum calcium can play multiple roles in each stage of the occurrence and progression of cerebral hemorrhage, as high blood calcium levels in patients with SHPT cause severe calcification of the vascular wall and trigger cerebral hemorrhage through several mechanisms (Xiao et al., 2017). Hypocalcemia is also associated with cerebral hemorrhage. Kitamura et al. showed that asymptomatic blood pressure increased in HD patients before cerebral hemorrhage; this increase in blood pressure was associated with frequent occurrences of lower serum calcium levels, but the exact mechanism of this phenomenon is unknown (Dandapat et al., 2019; Kitamura et al., 2020). In the perioperative period of parathyroidectomy (PTX), the patient is in a state of "calcium starvation" due to a sudden drop in PTH and an increase in skeletal mineralization (Liu et al., 2020), which leads to fluctuations in blood pressure due to sudden changes in multiple factors in the internal environment; these changes might promote cerebral hemorrhage. In patients with hemorrhagic stroke, moderate-to-severe CKD is associated with a 2.3-fold increase in the hematoma volume and a poor prognosis (Molshatzki et al., 2011; Nayak-Rao and Shenoy, 2017). Postoperative hypocalcemia should be aggressively treated by administering calcium supplements *via* oral and intravenous modalities.

3.2. Prevention and management of a cerebral hemorrhage in the perioperative period of parathyroid surgery

The hypercoagulable state of the blood in patients with SHPT increases the risk of hemorrhage. Before patients undergo surgery, cerebral hemorrhage and bleeding at the operative site should be prevented to reduce the risk of perioperative bleeding. Due to the multifactorial nature of cerebral hemorrhage, prevention and treatment options might include one or a combination of the following: dialysis, erythropoietin, cold precipitation, anticoagulation, antihypertensive drugs, and postsurgical correction of hypocalcemia (Hedges et al., 2007).

Patients should be examined as comprehensively as possible after admission. Besides the examinations related to the surgical area, examination of the cardiac, cerebral, and pulmonary conditions should be conducted to exclude contraindications to surgery. Preoperative precise localization is needed to decrease the operating time and the discomfort to the patient. Those receiving low-molecular heparin can be switched to heparin-free dialysis within 24 h before the operation. In recent years, the administration of sodium citrate as an anticoagulant in hemodialysis for patients with uremic cerebral hemorrhage has shown significant advantages. Through extracorporeal local anticoagulation without aggravating bleeding, it can ensure adequate hemodialysis, replenish blood sodium, reduce cerebral edema, promote hemorrhage absorption, and also reduce blood calcium due to the physical and chemical properties of sodium citrate. This can further reduce blood pressure and reduce inflammation and oxidative stress (Xun et al., 2021). The treatment can also be switched to anticoagulant dialysis with sodium citrate within 24 h before and after the operation. Hypertension in HD patients can prevent the risk of death, but the optimal predialysis blood pressure range remains undetermined. The risk of a new onset of cerebrovascular and arteriovenous fistula infarction increases with a decrease in blood pressure during the perioperative period. Additionally, the risk of cerebral hemorrhage decreases with better perioperative blood pressure control to reduce blood pressure fluctuations. According to the 2022 edition of the Expert Consensus, perioperative blood pressure should be regulated at a level of <180/100 mmHg (Dream et al., 2022). Since PTX was introduced in our hospital in 2010, only this patient died due to a postoperative cerebral hemorrhage. Although the risk of a cerebral hemorrhage is high, surgery can largely relieve clinical symptoms and improve the quality of life of the patients. Most patients benefit from surgery, and the incidences of patients with SHPT opting for surgery have increased.

3.3. Treatment and prognosis of a concurrent cerebral hemorrhage

If a cerebral hemorrhage occurs, treatment options and prognosis depend on several factors. For example, the treatment of patients with cerebral hemorrhage in uremia is divided into conservative medical treatment and surgical treatment. Surgery is the primary method for treating cerebral hemorrhage and is also the best option for patients on hemodialysis. However, the choice of surgical treatment and the type of procedure used remains undetermined. In most studies, mortality and disability rates after surgical treatment of cerebral hemorrhage were similar to those of patients treated non-surgically. Kim et al. (2013) reported three deaths in six surgically treated patients, and the other three were severely disabled. Therefore, patients with the uremic syndrome should be informed of the risks and adverse effects of surgery by neurosurgeons. (1) Surgery might be considered for patients with hemorrhage >35 mL in the basal ganglia or 50–60 mL in the cerebral lobes, but only for those who are stable and have no significant increase in hematoma on cranial CT reexamination; (2) surgery is generally not considered for patients with excessive hemorrhage (e.g., >100 mL) or patients >70 years; (3) preoperative and postoperative treatment should include active dialysis and preoperative or intraoperative transfusion of plasma, cold precipitation, or platelets to improve coagulation. Mannitol is a volumetric dehydrating agent that increases blood volume and relies on renal excretion. It can easily cross the fragile blood-brain barrier into the brain parenchyma and aggravate brain edema. To reduce intracranial pressure, glycerol fructose or albumin can be used instead of mannitol. In our study, the patient was a middle-aged female who had a combination of hypertension, cerebral infarction, and various underlying diseases. This led to a poor prognosis and increased the risk of death after the onset of a cerebral hemorrhage.

The reason for this is that ESRD is closely related to the occurrence of a cerebral hemorrhage in the perioperative period in patients with SHPT. Before patients with SHPT undergo surgery, surgeons should perform preoperative preparations and risk assessment predictions. The patients and their families should be informed of their condition and the risks and complications of surgery. In case of complications of a cerebral hemorrhage, timely diagnosis of the patients and early selection of appropriate treatment modalities might decrease their mortality and disability rate.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the 960th Hospital of People's Liberation Army. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

QH and PZ: contributed to the design and conception of this report. PZ and JX: data curation and writing-original

draft preparation. QH, DZ, and XL: supervision. QH and TY: writing-reviewing and editing. All authors read and approved the final manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2023.1153453/full#supplementary-material

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Long non-coding RNAs in intracerebral hemorrhage

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Intracerebral hemorrhage (ICH), a subtype of stroke, can lead to long-term disability and is one of the leading causes of death. Unfortunately, the effectiveness of pharmacological therapy for ICH is still uncertain. Long non-coding RNA (lncRNA) was defined as an RNA molecule that consists of more than 200nt without translational activity. As a vital class of diverse molecules, lncRNAs are involved in developmental and pathological processes and have been attractive for decades. LncRNAs have also become potential targets for therapies, as they were massively identified and profiled. In particular, emerging evidence has revealed the critical role of lncRNAs in ICH while attempts were made to treat ICH via regulating lncRNAs. But the latest evidence remains to be summarized. Thus, in this review, we will summarize the recent advances in lncRNA in ICH, highlighting the regulatory role of lncRNAs and their potential as therapeutic targets.

KEYWORDS

intracerebral hemorrhage, long non-coding RNA, pathology, therapeutic target, hemorrhagic stroke

1. Introduction

Intracerebral hemorrhage (ICH), a subtype of stroke, refers to the spontaneous rupture of injured small arteries or arterioles, leading to blood accumulation in cerebral parenchymal (Steiner et al., 2014; Gross et al., 2019). Although ICH counts for a smaller portion of all types of strokes (9–27%; Feigin et al., 2009; Sacco et al., 2009; Steiner et al., 2014), the global burden of ICH is higher than that of ischemic stroke (Krishnamurthi et al., 2013). A meta-analysis of nine studies showed that the mortality rate could be as high as 35.3% 3 months after ICH onset (Pinho et al., 2019).

Acute interventions for ICH, including medical therapies and minimally invasive surgery are likely to improve acute outcomes. Besides, sustained blood pressure control and optimized antithrombotic therapy are regarded as essential preventive strategies for improving longer-term outcomes in ICH (Hostettler et al., 2019). A better understanding of how the pathological mechanisms drive neurological injury in individuals is urgently required in order to develop therapies for acute and secondary progressive stages of ICH. Research on long non-coding RNA (lncRNA) has been attractive for decades. Up to now, a variety of lncRNAs have been proven to be involved in cerebral development and diseases, such as degeneration diseases of the central nerve system (Scheele et al., 2007; Chiba et al., 2009; Lagier-Tourenne et al., 2012; Ciarlo et al., 2013) and stroke (both ischemic and hemorrhagic stroke; Zhang and Wang, 2019). With the gain- and loss-of function methods, the functions of lncRNAs in ICH were further studied.

In this regard, it is crucial to reveal the current knowledge about the roles of lncRNAs in ICH. Therefore, we will briefly review the involvement of lncRNAs in the ICH-caused damages and their potential to be therapeutic targets and biomarkers will be discussed. The searching

strategies used in this article are summarized in Supplementary material.

2. The pathogenesis of acute and secondary insults after ICH

Quickly after vessel rupture, blood accumulates in the cerebral parenchymal, causing intracranial pressure elevation, perilesional edema, and structural damage, which may lead to brain hernia and can be fatal (Wilkinson et al., 2018). Mostly, bleeding stops shortly after ICH, but 14–22% of ICH patients may experience hematoma expansion in 6–24 h after ICH onset, which causes more severe structural damage and neurological deterioration, and leads to worse outcomes (Kazui et al., 1996, 1997; Fujii et al., 1998).

After the primary mechanical injury, complicated pathological responses will be triggered. The over-activated microglia release several cytokines and contributes to inflammation, blood brain barrier (BBB) breakdown, and edema in turn (Taylor and Sansing, 2013). Components of the complement system can pass through the damaged BBB and form membrane attack complex, enhancing the BBB injury and neurological damage (Xi et al., 2006). Heme and iron will be released during the erythrocyte elimination process (Wagner et al., 2003). The debris of blood enhances the production of free radicals and contributes to neurological injury, inflammation, BBB damage, and edema (Garton et al., 2016; Yang et al., 2016). Also, excitotoxicity molecules, such as glutamate was shown to anticipate in brain injury after intracerebral hemorrhage (Sharp et al., 2008). Although lots of efforts were made, the full picture of the ICH damage mechanisms, especially of the secondary injury, remains to be further explored.

After the acute stage, microglia polarize toward M2-like microglial, contributing to hematoma clearance (Lan et al., 2017). The hematoma breaks down with the invasion of macrophages and microvessel formation. The hemosiderin-stained scar, a cavity containing blood surrounded by fibrous tissue, and eventually gliosis will ultimately form (Fewel et al., 2003). And neuronal plasticity allows the brain to cope better with the indirect effects of brain damage resulting from ICH (Keep et al., 2012).

3. Insights into biological roles of lncRNAs in ICH

3.1. Introduction to lncRNA

Long non-coding RNA is commonly defined as an RNA molecule that consists of more than 200 nt without translational activity (Ponting et al., 2009; Nagano and Fraser, 2011). There are at least 170,000 lncRNAs found in humans and more than 130,000 lncRNAs identified in mice (Zhao et al., 2021). In accordance with the relationships between lncRNAs and their regulated genes, lncRNAs were classified as sense lncRNA, antisense lncRNA, intronic lncRNA, intergenic lncRNA, enhancer lncRNA, and circular lncRNA (Uchida and Dimmeler, 2015).

Long non-coding RNAs were long regarded as transcriptional garbage or transcriptional noise. Studies on lncRNA H19 and lncRNA Xist have initially revealed the biological functions of lncRNAs (Brannan et al., 1990; Wilusz et al., 2009; Gendrel and Heard, 2014). Briefly, lncRNAs can interact with proteins, DNA and RNA transcripts to control alternative splicing, chromosome remodeling, nuclear import and mRNA decay, and lncRNAs participate in almost every aspect of gene expression programs (Schmitz et al., 2010; Grote et al., 2013; Khorkova et al., 2015; Isoda et al., 2017).

In recent years, lncRNAs have been characterized are implicated in diverse diseases, including cardiovascular diseases (Uchida and Dimmeler, 2015), neurodegeneration diseases, ischemic stroke, and traumatic brain injury (Riva et al., 2016; Zhang and Wang, 2019; Ren et al., 2020).

3.2. LncRNAs play versatile roles in pathological processes underlying ICH

Several studies using high-throughput RNA-seq technique were conducted on mice or rat ICH model, and human samples. In a collagenase-induced mice ICH model, 31 lncRNAs were found to differentially express 24 h after modeling (Hanjin et al., 2018). And another study carried out on a similar model showed 625 dysregulated lncRNAs 21 days after ICH onset (Cao et al., 2020). A similar dynamic change of lncRNAs was observed in rats. Kim et al. (2019) found there were 83, 289, and 401 lncRNAs significantly upregulated and 52, 459, and 786 lncRNAs significantly downregulated 1, 3, and 7 days after collagenase-induced ICH modeling, respectively. These studies revealed the extensive involvement and the dynamic changes of lncRNAs in ICH.

RNA-sequencing data from GSE24265 (containing four human patients' RNA-seq data) were re-analyzed by Liu et al. (2021) and Yang et al. (2022). Liu et al. (2021) predicted that nine lncRNAs were associated with MAPK1 and may contribute to the progression of ferroptosis after ICH. Yang et al. (2022) found six hub lncRNAs and constructed the potential ceRNA network. In the peripheral blood of ICH patients, 211 lncRNAs dysregulated and were classified into16 lncRNA modules by weighted gene co-expression network analysis and some immune-related lncRNAs were also identified by using ceRNA network (Hao et al., 2022).

These studies supported the advantages of high-throughput techniques in the discovery, functional prediction, and key regulator identification of lncRNAs. However, the exact functions of dysregulated lncRNAs detected by RNA-seq remain to be explored and validated. And the different characteristics of lncRNA dysregulation between species, modeling methods and the less conserved characters of lncRNAs (Sharma and Carninci, 2020) require further study and more careful interpretation from preclinical study results.

Long non-coding RNA H19 (also known as H19 imprinted maternally expressed transcript) was one of the earliest identified lncRNAs (Brannan et al., 1990). H19 was observed to be one of the most stable lncRNAs in the gray matter of human brain and was extensively studied in the development and diseases of the central nerve system, including ischemic stroke, glioma, pituitary adenoma, neuroblastoma, degeneration, and trauma (Zhong et al., 2021). Recently, the role of H19 in ICH was extensively studied. In Kim's study, lncRNA H19 was the most upregulated lncRNA from day 1 through day 7 after ICH both in the ICH model induced by collagenase or autologous blood (Kim et al., 2019). Further bioinformatic analysis predicted that H19 was associated with type I interferon signaling pathway. Following this study, Chen and colleagues further studied the roles of H19 in ICH. After confirming the high expression level of H19 in the ICH cell model, Chen B. et al. (2021)

demonstrated that H19 targeted miR-106b-5p and thus regulated ACSL4, enhancing ferroptosis in brain microvascular endothelial cells (BMVECs) under oxygen and glucose deprivation hemin-treated (OGD/H-treated) condition, as validated by RNA pull-down and luciferase reporter gene assays. In ICH rat model induced by type IV collagenase, NF-κBp65 and IKKβ expression were significantly lower and IκBα was significantly higher in the sh-H19 group when compared with ICH model group, indicating that H19 may be associated with NF-κB pathway. Mao et al. (2022) also found that elevated level of H19 expression was associated with the levels of TNF-α, IL-6, IL-1β, ROS, and MDA, showing that H19 was also associated with inflammation and oxidative stress. Also, H19 was found to be associated with the risk of symptomatic ICH in ischemic stroke patients after recombinant tissue plasminogen activator treatment (Han et al., 2022). Collectively, H19 could interact with miRNA and was demonstrated to be associated with NF-κB pathway, inflammation, free radical production and cell death after ICH.

The lncRNA FOXF1 adjacent non-coding developmental regulatory RNA (FENDRR) increased in C57BL/6 mice with hypertensive ICH (Dong et al., 2018) and was demonstrated to target miR-126 by RNA immunoprecipitation and RNA pull-down. Via targeting miR-126, FENDRR regulated VEGFA and thus contributed to the apoptosis of human brain microvascular endothelial cells. Importantly, it was demonstrated that VEGFA was important in the activation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT; Ruan and Kazlauskas, 2012).

Xie et al. found that MEG3 elevated in collagenase-induced ICH rat brain tissues. The interaction of MEG3 and miR-181b was validated by Starbase analysis and dual-luciferase reporter assay. And they proved the interaction of MEG3 and miR-181b was associated with the activity of PI3K/AKT pathway. In this study, upregulation of MEG3 was associated with the release of inflammatory cytokines and oxidative stress, contributing to brain edema, neuronal apoptosis, and increased caspase3 activity, which can be reversed by miR-181b inhibition (Xie B. et al., 2021). More importantly, the relationship of MEG3 and miR-181b was confirmed in patients with severe ICH. The upregulation of MEG3 and the downregulation of miR-181b were also observed in serum from ICH patients (Wang H. et al., 2022).

Similarly, lncRNA FGD5-AS1 was also found to regulate PI3K/AKT pathway in collagenase-induced C57BL/6 mice ICH model, although via targeting miR6838-5p/VEGFA axis, which was validated by luciferase reporter gene and pull-down assays. The interaction between FGD5-AS1 and miR6836-5p led to inhibited cell proliferation, increased pro-inflammatory factors, and injured BBB. The proinflammatory effects of FGD5-AS1 were also verified in BMVECs. This study also detected the upregulation of FGD5-AS1 in the serum of ICH patients (Jiang et al., 2022).

GAS5, an extensively studied lncRNA in ischemic stroke, also played important roles in ICH. Wang and colleagues found that FoxO1 could enhance the expression of GAS5 by binding to its promoter. GAS5 bond miR-378a-5p and upregulated the expression of Hspa5, which caused a significant elevation of pro-inflammatory factors, brain edema and neurological injury (Wang B. et al., 2022). And FoxO1 was demonstrated to be a major PI3K-AKT downstream effector (Xing et al., 2018).

Chen and colleagues established the ICH model in mice by collagenase injection and in the cellular inflammation model by treating microglia with lipopolysaccharide. After sequencing RNAs obtained from mice, they found the expression of NONMMUT023599.2 was significantly upregulated. Thus, NONMMUT023599.2 knocking down was conducted in both ICH mice and cellular inflammation model, which strongly downregulated the expression of TRIF, p65 phosphorylation and the secretion of TNF-α and IL-1β, indicating the regulatory potential of NONMMUT023599.2 in NF-κB pathway. The elevation of miR-709, which was previously predicted to be the target of NONMMUT023599.2, was also observed. Authors concluded that elevated levels of NONMMUT023599.2 targeted miR-709, regulated NF-κB pathway, enhanced inflammatory cytokine secretion (such as TNF-α and IL-1β) and thus worsened the outcome after ICH in mice (Chen et al., 2020).

Nuclear factor-k-gene binding interacting lncRNA (NKILA) was first identified in breast cancer. NKILA binds to NF-κB/IκB, masks the phosphorylation motifs of IκB, and thereby inhibits IKK-induced IκB phosphorylation and NF-κB activation. The interaction of NKILA and NF-κB pathway signaling molecules prevented the over-activation of NF-κB pathway (Liu et al., 2015). Zhang et al. also proved that NKILA got involved in the pathological changes after ICH on a collagenase-induced rat model (Jia et al., 2018). NKILA was downregulated in the collagenase-induced rat ICH model. Interestingly, further inhibition of NKILA by siRNA activated NF-κB pathway, reduced endoplasmic reticulum stress, neuron autophagy and neurological deficits, but exacerbated brain edema, neuronal cell apoptosis, BBB breakdown, and promoted inflammatory cytokines release.

The c-Jun N-terminal kinase (JNK) signaling pathway also regulates both physiological and pathological processes, such as neurodegenerative diseases, and inflammatory diseases. Dual specificity phosphatases (DUSPs) were found to regulate the JNK pathway by dephosphorylating their substrates, e.g., DUSP6 binds to JNK2/3 and thus inhibits JNK phosphorylation (Ha et al., 2019). LncRNA TCONS_00145741 could disrupt the interaction between DUSP6 and JNK and stabilize JNK phosphorylation, which suppressed M2 differentiation of microglia after ICH (Wu et al., 2021).

Other studies also pointed out the important roles of some lncRNAs in ICH, although, lack specific targets. Zhang et al. found that SNHG3 expression was significantly induced both in OGD/H-treated BMVECs and in the collagenase-induced rat ICH model. The overexpression of SNHG3 upregulated TWEAK and its receptor Fn14, activating the downstream neuroinflammatory pathway STAT3 and enhancing the expression of matrix metallopeptidase 2/9, causing dysfunction of cerebral microvascular cells after ICH (Zhang et al., 2019). In the autologous blood injection ICH mice model, BLNC1 was upregulated in perihematomal edema, hematoma and microvessel. The elevation of BLNC1 enhanced the apoptosis of BMVECs, potentially by activating PPAR-γ/SIRT6/FoxO3 pathway (Xie L. et al., 2021).

The current knowledge about lncRNAs in ICH-induced secondary injury was summarized in Figure 1. The studies included in this section and the locations of the corresponding lncRNAs are summarized in Table 1. Although many of the lncRNAs were studied in mice or rats, most of them were named in their original articles with capital letters, so we named them with capital letters.

3.3. LncRNAs are implicated as diagnose markers and therapeutic targets for ICH

The pathological process of ICH is complicated, thus if the treatment targets versatile regulators, the outcomes of ICH might

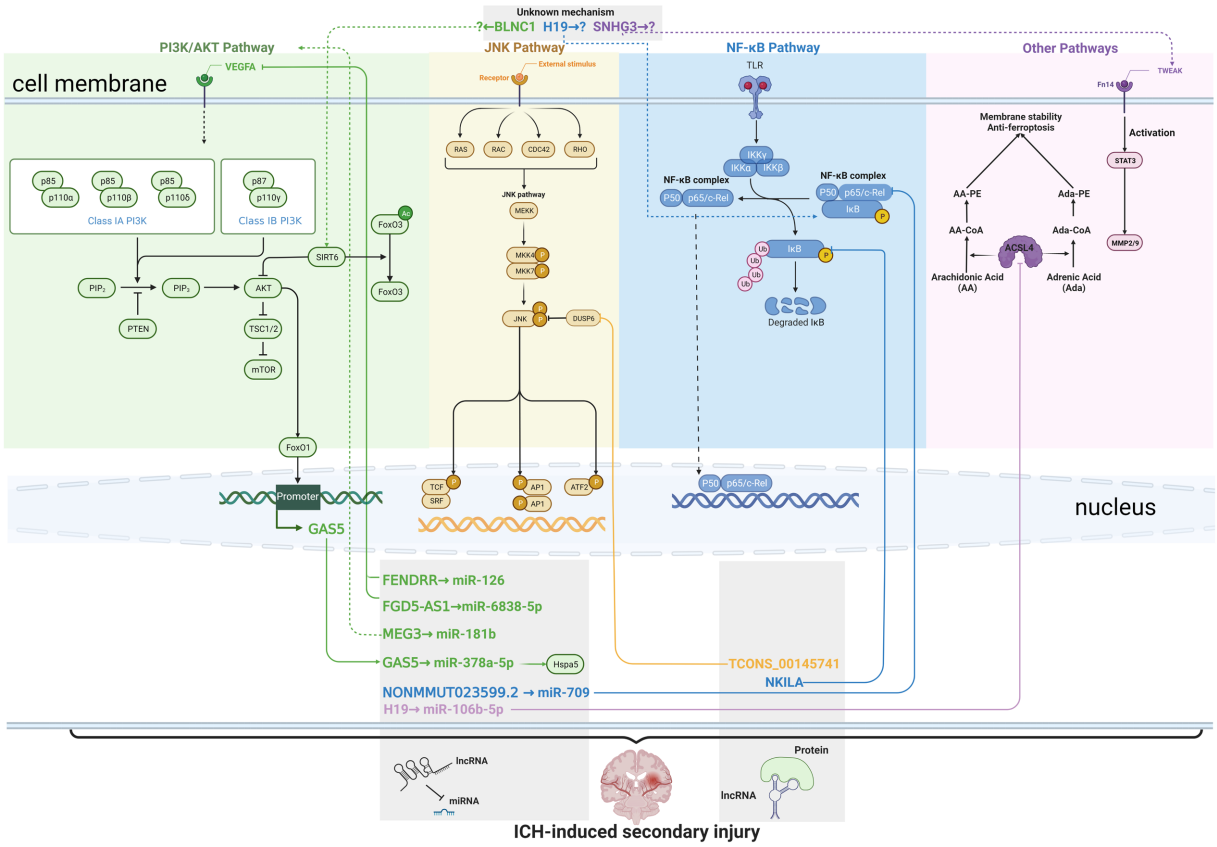

FIGURE 1
Current knowledge about long non-coding RNAs (lncRNAs) in intracerebral hemorrhage (ICH)-induced secondary injury. Solid lines indicate direct interaction, while dashed lines indicate unknown or indirect regulation. The lncRNAs are marked in green, orange, blue, and purple to indicate their regulation of (or modulation by) the PI3L/AKT pathway, JNK pathway, NF-κB pathway, and other pathways, respectively. The lncRNAs in the same gray box share the similar action mechanisms, such as interacting with miRNAs or proteins. FENDRR, FGD5-AS1, GAS5, NONMMUT023599.2, and H19 were reported to interact with miRNAs, while TCONS_00145741 and NKILA were reported to interact with proteins that contributed to pathological changes after the onset of ICH.

be improved. Therefore, lncRNAs were capable to be the targets. This assumption was proven right, as knockdown of H19 in ICH rats significantly reduced inflammatory response, oxidative injury, and improved neurological function (Mao et al., 2022). Several drugs were proved to alleviate ICH-induced injury via regulating lncRNAs. After treating ICH rats with Buyang Huanwu decoction, the traditional Chinese formula, Cui et al. found 18 lncRNAs related to ICH were regulated (Cui et al., 2018). Paeonol, a natural product derived from *Paeonia Suffruticosa Andr.*, was found to inhibit ferroptosis by regulating lncRNA HOTAIR/UPFI/ACSL4 axis and thus inhibit the progression of ICH (Jin et al., 2021). These two studies showed that lncRNAs involved in ICH could be regulated by drugs, and the regulation of lncRNAs ameliorated ICH-induced damage. More importantly, the results of Cui's study indicated that numerous lncRNAs can be regulated simultaneously and contributed to better outcomes.

Although we now know that lncRNAs may control the secondary injury in ICH, the specific targets were not extensively studied. lncRNAs can interact with several types of macromolecules, but only limited miRNAs and signaling molecules as targets were further analyzed and validated, while some studies even lacked the validation of targets and numerous lncRNAs were not studied. Lacking specific targets impede drug R&D, even when the transcriptomics data provide hints (Dugger et al., 2018). Identifying the targets and validating the exact functions of lncRNAs in ICH remain attractive and important for drug R&D. Also, efforts were made to use CRISPR/Cas9, siRNAs, etc. to treat diseases by targeting lncRNAs. Viral, liposomes and exosomes might be used to deliver lncRNAs to peri-hematoma tissue (Chen Y. et al., 2021). However, stable targeting delivery systems are still lacking. Although most pharmaceutical research is still far from clinical translation, these studies are creative, attractive and have the potential to be beneficial for ICH treatment.

As mentioned above, after ICH onset, lncRNAs dysregulated not only in brain tissue, but also in the circulation system, such as MEG3 and FGD5-AS1 (Hao et al., 2022; Jiang et al., 2022; Wang H. et al., 2022). Also, the association between H19 and the risk of symptomatic ICH in ischemic stroke patients treated with recombinant tissue plasminogen activator was established (Han et al., 2022). The presence of dysregulated lncRNAs in serum, which were also associated with ICH, provided an easier sampling routine and opportunities to use lncRNAs as biomarkers in diagnosing ICH.

On the other hand, the opposite treatments between ischemic stroke and ICH and the nature of rapid pathological changes of both ischemic stroke and ICH require accurate and quick differential diagnosis between these two diseases. With technique development,

TABLE 1 Summary of studies concerning lncRNAs in ICH.

lncRNA	Location*	Target	Subject	Modeling method/ Study design	Main finding	Author and publishing year
H19	11p15.5	Unknown	SD rat	cICH and bICH	H19 was the most upregulated lncRNA and was associated with type I interferon signaling pathway.	Kim et al. (2019)
		miR-106b-5p	BMVEC	OGD/H-treated	H19 targeted miR-106b-5p and thus regulated ACSL4, contributing to ferroptosis.	Chen Y. et al. (2021)
		Unknown	SD rat	cICH	H19 may be associated with NF-κB pathway.	Mao et al. (2022)
		Unknown	Human	Cohort study	H19 was associated with the risk of symptomatic ICH in ischemic stroke patients after recombinant tissue plasminogen activator treatment.	Han et al. (2022)
FENDRR	16q24.1	miR-126	C57BL/6 mice	hICH	FENDRR contributed to the apoptosis of BMVEC.	Dong et al. (2018)
MEG3	14q32.2	miR-181b	SD rat	cICH	MEG3 was associated with the release of inflammatory cytokines and oxidative stress.	Xie B. et al. (2021)
		Unknown	Human	Cross-sectional study	The upregulation of MEG3 and the downregulation of miR-181b were also observed in serum from ICH patients.	Wang H. et al. (2022)
FGD5-AS1	3p25.1	miR6838-5p	1. C57BL/6 mice	1. cICH	1. The interaction between FGD5-AS1 and miR6836-5p led to inhibited cell proliferation, increased pro-inflammatory factors and injured BBB. 2. The upregulation of FGD5-AS1 was observed in the serum of ICH patients.	Jiang et al. (2022)
			2. human	2. cross-sectional study		
GAS5	1q25.1	miR-378a-5p	C57BL/6 mice	bICH	GAS5 contributed to the significant elevation of pro-inflammatory factors, brain edema and neurological injury.	Wang B. et al. (2022)
NONMMUT023599.2	15qD1	miR-709	C57BL/6 mice	cICH	NONMMUT023599.2 regulated NF-κB pathway.	Chen et al. (2020)
NKILA	20q13.31	IκB	SD rat	cICH	Inhibition of NKILA after ICH activated NF-κB pathway, reduced neurological deficits, although exacerbated brain edema and BBB breakdown.	Jia et al. (2018)
TCONS_00145741	Not reported	DUSP6 and JNK	C57BL/6 mice	bICH	TCONS_00145741 stabilized JNK phosphorylation and suppressed M2 differentiation of microglia after ICH.	Wu et al. (2021)
SNHG3	1p35.3	Unknown	SD rat	cICH	SNHG3 upregulated TWEAK and its receptor Fn14, which were associated with neuroinflammatory pathway STAT3.	Zhang et al. (2019)
BLNC1	Not reported	Unknown	C57BL/6 mice	bICH	After ICH, Blnc1 activated PPAR-γ/ SIRT6/FoxO3 pathway and enhanced the apoptosis of BMVEC.	Xie L. et al. (2021)

BMVEC, brain microvascular endothelial cells; bICH, autologous blood-induced intracerebral hemorrhage; cICH, collagenase-induced intracerebral hemorrhage; hICH, hypertensive hypertension; OGD/H-treated, oxygen and glucose deprivation hemin-treated; and SD rat, sprague dawley rat. *In human chromosome.

RNA identification and quantification can be much faster (Carter et al., 2021), which aids the diagnosis and saves time when deciding on proper treatments, especially in rural hospitals (Kamtchum-Tatuene and Jickling, 2019).

Although previous studies demonstrated that mRNAs may have the potential to be biomarkers for the diagnosis and the differentially diagnose of ICH (Stamova et al., 2019), we still lack knowledge about the sensitivity, specificity, and the value of lncRNA for ICH therapy. Opportunities lie ahead, but studies are still needed.

4. Conclusion

Previous studies profiled the multi-functional roles of lncRNAs in ICH. lncRNAs are involved in ICH-induced secondary injury, such as inflammatory response, oxidative stress and cell death, etc., via targeting miRNAs or signaling molecules. We are at the infant stage and this field remains attractive. More studies could be conducted to validate the targets of lncRNAs, evaluate the value of lncRNAs as risk prediction for ICH, and develop drugs or special delivery systems to treat ICH by targeting lncRNAs.

Author contributions

CZ and YZ wrote the manuscript. QW and ZF drew the figure. MZ and XX conducted literature search. TX reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2023.1119275/full#supplementary-material

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Efficacy and safety of inclisiran in stroke or cerebrovascular disease prevention: a systematic review and meta-analysis of randomized controlled trials

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Aims: As the impact of inclisiran in stroke prevention in atherosclerotic cardiovascular disease (ASCVD) patients or those at high risk of ASCVD is still unclear, we conducted a systematic review and meta-analysis of randomized controlled trials (RCT) to quantify the effectiveness of inclisiran in stroke prevention in these patients.

Methods: Literature research was conducted in four electronic databases (PubMed, EMBASE, Web of Science, CENTRAL) and two clinical trials registers (ClinicalTrials.gov, WHO ICTRP) from the inception of the study to 17 October 2022, and was updated by the end of the study on 5 January 2023. Two authors independently screened the studies, extracted the data, and assessed the bias. The risk of bias was assessed using the Cochrane risk-of-bias tool for randomized trials (RoB 2). The intervention effect was estimated by calculating risk ratio (RR), weighted mean difference (WMD), and 95% confidence interval (CI) with R 4.0. 5. Sensitivity analysis by changing meta-analysis model was also performed to test the robustness of the pooled results. If this was not possible, a descriptive analysis was conducted.

Results: Four RCTs (n = 3,713 patients) were rated as high-risk bias. Meta-analysis of three RCTs (ORION-9, ORION-10, and ORION-11) showed that inclisiran reduced myocardial infarction (MI) risk by 32% (RR = 0.68, 95%CI = 0.48–0.96) but did not reduce stroke (RR = 0.92, 95%CI = 0.54–1.58) and major cardiovascular events (MACE) (RR = 0.81, 95%CI = 0.65–1.02) risk. Sensitivity analysis results were stable. Safety was similar to the placebo group but had frequent injection-site reactions (RR = 6.56, 95%CI = 3.83–11.25), which were predominantly mild or moderate. A descriptive analysis of one RCT (ORION-5) was conducted due to different study designs, and suggested that inclisiran might be given semiannually from the beginning.

Conclusion: Inclisiran is not beneficial for stroke or MACE prevention in ASCVD or patients at high risk of ASCVD but is associated with the reduction of MI. Given the limited number and quality of the available studies and the lack of a standardized definition for cardiovascular events, further studies are essential for confirming the results.

KEYWORDS
inclisiran, stroke, ASCVD, systematic review, meta-analysis, rct

1 Introduction

Stroke is a neurological disease in which brain tissue is damaged due to the sudden rupture of a blood vessel or a blood vessel embolism, which can lead to sudden death (Kuriakose and Xiao, 2020) and can also generate depression (Medeiros et al., 2020) and dementia (Pasi et al., 2012). Stroke is characterized by high incidence, high disability, high recurrence, high death, and high burden. According to the statistics, in 2021, stroke remained the second-leading cause of death and the third-leading cause of death and disability combined in the world. Atherosclerotic cardiovascular disease (ASCVD) refers to the accumulation of plaque in the artery, with a risk of bleeding within the plaque, necrotic core rich in lipids, and fibrous cap rupture (Deng et al., 2020), which can lead to the occurrence of acute coronary syndrome, angina pectoris, stroke, transient ischemic attack (TIA), and peripheral artery disease (Rogers and Baker, 2020). The risk of stroke is further increased when patients have GBD 2019 Stroke Collaborators. (2021), so it is particularly important to prevent the occurrence of stroke in ASCVD and in patients at high risk of ASCVD. Lipid, especially low-density lipoprotein cholesterol (LDL-C), is the most prominent risk factor for ASCVD (Khatana et al., 2020). Lipid detection in stroke patients showed that the levels of total cholesterol (TC), triglyceride (TG), LDL-C, apolipoprotein A (Apo A), apolipoprotein B (Apo B), apolipoprotein E (Apo E), and lipoprotein a (Lp [a]) were significantly higher, while high-density lipoprotein cholesterol (HDL-C) was significantly lower (Yuan et al., 2015). Moreover, low HDL-C (<0.90 mmol/L) and high TG (>2.30 mmol/L) were associated with a two-fold increased risk of death in stroke (Kuriakose and Xiao, 2020). The LDL-C level was positively correlated with the occurrence of ischemic stroke (Holmes et al., 2018) and associated with long-term post-stroke mortality (Xing et al., 2016). Therefore, lipid-regulating therapy may play a key role in stroke prevention, especially for patients with ASCVD or at high risk of ASCVD.

Currently, statins are recommended as the first choice to reduce LDL-C in patients with increased risk of stroke in stroke prevention guidelines (Amarenco et al., 2004), and the benefits of more intensive LDL-C-lowering statin-based therapies for recurrent stroke risk reduction might be more favorable than the less intensive LDL-C-lowering statin-based therapies (Lee et al., 2022). The preventive effect of non-statin drugs, such as ezetimibe and proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, on stroke also produced significant benefits in studies (Hackam and Hegele, 2022), and compared to low-risk populations, the effect was only seen in high-risk ASCVD populations who had received a maximum tolerated dose of statins or who were intolerant to statins (Khan et al., 2022). Statins combined with ezetimibe or PCSK9 monoclonal antibody could reduce the risk of stroke by 26% (Khan et al., 2022). A 2021 guideline for the prevention of stroke in patients with stroke and TIA (Kleindorfer et al., 2021) recommended that for patients with a very high risk of stroke who have been treated with the combination of a maximum tolerated dose of statins and ezetimibe but whose LDL-C level is still not up to the standard, PCSK9 monoclonal antibody is a feasible therapy to prevent cardiovascular events (CVEs). However, for some patients, even after treatment with the previously mentioned drugs, the lipid level still fails to reach the standard. Furthermore, as a disease requiring long-term drugs for prevention, the incidence of stroke is higher in low and middle-income countries, and one-third of patients discontinue the use of one or more prevention drugs approximately 1 year after stroke (2021). Therefore, the development of a new mechanism for lipid-regulating treatments with better economic effectiveness and compliance is of great significance.

The degradation of LDL-C requires the action of LDL receptor (LDL-R) in the liver, and PCSK9 can compete with LDL-C, bind, and cause the LDL-R to be degraded by lysosomes (Moustafa and Testai, 2021). This process reduces the density of LDL-R on the cell surface and increases the LDL-C level. Therefore, inhibiting the synthesis of PCSK9 is an important mechanism in the development of lipid-regulating drugs. PCSK9 monoclonal antibodies were designed to reduce LDL-C by preventing the combination of and interaction between PCSK9 and LDL-R (Go and Mani, 2012). Over the past few decades, the birth of ribonucleic acid (RNA) interference (RNAi)-based therapeutics has ushered in a new era of drug development (Gangopadhyay and Gore, 2022). Inclisiran, the first small interfering RNA (siRNA) drug in the cardiovascular field and a new PCSK9 inhibitor, is an example of nucleic acid therapeutics.

Inclisiran consists of a passenger strand and a guide strand, with a triantennary N-acetylgalactosamine (tri-GalNAc) conjugated to the end. As an established liver targeting technique, the tri-GalNAc can specifically bind to the asialoglycoprotein receptor, which is only highly expressed in the liver (Springer and Dowdy, 2018). In this way, after inclisiran is specifically introduced into liver cells, with the assistance of the passenger strand, the guide strand identifies the information of PCSK9 message RNA (mRNA) and forms RNA-induced silencing complexes (RISC) with some enzymes inside the cell. RISC performs the cleavage and degradation of PCSK9 mRNA to block the synthesis of PCSK9 and reduce the LDL-C level (Fitzgerald et al., 2017; Khvorova, 2017).

Inclisiran has now been proven to have effective and long-lasting effects, with a single subcutaneous injection reducing the LDL-C level for 6 months. Compared with PCSK9 monoclonal antibodies, inclisiran is closer to the source of dyslipidemia, and the administration schedule (twice a year) also allows healthcare providers to manage ASCVD patients during their regular visits and improve compliance (Soffer et al., 2022).

Inclisiran has been shown to have a strong and consistent lipid-lowering effect in some randomized controlled trials (RCT). RNAi therapy may be used if statins are not effective in reducing lipid levels or are intolerant. Therefore, inclisiran may be of great significance in stroke prevention. However, the efficacy and safety of inclisiran in stroke prevention in ASCVD or ASCVD high-risk patients remain unclear. Therefore, we conducted a systematic review and meta-analysis of the available evidence from RCTs to quantify the effectiveness of inclisiran in the

prevention of the risk of stroke in patients with ASCVD or at high risk of ASCVD.

2 Methods

This study was reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 statement (Page et al., 2021) (the PRISMA 2020 checklist is shown in Supplementary Table S1) (Shamseer et al., 2015). We have registered this study in the International Prospective Register of Systematic Reviews (PROSPERO) (registration number: CRD42022374280).

2.1 Literature search and inclusion criteria

The databases Pubmed, Embase, the Cochrane Central Register of Controlled Trials (CENTRAL), and Web of Science were researched from the study's inception to 17 October 2022 for potentially relevant studies, without language restrictions, using the search terms: exposure (Cardiovascular Diseases or Heart Disease Risk Factors or stroke or Cerebrovascular Disorders or Ischemic Attack), intervention (Inclisiran or ALN-60212 or ALN-PCS or ALN-PCSsc), and study (randomized controlled trial or controlled clinical trial or randomized). We also searched two clinical trials registers, ClinicalTrials.gov (https://ClinicalTrials.gov/) and WHO ICTRP (https://trialsearch.who.int/), for RCTs using the search terms: intervention (ALN-PCSsc or ALN-60212 or PCSK9si KJX-839 or inclisiran or small interfering RNA or RNAi or siRNA or RNA, Small Interfering) and filters (with results). By the end of the study (5 January 2023) and the revision of the study (6 March 2023), we retrieved and updated the inclusions. The complete search terms and records are provided in Supplementary Table S2. Additionally, we manually examined the reference lists of retrieved studies to identify additional relevant literature.

The studies were included if they met the following criteria: (1) the enrolled patients suffered from ASCVD or were at high risk of ASCVD (Supplementary Table S3); (2) the intervention was inclisiran used alone or in combination with other lipid-regulating drugs. The duration of inclisiran treatment met the current standard administration: 300 mg dosage of inclisiran sodium or 284 mg dosage of inclisiran administered as a single subcutaneous injection initially, then again at 3 months, and then every 6 months, all subjects receiving at least three doses; (3) the control group was given a placebo or other drugs; (4) the outcomes include at least one of the following: stroke, cerebrovascular disease, major adverse cardiovascular events (MACE), all-cause mortality, change in serum LDL-C, PCSK9, and other lipid parameters (TG, TC, HDL-C, et al.) from baseline to the last available follow-up, adverse events (AE), treatment-emergent adverse events (TEAE), TEAE leading to discontinuation of treatment, and serious adverse events (SAE) (the definition of SAE is shown in Supplementary Table S4 and is consistent with the Good Clinical Practice Guideline of International Conference on Harmonization); (5) the study was designed as an RCT. Studies were excluded if they met one of the following criteria: (1) the results were not yet available or the full text could not be accessed; (2) the articles were conference articles, letters, qualitative studies, reviews, commentaries, pilot studies, or protocols.

All titles and abstracts of the studies were downloaded and imported into Endnote X9. Study selection was independently conducted by two review authors (ML and YL) after deleting the duplications by automatic tool and by humans. The irrelevant studies were excluded by screening the titles and abstracts first and then reviewing the full text of each literature to select the included studies in conformity with the eligibility criteria. If there were discrepancies in any details of the literature, the third reviewer (XX) made the necessary decisions after discussion.

2.2 Data extraction and outcome assessments

Two authors (ML and YL) independently extracted data from eligible studies using a data extraction form set in advance. The contents of the data extraction form included author, published year, name of RCT, data source (from literature or clinical trials registers), ClinicalTrials.gov Identifier, study design, duration of follow-up, participants (sample size, diagnosis, background therapy), intervention group (age, sex, sample size, baseline LDL-C mg/dL), control group (age, sex, sample size, baseline LDL-C mg/dL), outcomes (primary endpoints, key secondary endpoints, prespecified exploratory endpoints, safety).

The primary outcomes of our study were the occurrence of stroke or cerebrovascular disease and MACE. The secondary outcomes were all-cause mortality, change in serum LDL-C and PCSK9 levels from baseline to the last available follow-up, change from baseline in other lipid parameters, and TEAEs, TEAE leading to discontinuation of treatment, and SAEs.

The primary outcomes were defined using the standardized Medical Dictionary for Regulatory Activities (MedDRA) queries (SMQs) from MedDRA version v20.1. Stroke and cerebrovascular disease were defined as central nervous system vascular conditions (SMQ), which can also be subdivided into "ischaemic central nervous system vascular conditions (SMQ)," "hemorrhagic central nervous system vascular conditions (SMQ)," and "central nervous system vascular disorders, not specified as hemorrhagic or ischaemic (SMQ)." MACE was defined as the composite of "cardiovascular cause death," "myocardial infarction (MI)," "stroke," "cardiac arrest," and "cardiac failure." The included SMQ and preferred term (PT) for each outcome can be found in Supplementary Table S4.

2.3 Risk of bias

The risk of bias in each included study was also independently assessed by two authors (ML and YL) using the Cochrane risk-of-bias tool for randomized trials (RoB 2) recommended in the Cochrane, 2022. The discrepancies were resolved by the third author (XX). RoB 2 is structured into a fixed set of domains of bias, focusing on different aspects of trial design, conduct, and reporting. It includes five domains: "bias arising from the randomization process," "bias due to deviations from intended

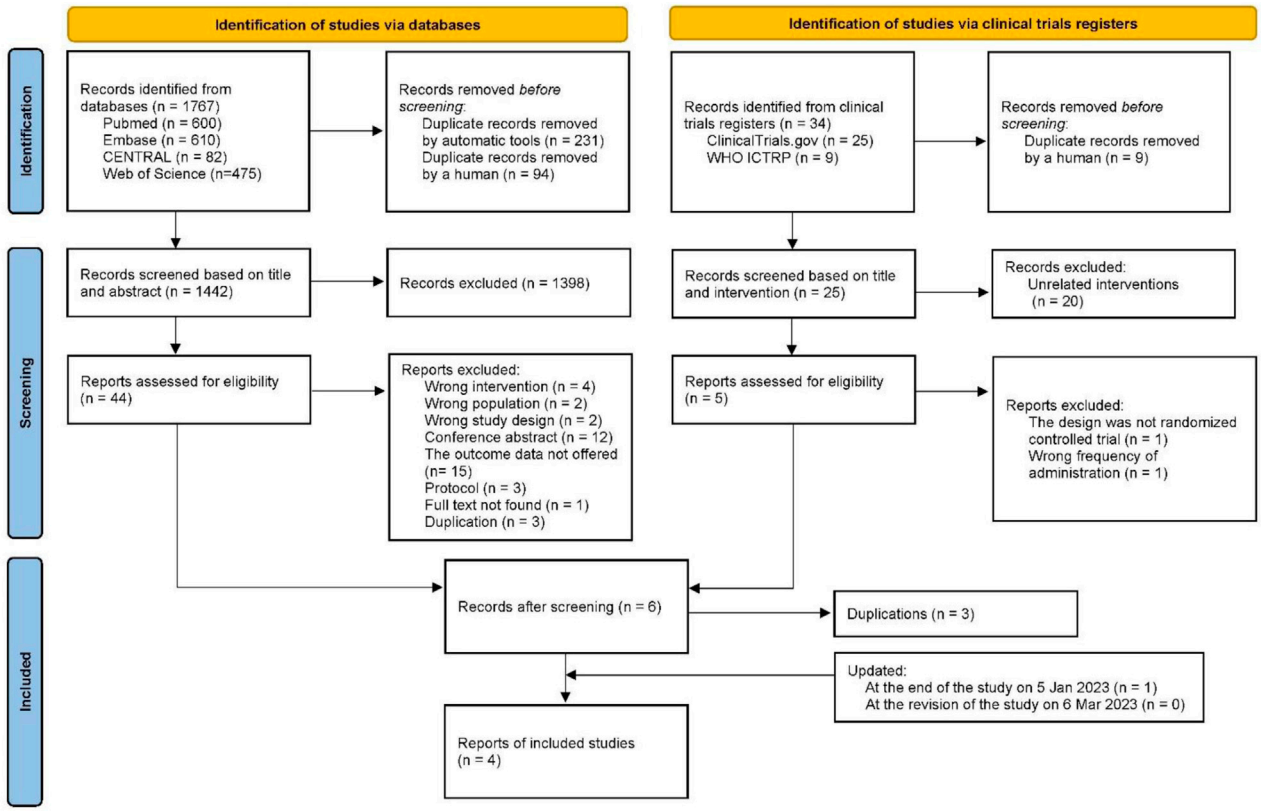

FIGURE 1
PRISMA flowchart.

interventions," "bias due to missing outcome data," "bias in measurement of the outcome," and "bias in selection of the reported result." Each domain has a series of signaling questions that need to be judged and responded to objectively by the authors based on the actual content of the studies. There are five response options in each domain: "Yes (Y)," "Probably yes (PY)," "Probably no (PN)," "No (N)," and "No information (NI)." Once the signaling questions are answered, a risk-of-bias judgment can be reached and one of three levels can be assigned to each domain: "Low risk of bias," "Some concerns," or "High risk of bias."

2.4 Data synthesis and analysis

The heterogeneity tests and meta-analysis were conducted with the "meta-package" of R statistical language version 4.0.5. Heterogeneity between studies was assessed using the I^2 statistic and Cochran's Q test, with I^2 >50% and p-value < 0.10 considered as having high heterogeneity. If high heterogeneity was present between studies, we used a random-effects model or provided a narrative overview. If heterogeneity was not identified, we computed pooled estimates of the treatment effect for each outcome under a fixed-effect model. For dichotomous outcome measures (such as cardiovascular outcomes), we calculated a pooled estimate of the treatment effect for each outcome across trials using the risk ratio (RR) and 95% confidence intervals (CI) according to the Mantel-Haenszel method. For continuous outcomes (such as LDL-C and PCSK9 levels), we used the weighted mean difference (WMD) with 95%CI. The overlap of intervention effects was shown using a forest plot, and differences with p values of <0.05 were considered statistically significant. In addition, we conducted a sensitivity analysis by changing pooled model to test the robustness and reliability of the pooled results. If the number of included studies was ≥10, a funnel plot or an Egger's test was used to assess publication bias (2016; Riley et al., 2019), otherwise, it was regarded as the existence of publication bias.

3 Results

3.1 Study selection

In the initial search, 1,767 and 34 records were retrieved from four electronic databases and two clinical trial registers, respectively. After removing 334 records using the automatic tool and manual de-duplication, 1,418 records were excluded according to the review of the titles, abstracts, and interventions. Subsequently, 44 records from databases and 5 records from registers underwent full-text review. Finally, three studies, ORION-9 (Raal et al., 2020), ORION-10 (Ray et al., 2020), and ORION-11 (Ray et al., 2020), were eligible for data extraction and quantitative analysis. The search and selection processes are shown in Figure 1. At the end of the

TABLE 1 The characteristics of included studies[a].

Author/ Published year/Name	ClinicalTrials.gov Identifier (NCT number)	Study design	Locations	Duration of follow-up (months)	Participants				Intervention group				
					Sample size (N)	Diagnosis	Background therapy	White people (N)	Age, mean (SD) (years)	Male/ Female	Sample size (N)	Treatment	Baseline LDL-C, mean (SD), mg/dL
Raal et al. (2020) ORION-9	NCT03397121	RCT (DB, PC)	United States, Canada, Europe, South Africa	18	482	HeFH	Maximally tolerated statin with/without other LLT	453	54.4 (12.48)	112/130	242	Inclisiran 300 mg at day 1, day 90, then every 6 months	151.4 (50.4)
Ray et al. (2020) ORION-10	NCT03399370	RCT (DB, PC)	United States	18	1561	ASCVD		1311	66.4 (8.9)	535/246	781	Inclisiran 300 mg at day 1, day 90, then every 6 months	104.5 (39.6)
Ray et al. (2020) ORION-11	NCT03400800	RCT (DB, PC)	Europe, South Africa	18	1617	ASCVD or an ASCVD risk equivalent		1587	64.8 (8.3)	579/231	810	Inclisiran 300 mg at day 1, day 90, then every 6 months	107.2 (41.8)
2022 ORION-5	NCT03851705	Part1: RCT (DB, PC)	Hong Kong, Israel, Russian Federation, Serbia, South Africa, Taiwan, Turkey, Ukraine	Part 1: 6 Part 2: 18	Part 1: 53 Part 2: 47	FoFH		48	Unclear	Total: 14/23	Part 1: 34 Part 2: 29	Part 1: Inclisiran 300 mg at day 1, day 90	Unclear
		Part 2: OL										Part 2: Inclisiran 300 mg at day 270, day 450, and day 630	

Author/ Published year/Name	Control group					Outcomes			
	Age, mean (SD) (years)	Male/ Female	Sample size (N)	Treatment	Baseline LDL-C, mean (SD), mg/dL	Primary endpoints	Key secondary endpoints	Prespecified exploratory endpoints	Safety
Raal et al. (2020) ORION-9	55.0 (11.81)	115/125	240	0.9% NaCl on day 1, day 90, then every 6 months	154.7 (58.0)	Percentage change in LDL-C from baseline to day 510 and the time-adjusted percentage change in LDL-C from baseline between day 90 and day 540	Absolute change from baseline to day 510 and time-adjusted absolute change from baseline between day 90 and day 540 in LDL-C; Percentage change in PCSK9, TC, Apo B, Non-HDL-C from baseline to day 510	Proportion of patients who met the lipid targets for their level of cardiovascular risk and the treatment response according to the underlying genotype of FH	Frequent AEs, Serious AEs, Other cardiovascular AEs (Prespecified exploratory cardiovascular event, Fatal or nonfatal MI, Fatal or nonfatal stroke), Protocol-defined injection-site reaction, Laboratory results et al
Ray et al. (2020) ORION-10	65.7 (8.9)	548/232	780	0.9% NaCl on day 1, day 90, then every 6 months	104.8 (37.0)			MedDRA defined cardiovascular basket of non-adjudicated terms, including	

(Continued on following page)

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TABLE 1 (*Continued*) The characteristics of included studies[a].

Author/ Published year/Name	Control group					Outcomes			
	Age, mean (SD) (years)	Male/ Female	Sample size (N)	Treatment	Baseline LDL-C, mean (SD), mg/dL	Primary endpoints	Key secondary endpoints	Prespecified exploratory endpoints	Safety
Ray et al. (2020) ORION-11	64.8 (8.7)	581/226	807	0.9% NaCl on day 1, day 90, then every 6 months	103.7 (36.4)			those classified within cardiac death, and any signs or symptoms of cardiac arrest, nonfatal MI, or stroke	
2022 ORION-5	Unclear	Total: 8/11	Part 1: 19 Part 2: 18	Part 1: 0.9% NaCl on day 1, day 90; Part 2: Inclisiran 300 mg on day 270, day 450, and day 630	Unclear	Percentage change in LDL-C from baseline to day 150	Percentage change and absolute change in LDL-C, PCSK9, TC, Apo B, Non-HDL-C, HDL-C, VLDL-C, Apo-A1, Lp(a), hsCRP from baseline to day 90, 150, 180, 330, 450, 510, 630, 690, and 720; Individual responsiveness of subjects (Number of subjects reaching on treatment LDL-C of <25 mg/dL, <50 mg/dL, <70 mg/dL, and <100 mg/dL up to Day 180 and 720); Proportional responsiveness (Number of participants in each group who attain global lipid targets for their indication) et al	Unclear	All-Cause Mortality, Serious AEs, Other (Not include serious) AEs

[a]In Table 1, RCT, randomized controlled trial; DB, double-blind; PC, placebo-controlled; OL, open-label; N, number; FH, familial hypercholesterolemia; HeFH, heterozygous FH; ASCVD, atherosclerotic cardiovascular disease; FoFH, homozygous FH; LLT, lipid-lowering therapy; SD, standard deviation; LDL-C, low-density lipoprotein cholesterol; PCSK9, proprotein convertase subtilisin/kexin type 9; TC, total cholesterol; Apo B, Apolipoprotein B; HDL-C, high-density lipoprotein cholesterol; Non-HDL-C, Non-HDL cholesterol; VLDL-C, Very-Low-Density Lipoprotein Cholesterol; Apo-A1, Apolipoprotein A-1; Lp(a) Lipoprotein(a); hsCRP, High-Sensitivity C-Reactive Protein; AEs, adverse events; MI, myocardial infarction.

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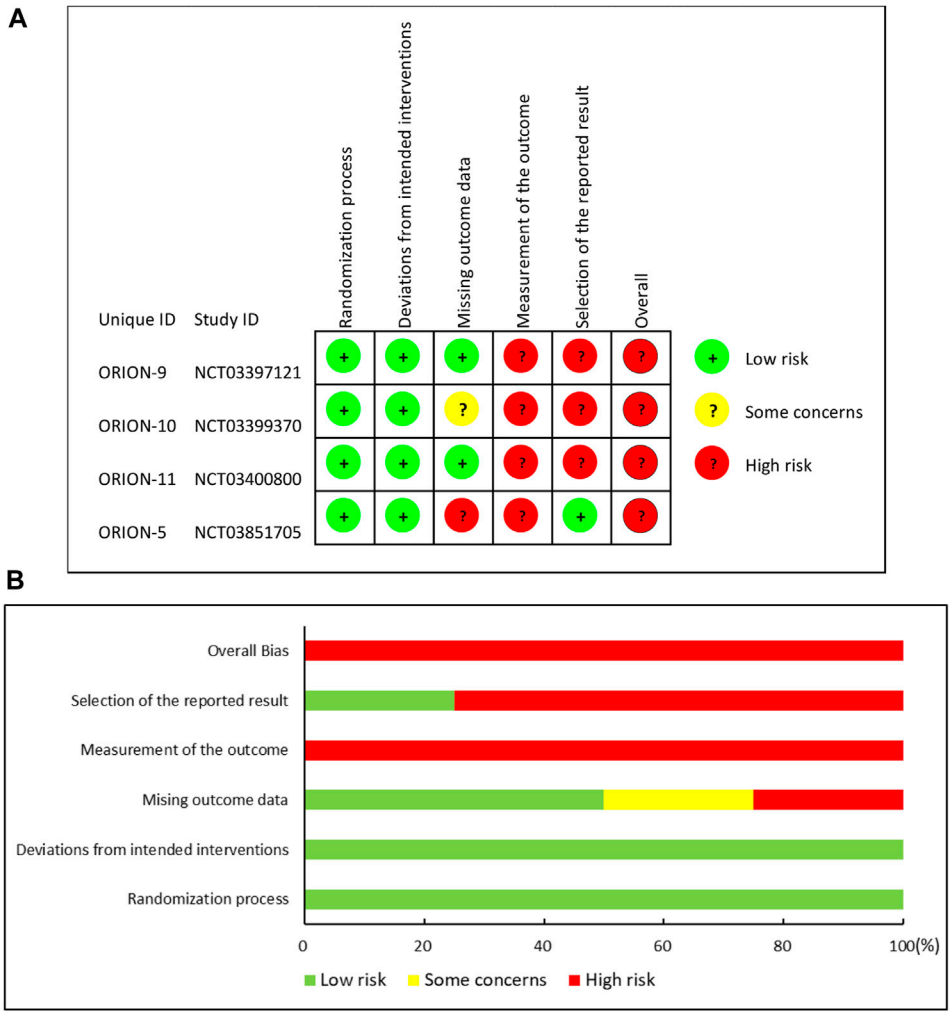

FIGURE 2
Risk of bias summary: **(A)** "traffic light" plots of the domains; **(B)** Weighted bar plots of the distribution of risk.

study on 5 January 2023, we searched the clinical registers again and found that a new RCT meeting the criteria, ORION-5, was included in our review. Additionally, we searched all databases again when we revised this study (6 March 2023), and no new RCTs were found that met the criteria.

3.2 Characteristics of included studies

The characteristics of the studies are reported in Table 1. Except for part 2 in ORION-5, the studies were double-blind, randomized, placebo-controlled international multicenter clinical trials conducted in many different countries or sites, and the outcomes were published between 2020 and 2022. In total, data from 3,713 patients were included. All included subjects were aged >18 years and were mainly middle-aged and elderly. The subjects were predominantly White (>80%). Patients in four studies had histories of disease involving diagnoses of heterozygous familial hypercholesterolemia (HeFH), ASCVD, ASCVD or an ASCVD risk equivalent, and homozygous familial hypercholesterolemia (HoFH), respectively, and their history of treatment involved the maximally tolerated statin with or without other lipid-lowering therapy. Interventions in ORION9 (Raal et al., 2020), ORION-10 (Ray et al., 2020), and ORION-11 (Ray et al., 2020) were inclisiran 300 mg on day 1 and day 90, then every 6 months, with an 18-month follow-up period. ORION-5 consisted of two parts. Part 1 was a 6-month double-blind period in which subjects were randomized to receive either inclisiran 300 mg or a placebo on day 1 and day 90. Part 2 was an 18-month open-label follow-up period, and all subjects from part 1, including the experimental group and control group received inclisiran 300 mg on day 180 and then every 6 months. Outcomes mainly included efficacy and safety outcomes. The efficacy outcomes mainly included the changes in LDL-C and other lipid levels and PCSK9 levels. ORION-9 (Raal et al., 2020), ORION-10 (Ray et al., 2020), and ORION-11 (Ray et al., 2020) also included exploratory cardiovascular outcomes. According to the different interventions, a meta-analysis was conducted in ORION-9, ORION-10, and ORION-11, and a descriptive analysis was conducted in ORION-5.

3.3 Risk of bias

According to RoB 2, the included RCTs all showed a high overall risk of bias. The assessment in each domain and the summary of the risk of bias are presented in Figure 2.

More specifically, three RCTs [ORION-9 (Raal et al., 2020), ORION-10 (Ray et al., 2020), and ORION-11 (Ray et al., 2020)] and part 1 of ORION-5 were all double-blind, randomized, placebo-controlled trials. Researchers clearly described their randomization method and allocation concealment: randomization was conducted via an automated interactive response technology to assign subjects to investigational products. Study medication was blinded before distribution to the site. Each investigational product vial contained a yellow shroud to blind it. Four studies (Raal et al., 2020; Ray et al., 2020) did not mark out the differences between the patients at baseline, and intent-to-treat (ITT) analysis was used to conduct the analysis. Although part 2 in ORION-5 was an open-label follow-up period, the subjects were the continuation in part 1, and the treatment of the intervention group and control group were the same, so we considered that even if part 2 did not use blinding, the bias in the outcomes would be negligible. Therefore, they were rated as having a low risk of bias in the randomization process and deviations from intended interventions. In the assessment of bias due to missing outcome data, two studies (Raal et al., 2020; Ray et al., 2020) were rated as low risk because the data missing rate was less than 5% and the number of dichotomous outcome events was significantly greater than the missing data. The remaining two studies (Ray et al., 2020) were rated as being of some concern or being at high risk of bias because they did not meet the above conditions and the reason for missing data in the intervention and control groups did not match. In addition, four studies (Raal et al., 2020; Ray et al., 2020) were rated as having a high risk of outcome measurement bias because they did not describe the blinding to outcome assessors, and as they were all international multicenter clinical trial studies, the subjective judgment of different outcome assessors might lead to bias. Finally, three studies (Raal et al., 2020; Ray et al., 2020) were rated as having a high risk of bias in the selection of the reported result because the supplementary appendix indicated that several analysis techniques were utilized to assess the efficacy of inclisiran. Treatments were compared utilizing two-sample t-tests, analysis of covariance models (ANCOVA), and mixed models for repeated measures. However, only ANCOVA results were reported. One study was rated as having a low risk due to the match of outcomes reported and the statistical methods they published.

Generally, the overall risk of bias in included studies was assessed as a high risk of bias.

3.4 Meta-analysis results

3.4.1 Primary outcomes

We extracted data on stroke and MACE from safety reports. Since the intervention method of ORION-5 was different from others, we only conducted a meta-analysis on the data of ORION-9 (Raal et al., 2020), ORION-10 (Ray et al., 2020), and ORION-11 (Ray et al., 2020), and a descriptive analysis was conducted for ORION-5, 2023. The safety population in three studies included a total of 3,655 patients [inclisiran (n = 1,833); placebo (n = 1,822)].

Events of stroke were reported in all three studies. Stroke occurred in 25 (1.3%) patients in the experimental group and 37 (1.5%) patients in the control group. Due to a low level of heterogeneity (I^2 = 35% < 50%, p = 0.22 > 0.10), we used the common-effect model to pool and analyze the data. The results showed that inclisiran did not reduce the risk of stroke (RR = 0.92, 95%CI = 0.54–1.58, p = 0.76) (Figure 3A). The pooled RR of ischaemic stroke and hemorrhagic stroke were 1.33 (95%CI = 0.72–2.34, p = 0.36) and 0.62 (95%CI = 0.23–1.63, p = 0.33), respectively. Sensitivity analysis showed that using the random-effect model did not reverse the pooled results, indicating that the results were stable (Supplementary Figure S1).

We conducted a meta-analysis of MACE and its subdivided components. MACE occurred in a total of 131 (7.10%) patients in the experimental group and 160 (8.8%) patients in the control group. As there was a low level of heterogeneity (I^2 = 2% < 50%, p = 0.36 > 0.10), the data were pooled using a common-effect model and showed that inclisiran intervention did not significantly reduce the risk of MACE (RR = 0.81, 95%CI = 0.65–1.02, p = 0.07) (Figure 3B). Sensitivity analysis showed stability due to the consistent result after changing to the random-effect model.

In the component events of MACE, 17 (0.93%) and 15 (0.82%) patients in the experimental group and control group had CVE death, 52 (2.80%) and 76 (4.20%) patients had MI, 25 (1.30%) and 37 (1.50%) patients had stroke, 6 (0.33%) and 1 (0.05%) patient had cardiac arrest, and 31 (1.70%) and 41 (2.30%) patients had heart failure, respectively. In these events, the heterogeneity of the studies was low, so the common-effect model was used for analysis. Overall, inclisiran was a protective factor for MI, reducing the risk of MI by 32% (RR = 0.68, 95%CI = 0.48–0.96, p = 0.03) (Figure 3C), but had no significant effect on other events. Sensitivity analysis showed the results were stable in all events. The pooled results and sensitivity analysis of other events are displayed in Supplementary Figure S1.

In general, our study indicates that inclisiran has no significant effect on stroke or MACE but can reduce the risk of MI by 32%.

3.4.2 Secondary outcomes

Inclisiran has significant lipid-lowering effects. In particular, it reduced the percentage change and absolute change of LDL-C by approximately 50% and 50 mg/dL. It also has a significant benefit in reducing PCSK9, TC, Apo B, and Non-HDL-C levels. The pooled effects of inclisiran on blood lipid and PCSK9 levels are shown in Table 2.

The safety populations were 1,833 in the inclisiran group and 1,822 in the placebo group. We performed a meta-analysis of TEAE, SAE, injection-site reaction, and some laboratory results in two groups. The pooled results showed that the inclisiran group had more significant injection-site reactions (n = 99) than the placebo group (n = 15) (RR = 6.56, 95%CI = 3.83–11.25), but they were mainly mild or moderate. There was no significant difference in other outcomes. The pooled results of safety outcomes are summarized in Table 3.

3.4.3 Descriptive analysis of ORION-5

ORION-5 was a two-part multicenter study to evaluate the safety, tolerability, and efficacy of inclisiran in subjects with

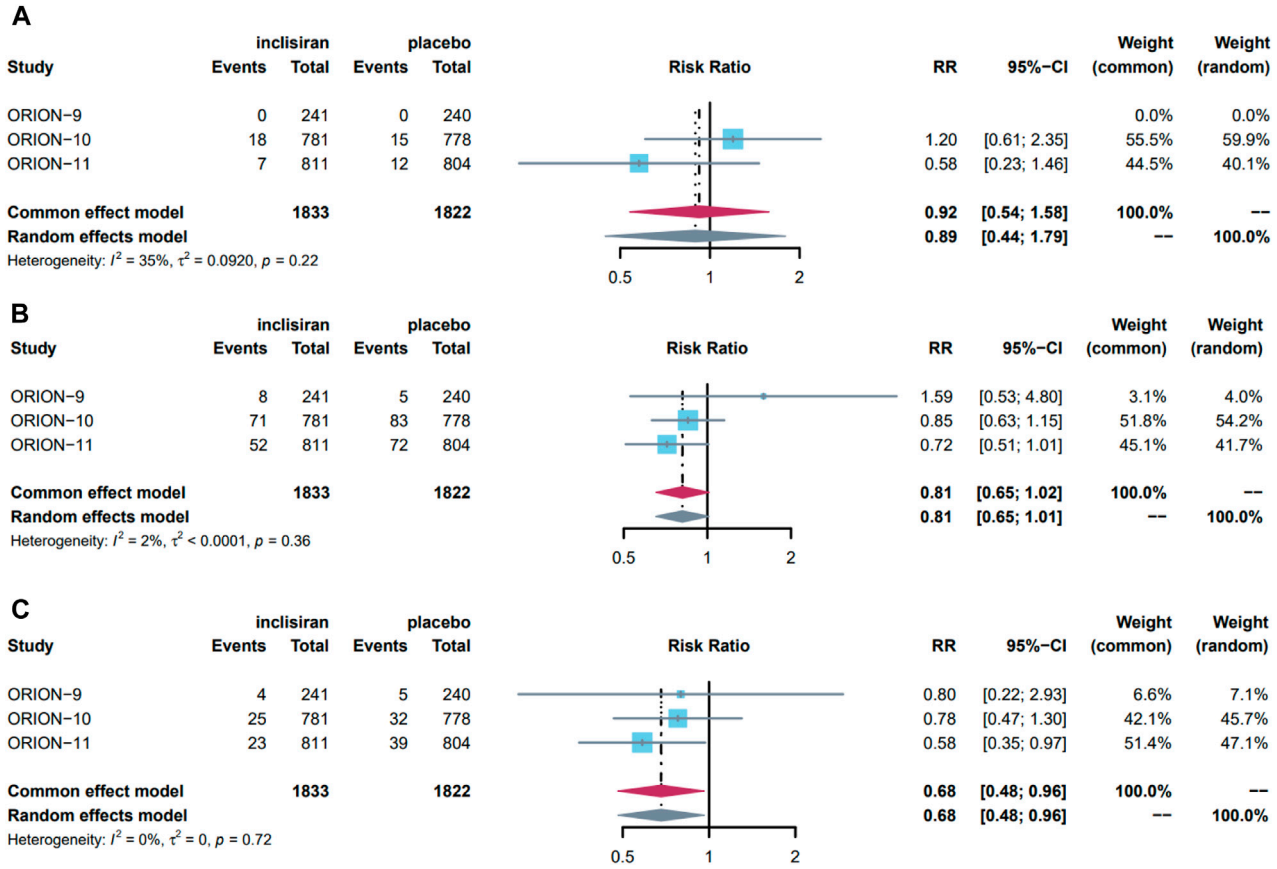

FIGURE 3
Forest plot of the effect of inclisiran in stroke, MACE, and MI, pooled using common-effects meta-analysis: **(A)** effect on stroke; **(B)** effect on MACE; **(C)** effect on MI. Overall, three studies were included in this meta-analysis. The maroon diamond represents the pooled difference using a random effects model for each subgroup and for the total. Heterogeneity of outcomes is represented by I^2 values (%) with p values reported for the χ^2 test for heterogeneity. The gray diamond represents the result of changing pooled model used for sensitive analysis. MACE major cardiovascular events, MI myocardial infarction, RR risk ratio, CI confidence interval.

HoFH. Subjects were randomized 2:1 to inclisiran: placebo. A total of 56 adults were enrolled, 34 being female (60.70%) and 48 being White (85.70%). There were 37 patients in the experimental group (34 completed part 1, 29 completed part 2) and 19 patients in the control group (all completed part 1, 18 completed part 2).

All subjects randomized into the study comprised the ITT population for outcomes analysis. The primary outcome was a percentage change in LDL-C from baseline to day 150; the result was 0.70 (95%CI = −14.03–15.44) in the inclisiran group and 2.39 (95%CI = −19.98–24.75) in the placebo group, with a mean difference of −1.68 (95%CI = −29.19–25.83, p = 0.98). The secondary outcomes included absolute and percentage changes of lipids (LDL-C, TC, HDL-C, etc.) and PCSK9 levels at follow-up time points. The results from Part 1 demonstrated that, other than significant differences observed in both the percentage change and absolute change of PCSK9 between the two groups, no significant differences in blood lipid. In part 2, only mean and standard deviation were reported, so we conducted an independent samples t-test and showed no significant differences ($p > 0.05$) in all lipid and PCSK9 levels.

In terms of safety conditions, for part 1, two SAEs (5.41%) and 12 other AEs (32.43%) occurred in the inclisiran group, and one SAE (5.26%) and six other AEs (31.58%) occurred in the placebo group. In part 2, 11 (20.75%) SAEs and 29 (54.72%) other AEs occurred, with one (1.89%) stroke and cerebrovascular accident event (carotid arteriosclerosis) and five (9.40%) MACE (angina unstable, carotid arteriosclerosis, cardiac failure, pulmonary edema, and sudden cardiac death). In addition, three subjects died in part 2.

4 Discussion

This systematic review and meta-analysis of 3,713 patients with ASCVD or at high risk of ASCVD showed that for those with a background of treatment with a maximum tolerated dose of statins or other lipid-regulation therapy, using inclisiran over 18 months can significantly reduce lipid and PCSK9 levels, with a 50% reduction in LDL-C and an 80% reduction in PCSK9. Nevertheless, the research findings revealed that while inclisiran was able to reduce the risk of MI by 32%, it did not demonstrate a

TABLE 2 Pooled effect of secondary outcomes[a].

Secondary outcomes	Heterogeneity (I^2, *p*-value)	Statistical model	WMD (95%CI)
Percentage Change in LDL-C From Baseline to Day 510 (%)	I^2 = 72%, p = 0.03	REM	−53.98 (−58.30, −49.65)
Time-adjusted Percentage Change in LDL-C From Baseline After Day 90 and up to Day 540 (%)	I^2 = 88%, p < 0.01	REM	−49.29 (−54.52, −44.07)
Absolute Change in LDL-C From Baseline to Day 510 (mg/dL)	I^2 = 86%, p < 0.01	REM	−57.63 (−67.41, −47.86)
Time-adjusted Absolute Change in LDL-C From Baseline After Day 90 and up to Day 540 (mg/dL)	I^2 = 89%, p < 0.01	REM	−54.52 (−62.09, −46.95)
Percentage Change in PCSK9 From Baseline to Day 510	I^2 = 0%, p = 0.41	CEM	−79.67 (−81.89, −77.45)
Percentage Change in TC From Baseline to Day 510	I^2 = 60%, p = 0.08	REM	−31.50 (−33.76, −29.23)
Percent Change in Apo B From Baseline to Day 510	I^2 = 81%, p < 0.01	REM	−39.57 (−43.46, −35.69)
Percentage Change in Non-HDL-C From Baseline to Day 510	I^2 = 61%, p = 0.08	REM	−44.63 (−47.71, −41.54)

[a]In Table 2, REM, random effect model; CEM, common effect model; WMD, weighted mean difference; CI, confidence interval; LDL-C, low-density lipoprotein cholesterol; PCSK9, proprotein convertase subtilisin/kexin type 9; TC, total cholesterol; Apo B, Apolipoprotein B; Non-HDL-C, non-high-density lipoprotein cholesterol.

significant correlation with the occurrence of stroke (be it compound stroke, ischaemic stroke, or hemorrhagic stroke) and MACE.

In addition, compared with the placebo, inclisiran did not increase the overall occurrence of AEs but caused higher injection-site reactions. Most of the injection-site reactions were mild and moderate, and no severe and sustained reactions occurred.

Another study (Ray et al., 2022) also conducted a meta-analysis of the same trials but came to a different conclusion. Inclisiran was found to reduce the risk of MACE by 26% (relative risk [OR] = 0.74, 95%CI = 0.88–0.94) but was not associated with the occurrence risk of stroke (OR = 0.80, 95%CI = 0.50–1.27) and MI (OR = 0.86, 95% CI = 0.41–1.81). Through comparison, we found that the main reason was that the definitions of CVEs were different. Their definitions of stroke, MACE, and MI events are attached to Supplementary Table S5.

MACE, a common endpoint in cardiovascular studies, is a composite of clinical events, usually including endpoints reflecting safety and efficacy, which can reduce or eliminate the multiplicity problem of testing multiple endpoints. Additionally, accumulating evidence from individual endpoints to a composite endpoint can improve study power and reduce study size and trial duration (Huque et al., 2011). Due to the individual outcomes used to make this endpoint vary between studies, there was no standard definition of MACE. Therefore, the difference in the MACE definition among the studies and the unclear and incomplete reports make it impossible to compare, replicate, and summarize the study results (Bosco et al., 2021). Studies have shown that different definitions of MACE might lead to opposite results and conclusions (Kip et al., 2008). In addition, there was some variation in stroke from the statistic results of the most commonly used components of MACE, possibly due to differences in the definition of stroke, especially whether acute ischemic stroke with TIA, cerebral hemorrhage, or subarachnoid hemorrhage was included (Bosco et al., 2021).

In our study, we used SMQs to define CVEs. MedDRA is a medical dictionary for regulatory activities developed by The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. SMQs are formed by defining clusters of MedDRA terms that are highly relevant to medical conditions (Bill et al., 2012). Studies have confirmed that in the identification process of adverse events, SMQs can achieve higher sensitivity compared to PT and high-level term (HLT) (Pearson et al., 2009), so we adopted SMQs for the definition of CVEs. In addition, unlike the K et al. study, we analyzed both the overall event and its components. For example, stroke contains both hemorrhagic and ischemic events. Although it is still controversial whether lipid-regulating drugs cause hemorrhagic events, if inclisiran can reduce the occurrence of ischemic events and increase the occurrence of hemorrhagic events by lowering lipids, then mixing two events with opposite outcomes might have slashed the significance of the results. With that in mind, it makes sense that such effects could be avoided by analyzing and reporting hemorrhagic and ischemic events separately.

Nevertheless, even though the definitions of CVEs differed, the study by K et al. (Ray et al., 2022) and our study both show that inclisiran does not appear to contribute to the prevention of stroke.

A meta-analysis of other lipid-regulating treatments (non-inclisiran) (Lee et al., 2022) suggests that compared with less intensive LDL-C-lowering statin-based therapies (final mean LDL-C level = 119 mg/dL), more intensive therapies (final mean LDL-C level = 79 mg/dL) might be more favorable for stroke prevention (RR = 8.1% vs. 9.3%), especially for patients with evidence of atherosclerosis. In addition, lowering the LDL-C level was found to increase the risk of hemorrhagic stroke (RR = 1.46, 95%

TABLE 3 Pooled effect of safety outcomes[a].

No. of patients	Inclisiran (*n* = 1833)	Placebo (*n* = 1822)	Risk ratio (95%CI)	*p*-Value
TEAE				
Patients with≥1 TEAE	1430	1409	1.01 (0.97–1.05)	0.62
Patients with≥1 TEAE leading to discontinuation of trial intervention	45	35	1.28 (0.83–1.98)	0.27
SAE				
Patients with≥1 SAE	383	401	0.95 (0.84–1.08)	0.41
All-Cause Mortality	27	27	0.99 (0.59–1.69)	0.98
Cancer-related death	4	6	0.66 (0.19–2.34)	0.52
Cardiovascular cause	17	15	1.13 (0.56–2.25)	0.74
New worsening or compound cancer	44	49	0.89 (0.60–1.30)	0.58
Protocol-defined injection-site reaction				
Any event*	99	15	6.56 (3.83–11.25)	<0.001
Mild*	73	14	5.18 (2.94–9.15)	<0.001
Moderate*	26	1	25.84 (3.51–190.24)	0.001
Severe	0	0	-	-
Persistent	0	0	-	-
Laboratory results				
Liver function				
Alanine aminotransferase >3× ULN	7	5	1.39 (0.44–4.37)	0.57
Aspartate aminotransferase >3× ULN	8	10	0.80 (0.32–2.01)	0.63
Alkaline phosphatase >3× ULN	8	5	1.59 (0.52–4.85)	0.42
Bilirubin >2× ULN	14	14	0.99 (0.48–2.08)	0.99
Kidney function				
Creatinine >2 mg/dL	36	42	0.85 (0.55–1.32)	0.48
Muscle				
Creatine kinase >5× ULN	24	22	1.08 (0.61–1.93)	0.78
Hematology				
Platelet count <75,000 per mm^3	1	2	0.50 (0.05–5.48)	0.57

*Has significant difference ($p < 0.05$).

[a]In Table 3, TEAE, treatment-emergent adverse event; SAE, serious adverse event; ULN, upper limits of normal; CI, confidence interval.

CI = 1.11–1.91). The mean follow-up duration of RCTs in this study was 4 years, while in our study, it was 1.5 years. Subgroup analysis of study duration based on the risk of compound stroke in the above study suggested there was no significant difference between the study durations of <3 years (RR = 0.92, 95%CI = 0.73–1.16) and ≥3 years (RR = 0.87, 95%CI = 0.79–0.96). Another study (Koskinas et al., 2018) found that compared to its significant LDL-C reduction, the reduction in the risk of CVEs with PCSK9 inhibitor treatment was within the expectations but increased after using Kaplan-Meier curves to extend the follow-up duration to be consistent with other RCTs. Since stroke is a chronic condition and prolonged follow-up duration may result in more cases and affect the outcome, we continue to believe that the length of follow-up influences outcomes. However, in the absence of long-term outcome data of inclisiran, this is merely a hypothesis. ORION-4, 2023 and VICTORION-2P, 2023 PREVENT were two large ongoing randomized, double-blind, placebo-controlled studies that investigated the impact of inclisiran on patients with ASCVD. They each expected to enroll 15,000 subjects with a follow-up duration of ≥5 years. Ischemic stroke was one of the primary outcomes. A larger number of subjects, longer follow-up duration, and more specific cardiovascular efficacy outcomes may clarify the association of inclisiran with stroke in ASCVD patients.

For hemorrhagic stroke, although the latest American College of Cardiology/American Heart Association guideline on the management of blood cholesterol (Grundy et al., 2019) states that it is not a statin-related AE, given conflicting literature data, the risk of hemorrhagic stroke might vary due to different lipid-regulating therapies (such as statins and PCSK9 inhibitors) or ethnicities (the association between lower LDL-C and a higher incidence of hemorrhagic stroke appears to be stronger in Asian people), it is unclear whether lower LDL-C is associated with a higher incidence of hemorrhagic stroke (Karagiannis et al., 2021).

ORION-5 indicated that compared with patients who were given the first two doses at a 3-month interval and then all further doses at 6-month intervals, there was no significant difference in the reduction of blood lipid and PCSK9 levels in those who were initially given inclisiran every

6 months. This suggests that inclisiran might be given semiannually from the beginning, rather than at 3-month intervals for the first and second doses. Further research is expected to verify this hypothesis.

We acknowledge that our study has some limitations. Firstly, the number and quality of the included studies were limited. All the included studies were assessed as having a high risk of bias, mainly because of detection bias and incomplete data reports. In addition, publication bias existed because of the small number of included trials, all of which were funded by medicine companies. Secondly, subgroup analysis was not conducted because the characteristics of included studies were similar and the data were insufficient in the number of CVEs in different populations. The countries and regions distribution (Table 1) of included clinical trials were different, and the impact was not explored in our study due to the limited data. Thirdly, the change in the definition of disease, such as stroke or MI, may impact the data, and the detection of stroke or MI may become more sensitive with the progression of time due to increased incidence. We cannot exclude the effects of definition and duration. Fourthly, we only updated the included studies at the end of the study; therefore, some studies published after this analysis might not be analyzed. Finally, as inclisiran is a new drug, there are few clinical studies and post-marketing studies with published results, so it is an objective fact that there is publication bias in this study. However, inclisiran has been approved by the European Union and FDA for the treatment of adults with HeFH or clinical ASCVD who require additional lowering of LDL-C and has already been used in the clinical setting. Clinical trials in other populations are also being steadily registered and are underway. As the results of clinical trials, post-marketing monitoring, and real-world studies are published, the efficacy and safety of inclisiran for specific populations will become clearer. We will continue to follow up and actively update the outcomes of the systematic review. We look forward to more updated and high-quality studies with larger samples in the future to validate the results and reach more convincing conclusions.

5 Conclusion

Lipid regulation is important for the prevention of stroke and cerebrovascular events in patients with ASCVD or at high risk of ASCVD. As a novel lipid-regulating drug, inclisiran has a significant effect in lowering blood lipids and PCSK9 levels. Our systematic review and meta-analysis showed that in patients with a history of treatment with a maximum tolerated dose of statins or other lipid-regulation therapy, using inclisiran is not beneficial for the prevention of stroke or cerebrovascular disease and MACE but is associated with a reduced risk of MI. However, due to the insufficient quantity and quality of literature and the non-standard definition of CVEs, further studies are expected to provide more details.

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 authors.

Author contributions

Conceptualization: ML and YL; Methodology: ML and YL; Software: XX and YL; Validation: ML; Formal analysis, investigation, and data curation: ML, YL, and XX; Writing—original draft: ML and YL; Writing—review and editing: ML, YL, XX, KL, CS, HH, ZH, and FW; Visualization: ML, CS, and HH; Supervision, project administration, and funding acquisition: ZH and FW. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1158274/full#supplementary-material

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Exploring the mechanisms under Zuogui Pill's treatment of ischemic stroke through network pharmacology and *in vitro* experimental verification

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Due to its high mortality, incidence and disability rates, ischemic stroke poses heavy economic burdens to families and society. Zuogui Pill (ZGP) is a classic Chinese medicine for tonifying the kidney, which is effective for the recovery of neurological function after ischemic stroke. However, Zuogui Pill has not been evaluated for its potential effects on ischemic strokes. Using network pharmacology, the research aimed to explore the mechanisms of Zuogui Pill on ischemic stroke, which were further validated in SH-SY5Y cells injured by oxygen and glucose deprivation/reperfusion (OGD/R). Network analysis of Zuogui Pill identified 86 active ingredients and 107 compound-related targets correlated with ischemic stroke. Additionally, 11 core active compounds were obtained, such as Quercetin, beta sitosterol, and stigmasterol. Most of the compounds have been proven to have pharmacological activities. Based on pathway enrichment studies, Zuogui Pill may exert neuroprotection through MAPK signaling, PI3K-Akt signaling and apoptosis, as well as enhance neurite outgrowth and axonal regeneration effect via mTOR signaling, p53 signaling and Wnt signaling pathways. *In vitro* experiment, the viability of ischemic neuron treated with Zuogui Pill was increased, and the ability of neurite outgrowth was significantly improved. Western blot assays shown that the pro-neurite outgrowth effect of Zuogui Pill on ischemic stroke may be relate to PTEN/mTOR signal pathway. The results of the study provided new insights into Zuogui Pill's molecular mechanism in treatment of ischemic stroke, as well as clinical references for its use.

KEYWORDS

zuogui pill, ischemic stroke, network pharmacology, neurite outgrowth, SH-SY5Y cells

Introduction

Chinese adults suffer from ischemic stroke most often, which leads to death and disability (Sun et al., 2021). It is estimated that approximately 13.7 million people suffer from stroke each year globally, and about 87% are related to ischemia (Saini et al., 2021). In acute ischemic stroke patients within the time window, intravenous thrombolysis and

intravascular therapy are the most effective treatment measures (Suzuki et al., 2021). However, fewer than 5% of the patients were able to undergo effective thrombolysis and thrombectomy limited by the relatively short treatment time window (Saini et al., 2021). The majority of survivors suffer from serious neurological deficit symptoms, which severely impair their quality of life and burden society as a whole. In addition to vascular recanalization and neuroprotective strategies in the acute phase (Paul and Candelario-Jalil, 2021), neuronal regeneration and axonal repair run through the whole process of ischemic stroke, and play an even more important role during the recovery process. The prognosis of ischemic stroke can be significantly improved by promoting axonal sprouting, forming new collateral and synapse of damaged neurons, and reconstructing neural networks (Regenhardt et al., 2020; Takase and Regenhardt, 2021). However, no effective drug existed to promote nerve regeneration. The development of novel, safe and efficient medications for the regeneration and remodeling of ischemic stroke is highly required.

Traditional Chinese medicine categorizes ischemic stroke as "stroke" (Yu et al., 2016). The main pathogenesis is deficiency of yin and essence of the liver and kidney. On this basis, wind, fire, phlegm, Qi and blood and other pathological products cause cerebrovascular obstruction and brain marrow damage (Yu et al., 2016). Tonifying the kidney to generate the marrow, and filling the brain with marrow can promote the repair of the brain marrow and the recovery of neurological function (Hu et al., 2012; Luo et al., 2020). Zuogui pill (ZGP) is a traditional medicine prescription for tonifying kidneys and yins. Zhang Jingyue, a physician during the Ming Dynasty, created it to nourish the kidney, fill the essence, nourish the kidney and generate marrow (Chen et al., 2010). In the previous clinical and experimental studies, ZGP has shown definited effects on ischemic stroke (Li et al., 2019; Liu et al., 2022). The research about the mechanism of ZGP showed that it has the effects of protecting brain cells (Liu et al., 2022), promoting nerve regeneration and remodeling after ischemic stroke (Wang et al., 2011). However, there is still not a clear understanding of the material basis of ZGP and the potential molecular mechanisms that underlie its ability to promote neural regeneration and functional recovery.

The technology of network pharmacology focuses on the relationship between drug molecules, action mechanisms, and disease targets based on the construction of biological networks (Wang et al., 2021). Integrity and systematicity are key characteristics of this technology, which are in sync with traditional Chinese medicine's holistic approach and personalized treatment based on symptoms (Jiao et al., 2021). Therefore, it is widely used to predict the correlation between the pharmacodynamic components and the action mechanism of complex Traditional Chinese medicine (Wang et al., 2021; Yang et al., 2022). The study explored ZGP's mechanism in multi-target, multi-channel treatment of ischemic stroke using the network pharmacology method. Additionally, part of the mechanism was confirmed by *in vitro* experiments, which provided a biological basis for treating ischemic stroke with "Bushen Shengsui" Chinese medicine.

Materials and methods

Database construction of ZGP

Compounds in ZGP (Rehmannia glutinosa, Yam, Cornus officinalis, Achyranthes bidentata, Medlar, Dodder, Tortoise shell glue, Antler glue) were search in TCMSP (http://lsp.nwu.edu.cn/tcmsp.php) and BATMAN-TCM (http://bionet.ncpsb.org/batman-tcm/). The rate and degree of drug absorption in the body is referred to as oral bioavailability (OB). A compound's drug-likeness (DL) is defined as its similarity to a known drug. For the purpose of screening potential compounds, the active components with OB ≥ 30% and DL ≥ 0.18 were chosen. Figure 1 depicts the procedure for elucidating the ZGP mechanism.

Prediction of compound targets for ZGP

Search compound targets through TCMSP database, screen and eliminate the duplicate targets. The gene names and UniProt numbers of each target were converted into target abbreviations using the UniProt database (http://www.uniprot.org/uniprot/). Get rid of targets that are repetitive, not human or standard. Cytoscape 3.8 software was used to import the ZGP's primary active components and their targets to create a "Component-Target" network diagram. The targets and chemical components that were represented by the nodes in the network diagram. Interactions were represented by the edges. The degrees reflected the number of edges that combined a node, and function and degree values were positively associated. The information regarding degree centrality (DC), medium centrality (BC), and near centrality (CC) was obtained by evaluating the topological properties with the "CytoNCA" function.

Targets of ZGP against ischemic stroke prediction

By utilizing the phrase "ischemic stroke," disease targets were found in the TTD database (http://db.idrblab.net/ttd/), DisGeNET database (https://www.disgenet.org), GeneCards database (https://www.genecards.org), and OMIM database (http://omim.org/). With UniProt, duplicate targets were eliminated. The targets of the active ZGP components were compared to the targets of ischemic stroke by using the Venny platform (http://bioinfogp.cnb.csic.es/Tools/Venny/), and the targets of ZGP for treating ischemic stroke were determined.

Construction of drug-component-target-disease network

Each drug and drug active ingredient of ZGP was defined as "drug-component network" in Excel file format. The drug active ingredient and active ingredient target were defined as "component-target network" in Excel file format, and the disease and disease potential target were defined as "disease-target network" in Excel file

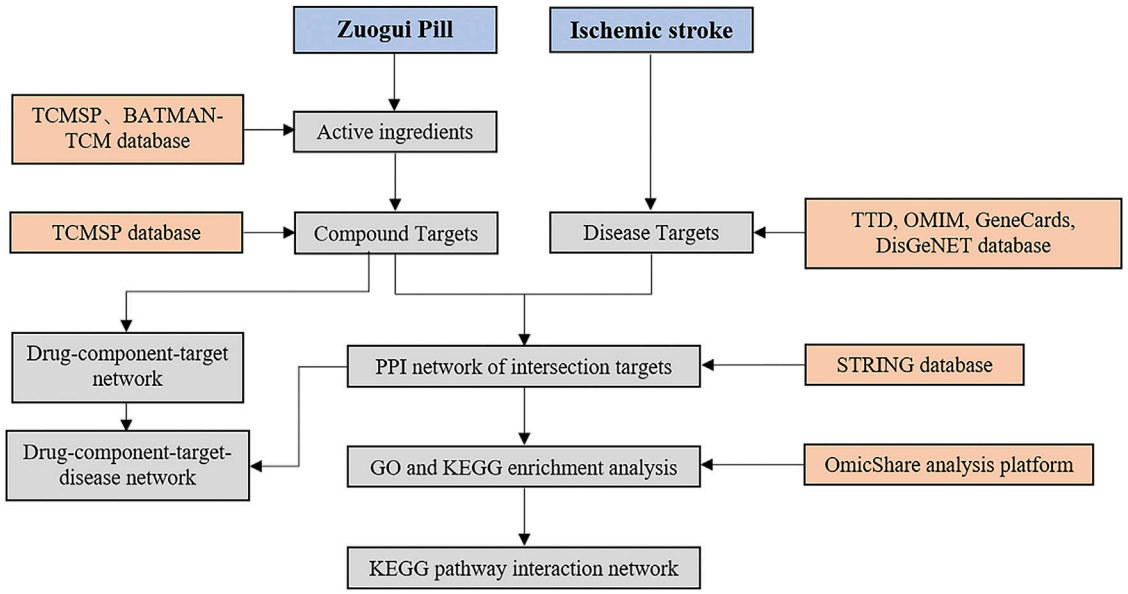

FIGURE 1
The work procedure to explain the mechanism of ZGP.

format. The above three files were imported into Cytoscape 3.8 software, and "drug-component-target-disease network" of ZGP was obtained by using merge function of the software. The "CytoNCA" function was used to analyze the topological properties.

Analysis of the protein-protein interaction (PPI) network

For the purpose of PPI analysis, the common targets of ZGP and ischemic stroke were imported into the STRING database (version 11.0, https://string-db.org/). For the PPI network construction, the interacting proteins with a high confidence score of ≥0.700 were chosen. Cytoscape 3.8 was used to import the aforementioned data, create a target PPI network diagram, and screen the core network. In order to identify potential hub targets, the "CytoNCA" function was utilized to examine the topological properties.

GO and KEGG enrichment analysis

For further analysis, the intersection targets were uploaded to the OmicShare analysis platform. OmicShare was used to analyze gene ontology (GO) data, the Kyoto Encyclopedia of Genes and Genomics (KEGG) pathway. The biological process (BP), the cellular component (CC), and the molecular function (MF) are all included in the GO enrichment analysis. By setting the *p*-value, the GO enrichment results were obtained, and the top 20 categories were chosen for visualization. The KEGG pathway interaction network was established for the enrichment results of KEGG pathways, and its topological properties were analyzed by using the function of "CytoNCA" to uncover the potential key pathways.

Molecular docking analysis

Molecular docking aims to simulate the docking between small molecule ligands and large molecule proteins, and the docking results are evaluated by binding energy (affinity). Lower binding energy indicates better molecular docking binding effect. The 3D structure of the compounds were obtained from TCMSP database (http://tcmspw.com/tcmsp.php), and the structure of target proteins were obtained from RCSB PDB (https://www.rcsb.org/). AutoDock Tools 1.5.6 was applied to perform molecular docking and predict the binding energy of compounds and proteins. Finally, PyMOL software was used to visualize the optimal docking results.

Chemicals and reagents for pharmacological verification

One prescription of ZGP comprises Rehmannia glutinosa 24 g, Fried yam 12 g, Lycium barbarum 12 g, Cornus officinalis 12 g, Sichuan Achyranthes 9 g, Dodder seed 12 g, Antler gum 12 g, and Tortoiseshell gum 12 g. The ratio of these herbs was 8:4:4:4:3:4:4:4. All of these ingredients were acquired from China and obtained from the pharmacy of Jiangsu Province Hospital of Chinese Medicine. The Chinese Pharmacopoeia 2015 was employed to verify the ZGP. A voucher specimen (2021–0527) was placed at the Central Laboratory of Jiangsu Province Hospital of Chinese Medicine. The detailed approaches for preparing and quality assurance of ZGP were as follows: A total of 10 prescriptions of ZGP (1050 g) were prepared, mixtures of components in ZGP were soaked with 1000 mL water for 30 min, then decocted twice for 30 min. The liquid was decocted twice, collected, concentrated to a density of about 1.3, and dried under vacuum at 60°C. Finally, the extract was crushed to obtain ZGP-dried powder (362 g). The powder was preserved in aliquots at −20°C and

TABLE 1 The active components of ZGP.

Mol ID	Molecule name	OB (%)	DL	Source
MOL000098	quercetin	46.4	0.275	CHUANNIUXI, GOUQIZI, TUSIZI
MOL000184	NSC63551	39.3	0.759	TUSIZI
MOL000310	Denudatin B	61.5	0.378	SHANYAO
MOL000322	Kadsurenone	54.7	0.378	SHANYAO
MOL000354	isorhamnetin	49.6	0.306	TUSIZI
MOL000358	beta-sitosterol	36.9	0.751	SHANZHUYU, CHUANNIUXI, GOUQIZI, TUSIZI
MOL000359	sitosterol	36.9	0.751	SHUDIHUANG, SHANZHUYU
MOL000422	kaempferol	41.9	0.241	TUSIZI
MOL000449	Stigmasterol	43.8	0.757	SHUDIHUANG, SHANZHUYU, SHANYAO, GOUQIZI
MOL000546	diosgenin	80.9	0.810	SHANYAO
MOL000554	gallic acid-3-O-(6′-O-galloyl)-glucoside	30.3	0.675	SHANZHUYU
MOL000953	CLR	37.9	0.677	SHANYAO, GOUQIZI, TUSIZI
MOL001323	Sitosterol alpha1	43.3	0.784	GOUQIZI
MOL001494	Mandenol	42.0	0.193	SHANZHUYU, GOUQIZI
MOL001495	Ethyl linolenate	46.1	0.197	SHANZHUYU, GOUQIZI
MOL001558	sesamin	56.5	0.827	TUSIZI
MOL001559	piperlonguminine	30.7	0.180	SHANYAO
MOL001736	(−)-taxifolin	60.5	0.273	SHANYAO
MOL001771	poriferast-5-en-3beta-ol	36.9	0.750	SHANZHUYU
MOL001979	LAN	42.1	0.748	GOUQIZI
MOL002320	Gamma-Sitosterol	36.9	0.750	GOUQIZI
MOL002773	Beta-Carotene	37.2	0.580	GOUQIZI
MOL002879	Diop	43.6	0.392	SHANZHUYU
MOL002883	Ethyl oleate (NF)	32.4	0.191	SHANZHUYU
MOL003137	Leucanthoside	32.1	0.781	SHANZHUYU
MOL003578	Cycloartenol	38.7	0.781	GOUQIZI
MOL005043	campest-5-en-3beta-ol	37.6	0.715	TUSIZI
MOL005360	malkangunin	57.7	0.626	SHANZHUYU
MOL005406	atropine	46.0	0.193	GOUQIZI
MOL005429	hancinol	64.0	0.373	SHANYAO
MOL005430	hancinone C	59.0	0.390	SHANYAO
MOL005435	24-Methylcholest-5-enyl-3belta-O-glucopyranoside_qt	37.6	0.717	SHANYAO
MOL005438	campesterol	37.6	0.715	SHANYAO, GOUQIZI, TUSIZI
MOL005440	Isofucosterol	43.8	0.758	SHANYAO, TUSIZI
MOL005458	Dioscoreside C_qt	36.4	0.871	SHANYAO
MOL005461	Doradexanthin	38.2	0.537	SHANYAO
MOL005463	Methylcimicifugoside_qt	31.7	0.237	SHANYAO

(Continued on following page)

TABLE 1 (*Continued*) The active components of ZGP.

Mol ID	Molecule name	OB (%)	DL	Source
MOL005465	AIDS180907	45.3	0.773	SHANYAO
MOL005481	2,6,10,14,18-pentamethylicosa-2,6,10,14,18-pentaene	33.4	0.240	SHANZHUYU
MOL005486	3,4-Dehydrolycopen-16-aL	46.6	0.491	SHANZHUYU
MOL005489	3,6-Digalloylglucose	31.4	0.663	SHANZHUYU
MOL005503	Cornudentanone	39.7	0.327	SHANZHUYU
MOL005530	Hydroxygenkwanin	36.5	0.272	SHANZHUYU
MOL005531	Telocinobufagin	70.0	0.793	SHANZHUYU
MOL005552	gemin D	68.8	0.561	SHANZHUYU
MOL005557	lanosta-8,24-dien-3-ol,3-acetate	44.3	0.824	SHANZHUYU
MOL005944	matrine	63.8	0.249	TUSIZI
MOL006209	cyanin	47.4	0.759	GOUQIZI
MOL006649	sophranol	55.4	0.282	TUSIZI
MOL007449	24-methylidenelophenol	44.2	0.753	GOUQIZI
MOL008173	daucosterol_qt	36.9	0.753	GOUQIZI
MOL008400	glycitein	50.5	0.238	GOUQIZI
MOL008457	Tetrahydroalstonine	32.4	0.813	SHANZHUYU
MOL009604	14b-pregnane	34.8	0.337	GOUQIZI
MOL009612	(24R)-4alpha-Methyl-24-ethylcholesta-7,25-dien-3beta-ylacetate	46.4	0.840	GOUQIZI
MOL009615	24-Methylenecycloartan-3beta,21-diol	37.3	0.798	GOUQIZI
MOL009617	24-ethylcholest-22-enol	37.1	0.751	GOUQIZI
MOL009618	24-ethylcholesta-5,22-dienol	43.8	0.756	GOUQIZI
MOL009620	24-methyl-31-norlanost-9 (11)-enol	38.0	0.751	GOUQIZI
MOL009621	24-methylenelanost-8-enol	42.4	0.768	GOUQIZI
MOL009622	Fucosterol	43.8	0.757	GOUQIZI
MOL009631	31-Norcyclolaudenol	38.7	0.814	GOUQIZI
MOL009633	31-norlanost-9 (11)-enol	38.4	0.725	GOUQIZI
MOL009634	31-norlanosterol	42.2	0.730	GOUQIZI
MOL009635	4,24-methyllophenol	37.8	0.750	GOUQIZI
MOL009639	Lophenol	38.1	0.714	GOUQIZI
MOL009640	4alpha,14alpha,24-trimethylcholesta-8,24-dienol	38.9	0.758	GOUQIZI
MOL009641	4alpha,24-dimethylcholesta-7,24-dienol	42.7	0.753	GOUQIZI
MOL009642	4alpha-methyl-24-ethylcholesta-7,24-dienol	42.3	0.783	GOUQIZI
MOL009644	6-Fluoroindole-7-Dehydrocholesterol	43.7	0.722	GOUQIZI
MOL009646	7-O-Methylluteolin-6-C-beta-glucoside_qt	40.8	0.305	GOUQIZI
MOL009650	Atropine	42.2	0.193	GOUQIZI
MOL009651	Cryptoxanthin monoepoxide	47.0	0.561	GOUQIZI
MOL009653	Cycloeucalenol	39.7	0.794	GOUQIZI
MOL009656	(E,E)-1-ethyl octadeca-3,13-dienoate	42.0	0.194	GOUQIZI

(Continued on following page)

TABLE 1 (*Continued*) **The active components of ZGP.**

Mol ID	Molecule name	OB (%)	DL	Source
MOL009660	methyl (1R,4aS,7R,7aS)-4a,7-dihydroxy-7-methyl-1-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-1,5,6,7a-tetrahydrocyclopenta [d]pyran-4-carboxylate	39.4	0.466	GOUQIZI
MOL009662	Lantadene A	38.7	0.574	GOUQIZI
MOL009664	Physalin A	91.7	0.272	GOUQIZI
MOL009665	Physcion-8-O-beta-D-gentiobioside	43.9	0.624	GOUQIZI
MOL009677	lanost-8-en-3beta-ol	34.2	0.740	GOUQIZI
MOL009678	lanost-8-enol	34.2	0.742	GOUQIZI
MOL009681	Obtusifoliol	42.6	0.757	GOUQIZI
MOL010234	delta-Carotene	31.8	0.546	GOUQIZI
MOL012286	Betavulgarin	68.7	0.394	CHUANNIUXI
MOL012298	Rubrosterone	32.7	0.466	CHUANNIUXI
MOL012888	Citrostadienol	43.3	0.790	GOUQIZI

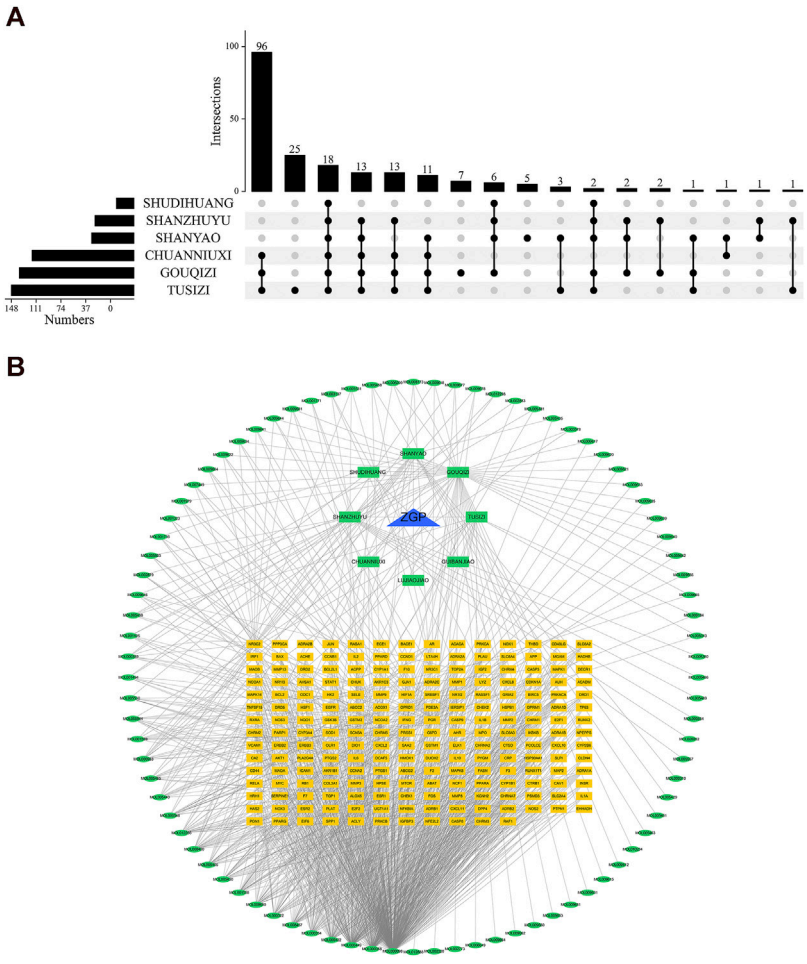

FIGURE 2
The compound-target network and analysis of ZGP. **(A)** Upset plot; **(B)** The potential targets are depicted by the yellow rectangles, the herbs by the green rectangles, the compounds by the green ellipses, and ZGP by the blue triangle.

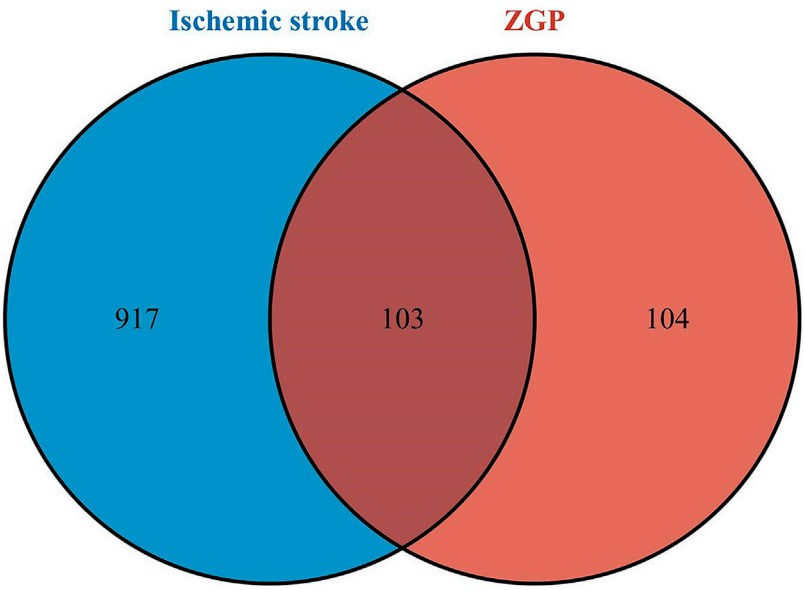

FIGURE 3
Venn diagram. The intersection of the potential ZGP targets and ischemic stroke targets.

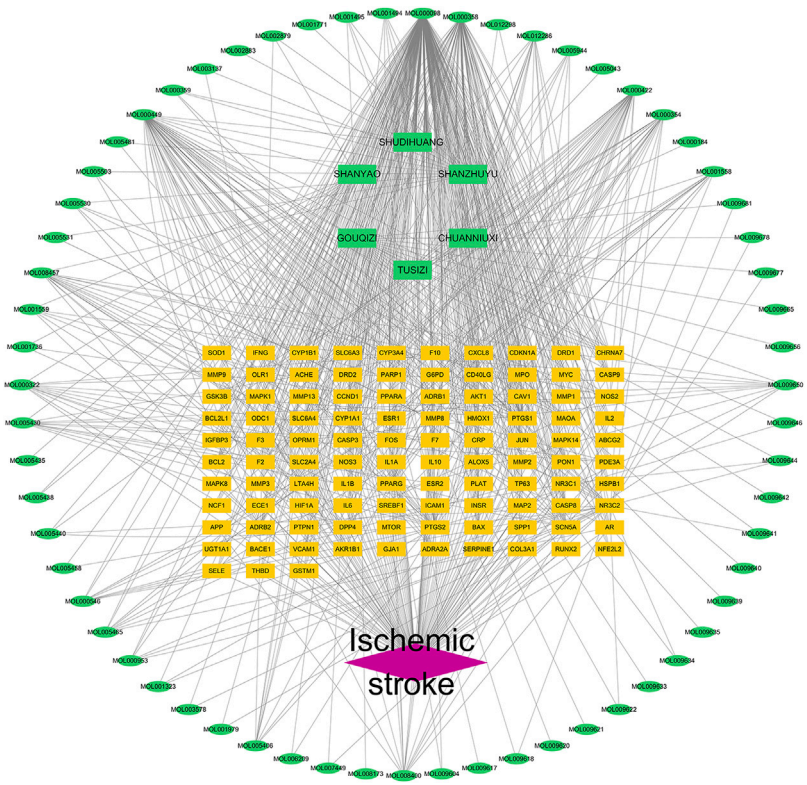

FIGURE 4
Drug component target disease network of ZGP. Network of the targets shared by ZGP and ischemic stroke. The ischemic stroke is represented by the red diamond, the potential targets are represented by the yellow rectangles, the herbs are represented by the green rectangles, and the compounds are represented by the green ellipses.

dissolved in a cell culture medium to create working solutions for bioassays. SH-SY5Y human neuroblastoma cells were supplied by the Chinese Academy of Sciences Stem Cell Bank. All-trans-retinoic acid (RA) was purchased from SIGMA (Unite State). Brain-derived neurotrophic factor (MCE, Unite State). The anti- GAP43, p-S6, mTOR, PTEN, and GAPDH were purchased from CST (Unite State).

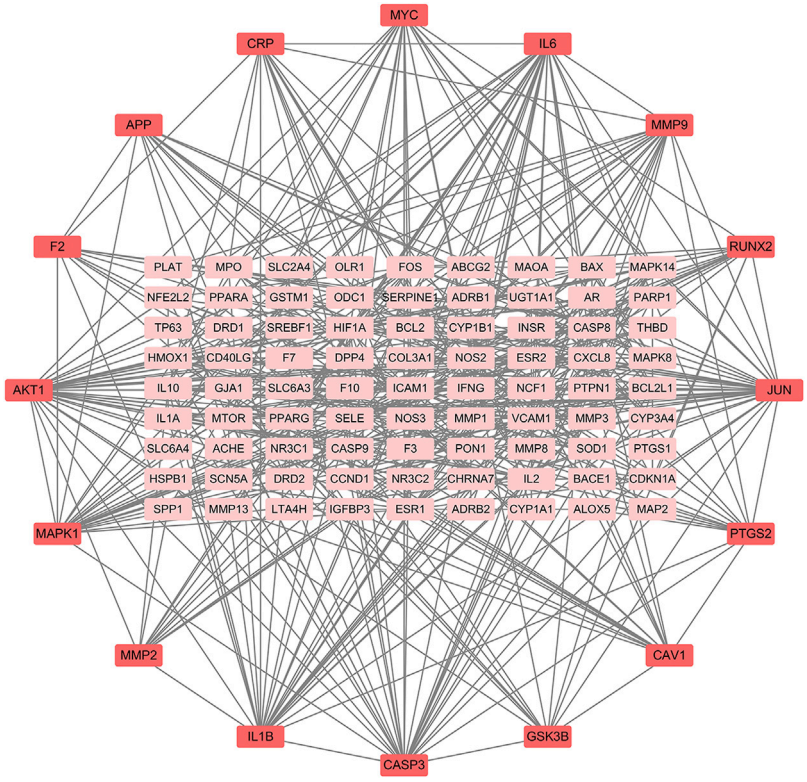

FIGURE 5
ZGP PPI networks in ischemic stroke treatment. Proteins are signified by network nodes; The targets' abbreviations are written beside the nodes, and the straight lines represent their connections. The correlation is greater the darker the color.

TABLE 2 Core targets in the PPI network.

Name	Target	DC	BC	CC
AKT1	RAC-alpha serine/threonine-protein kinase	41	1113.8	0.4948
JUN	Transcription factor AP-1	36	987.1	0.5134
IL6	Interleukin-6	32	635.5	0.4706
CASP3	Caspase-3	28	463.5	0.4660
IL1B	Interleukin-1 beta	28	501.8	0.4528
MAPK1	Mitogen-activated protein kinase 1	25	833.1	0.4848
MYC	Myc proto-oncogene protein	21	374.0	0.4305
MMP9	Matrix metalloproteinase-9	21	498.7	0.4593
PTGS2	Prostaglandin G/H synthase 2	17	562.5	0.4305
CAV1	Caveolin-1	17	446.6	0.3967
RUNX2	Runt-related transcription factor 2	16	245.2	0.4192
CRP	C-reactive protein	15	276.0	0.3887
GSK3B	Glycogen synthase kinase-3 beta	13	213.1	0.4085
MMP2	72 kDa type IV collagenase	13	223.8	0.4211
APP	Amyloid beta A4 protein	13	922.9	0.4156
F2	Prothrombin	12	830.8	0.4229

Cell culture and treatment

The Chinese Academy of Sciences Stem Cell Bank supplied the SH-SY5Y human neuroblastoma cells. In DMEM-F12, SH-SY5Y cells were treated with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin and cultivated at 37°C in a 5% CO2 environment. SH-SY5Y cells were cultivated in 6 cm culture plates at a density of 1.5×10^6 and separated into control and all-trans-retinoic acid (RA) induction cohort. The control cohort was cultured normally, while the RA induction cohort was treated with 5 μmol/L RA to induce cells differentiation into mature neurons (Jahn et al., 2017). The culture medium was changed once a day for three consecutive days. Then, oxygen-glucose deprivation/reoxygenation (OGD/R) procedure was used to treat the differentiated SH-SY5Y cells to mimic cerebral ischemia reperfusion injury *in vitro* (Zhi et al., 2020; Sun et al., 2022). The differentiated SH-SY5Y cells induced by RA were maintained in glucose-free EBSS (Early's Balanced Salt Solution) medium and kept in a three-gas incubator (O2 1%, CO2 5%, N2 94%) for 4 h. Subsequently, the cells were relocated to a normal culture medium and preserved in a normal incubator for the following experiments. ZGP was dissolved in normal culture medium and prepared into working solution with a series of concentrations (0.14–5.12 mg/mL). The cells were divided into the following cohorts for different experimental purposes: normal control (NC),

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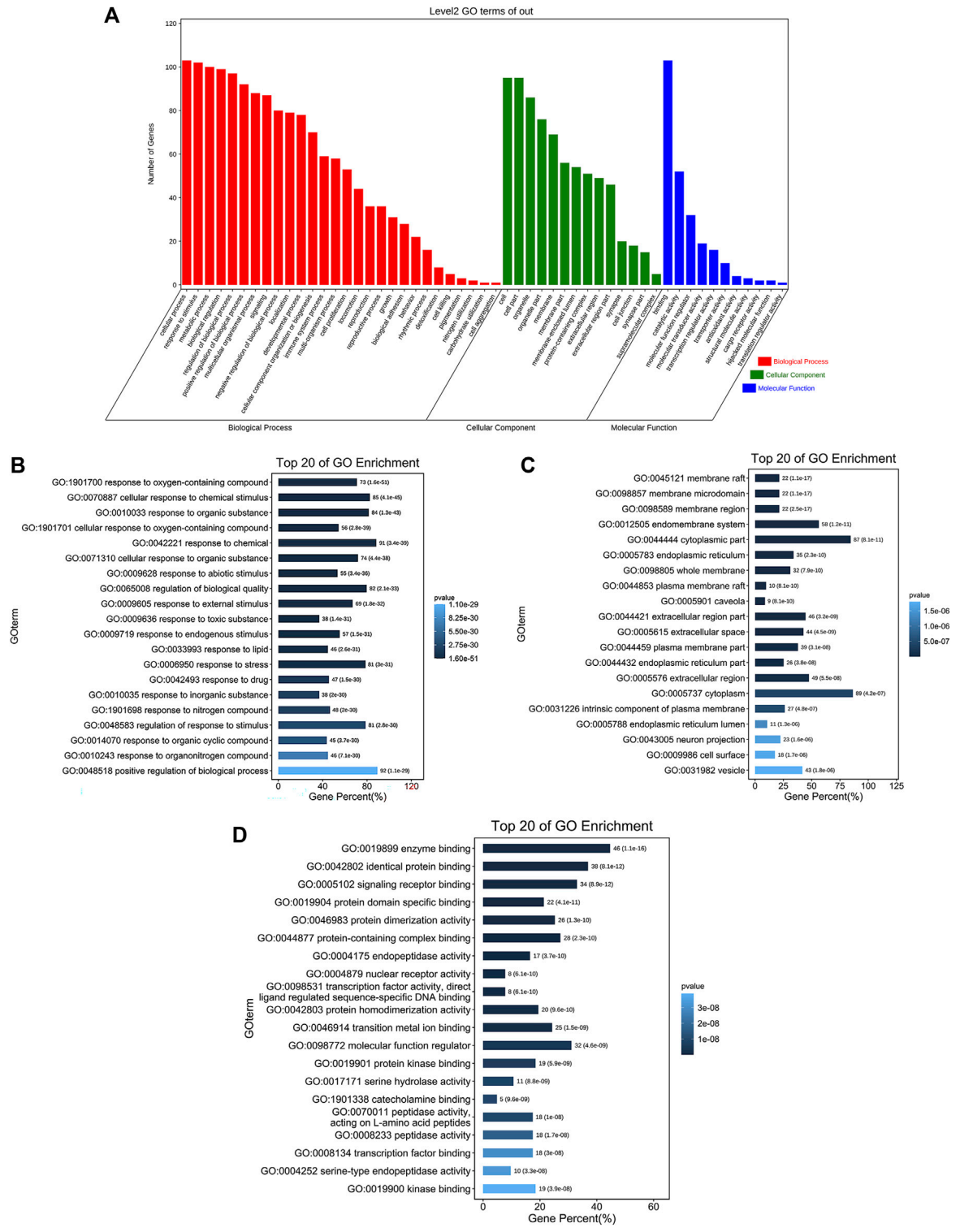

FIGURE 6
The findings of GO enrichment analysis. **(A)** On top of each bar was the number of genes in each category. The corresponding biological process (BP), cellular component (CC), and molecular function (MF) are represented by the colors red, green, and blue. **(B)** The top 20 of biological process. **(C)** The top 20 of cellular component. **(D)** The top 20 of molecular function.

differentiated SH-SY5Y cells induced by RA (RA), oxygen-glucose deprivation/reoxygenation (OGD), OGD + ZGP. To detect the impacts of ZGP on cell viability, the cells were treated with ZGP for 24 h. To observe the impacts of ZGP on neurite outgrowth and expression of target protein, the cells were supplemented with them for 72 h.

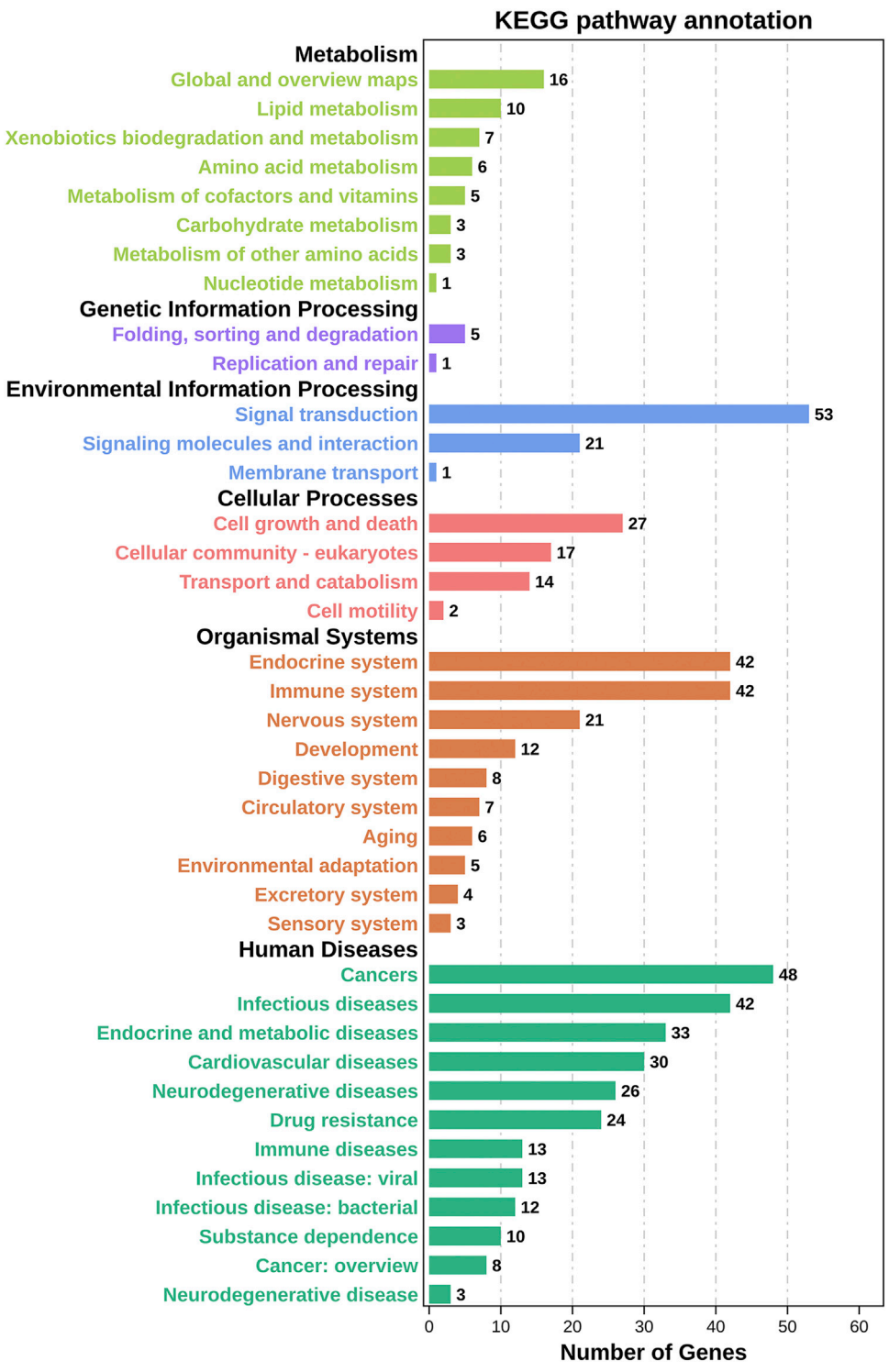

FIGURE 7
Results of KEGG enrichment analysis. On top of each bar was the number of genes in each category.

Cell viability assay

The viability of the cells was determined with the help of the CCK-8 test. In 96-well plates, the differentiated SH-SY5Y cells were seeded at a density of 3×10^4 cells per well in 100 μL of media. The cells were distributed into four cohorts: normal control (NC), differentiated SH-SY5Y cells induced by RA (RA), oxygen-glucose deprivation/reoxygenation (OGD), OGD + ZGP. For the ZGP cohorts, the cells were supplemented with ZGP at a density from 0.14 to 5.12 mg/mL for 24 h. The cells

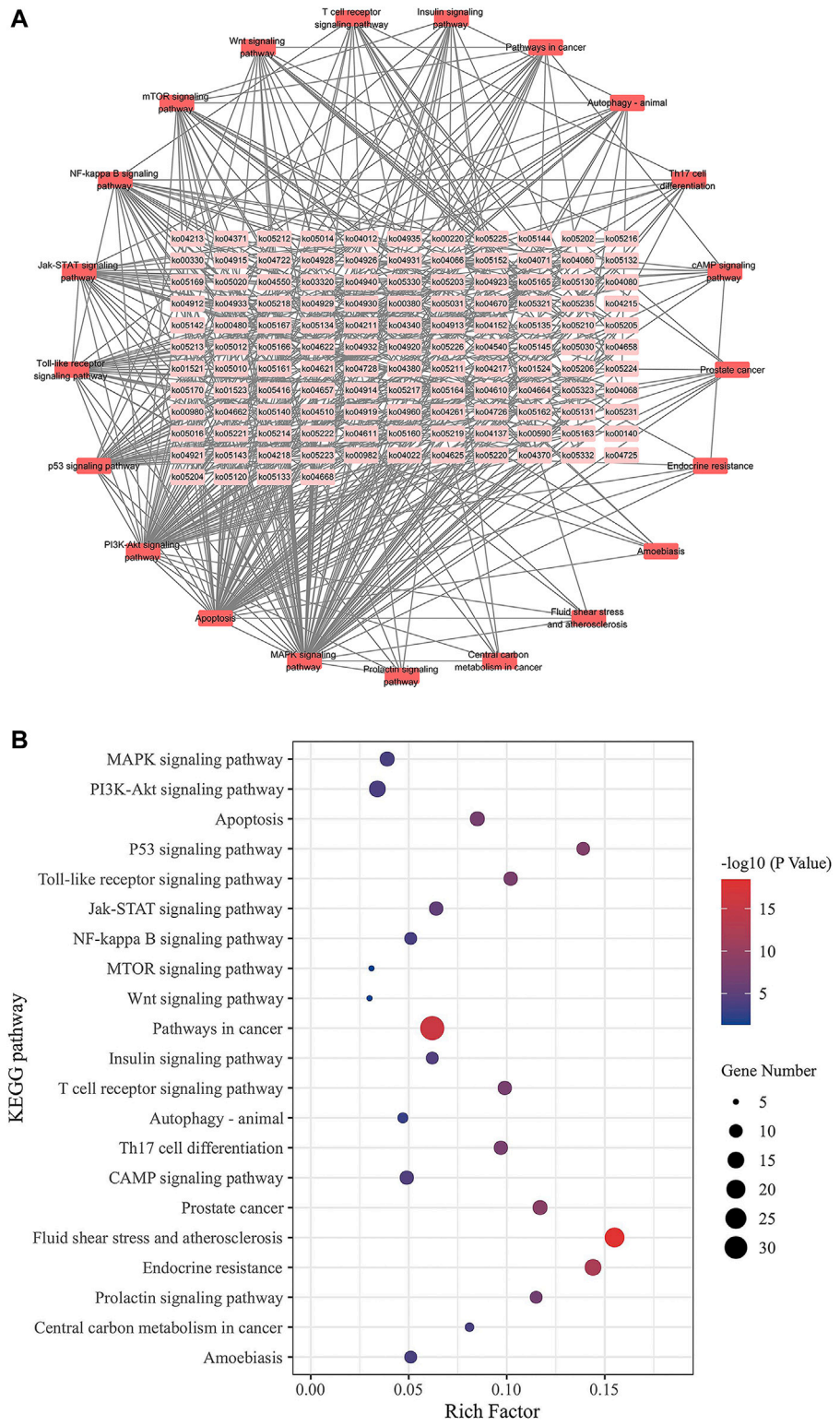

FIGURE 8
KEGG pathway interaction network. **(A)** Pathways are represented by network nodes; The annotates of the pathways are written on the nodes. Associations between the pathways are represented by straight lines. The correlation is stronger when the hue is darker. **(B)** Ischemic stroke-related key pathways.

received 10 μL of CCK-8 reagent after undergoing a variety of treatments, and the optical density (OD) was measured at 490 nm after an additional 4 h of cultivation. Cell viability was obtained by calculating the ratio of OD value of each cohort to that of control cohort (Cell viability = OD value of all cohorts/OD value of the NC cohort ×100%).

TABLE 3 21 key pathways related to ischemic stroke in KEGG pathway interaction network.

ID	Pathway	DC	BC	CC
ko04010	MAPK signaling pathway	82	7167.5	0.6682
ko04151	PI3K-Akt signaling pathway	62	3451.7	0.6067
ko04210	Apoptosis	62	4839.3	0.6092
ko04115	p53 signaling pathway	34	1012.0	0.5124
ko04620	Toll-like receptor signaling pathway	32	1111.0	0.5124
ko04630	Jak-STAT signaling pathway	31	612.5	0.5179
ko04064	NF-kappa B signaling pathway	28	654.7	0.4849
ko04150	mTOR signaling pathway	24	386.4	0.4770
ko04310	Wnt signaling pathway	18	250.0	0.4531
ko05200	Pathways in cancer	15	296.6	0.4817
ko04910	Insulin signaling pathway	15	375.0	0.4739
ko04660	T cell receptor signaling pathway	15	323.9	0.4517
ko04140	Autophagy - animal	14	433.3	0.4421
ko04659	Th17 cell differentiation	10	219.0	0.4421
ko04024	cAMP signaling pathway	10	259.8	0.4448
ko05215	Prostate cancer	9	717.0	0.4708
ko05418	Fluid shear stress and atherosclerosis	6	236.4	0.4545
ko01522	Endocrine resistance	6	345.3	0.4574
ko04917	Prolactin signaling pathway	6	634.1	0.4367
ko05230	Central carbon metabolism in cancer	6	444.7	0.4315
ko05146	Amoebiasis	6	290.1	0.4155

Neurite outgrowth analysis

The SH-SY5Y cells were seeded at a density of 5 $\times 10^5$ cells per well in 500 μL of media in 24-well plates. After inducing differentiation by ATRA and undergoing OGD/R injury, the cells were divided into the following groups: normal control (Control), oxygen-glucose deprivation/reoxygenation (OGD), OGD + ZGP (0.16, 0.32, 0.64 mg/mL). A treatment with 50 ng/mL of brain-derived neurotrophic factor (BDNF) was used as positive controls for neurite outgrowth (Dedoni et al., 2012; Agapouda et al., 2022). After being treated with different methods for 72h, the growth status of cell neurites was analyzed by measuring the length of neurites. The images of cells and neurites were obtained using a ZEISS LSM-710 Confocal Microscope (ZEISS Microsystems, Germany). Six high-power fields (40×) were detected from each well of different cohorts. The length of 10 neurites was measured per visual field using the ImageJ software, and the quantitative analysis was performed using Prism 9.0 software.

Western blot analysis

For Western blotting, the protein was extracted from cells to detect the target protein expression. After being treated with different methods, the cells were retrieved and extracted protein with RIPA lysate. The BCA method was used to determine the protein concentration. Conditions for SDS-PAGE gel electrophoresis: 80 V for 30 min and 120 V for 60 min. Conditions for membrane conversion: 250 mA of constant current for 90 min, completed on ice, and sealed for 1 hour at room temperature (25°C) with skimmed milk powder. A primary rabbit anti-GAP43 (1:1000), rabbit anti-p-S6 (1:1000), mouse anti-mTOR (1:5000), GAPDH (1:20,000), and rabbit anti-PTEN (1:2000) were used to preserve the membranes overnight at 4°C. After being treated with secondary goat anti-rabbit IgG (1:5000) or goat anti-mouse IgG (1:2000) antibodies for 1 hour and electrochemiluminescence (ECL) reagents for 30 s to 2 minutes, the membranes were exposed to Kodak film (Japan). The IOD proportion was measured and evaluated employing the ImageJ program to display the data.

Statistical analysis

For statistical analysis, the software SPSS26.0 and Graphpad Prism 8 were utilized. All data were tested for normality using the Shapiro-Wilk test. Normally distributed parameters were expressed as mean±S.D. The one-way ANOVA and the Tukey multiple comparisons test were used to identify group differences. LSD-t test was used for pairwise comparison. The statistical significance was described as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Results

ZGP active components screening

Eighty-six active components of ZGP were retrieved from the TCMSP and TCMID databases in accordance with the two screening criteria of OB value (≥30%) and DL index (≥0.18). These components include 2 components in SDH (Rehmannia glutinosa), 16 components in SY(Fried yam), 20 components in SZY(Cornus officinalis), 4 components in CNX (Sichuan Achyranthes), 48 components in GQ (Lycium barbarum), and 12 components in TSZ (Dodder seed). The six main components in GBJ (Tortoiseshell gum) and the two main components in CNX (Sichuan Achyranthes) were removed because they did not meet the screening requirements. The most important ZGP active components were shown in Table 1.

ZGP active component-target gene interaction network

TCMSP and PubChem gathered the 86 active components for target gene prediction. SDH (29), SY (121), SZY (112), CNX (182), GQZ (311), and TSZ (286) were among the 1041 predicted targets that were discovered. 207 relevant human gene targets were found after eliminating gene targets for other species and duplicate values. The relationship between drug composition and target was shown in Figure 2A. An herb-constituent-target network of ZGP was developed with the use of Cycloscape 3.7.1 program to make clear the relationships among the herbs. The outcomes are introduced in Figure 2B. The "CytoNCA" function was used to

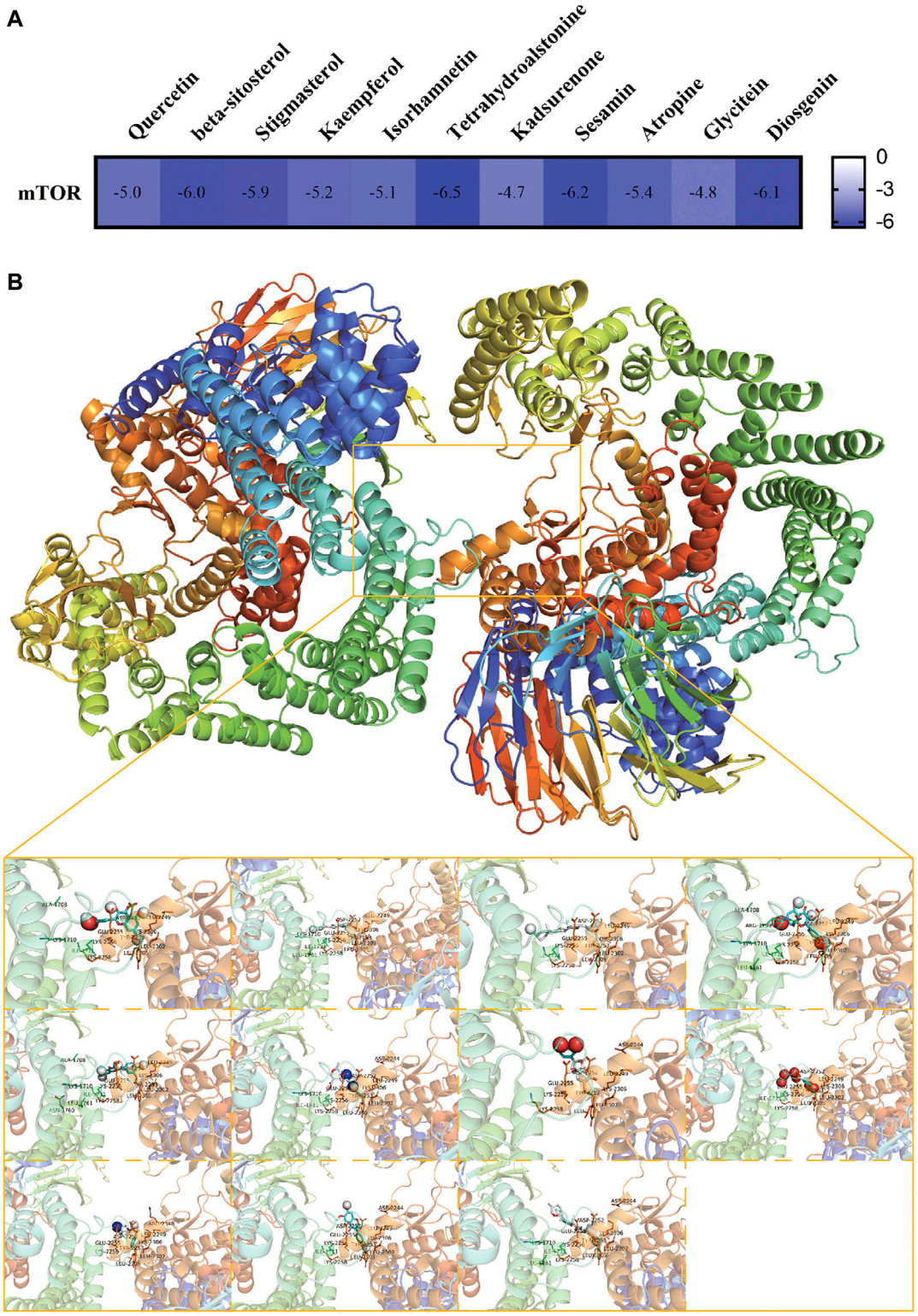

FIGURE 9
Molecular Docking Results. **(A)** The affinity of optimal docking results; **(B)** The visualization of optimal docking results (Middle: mTOR protein and its docking pocket. From left to right, top to bottom: Quercetin, beta-sitosterol, stigmasterol, kaempferol, isorhamnetin, tetrahydroalstonine, kadsurenone, sesamin, atropine, glyctein and diosgenin).

sort out the core components whose DC, BC, and CC were higher than the average during the topological properties analysis. Degree and intermediate centrality analyses yielded eleven core active compounds, including quercetin (MOL000098), beta sitosterol (MOL000358), stigmasterol (MOL000449), kaempferol (MOL000422), isorhamnetin (MOL000354), tetrahydroalstone

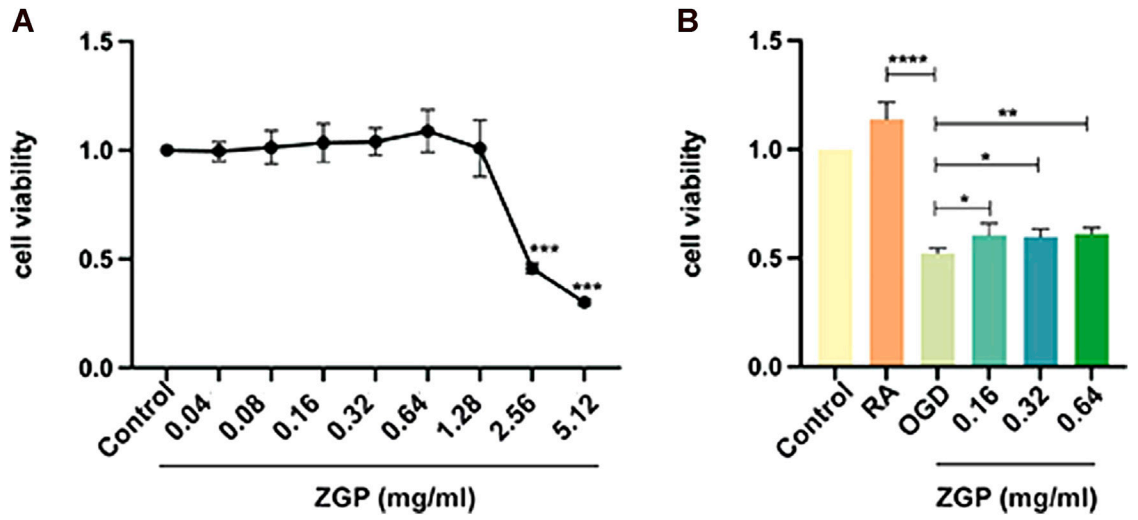

FIGURE 10
Impacts of ZGP on cell viability. The CCK-8 test was used to determine the cell viability, which was expressed as the OD value. **(A)** The cytotoxic effects of ZGP with different concentrations on differentiated SH-SY5Y cells. **(B)** ZGP increased the cell viability of differentiated SH-SY5Y cells after OGD/R. Each point denotes mean ± SD (n = 6). *$p < 0.05$, compared with OGD cohort; **$p < 0.01$, compared with OGD cohort; ****$p < 0.0001$, compared with RA cohort.

(MOL008457), kadsurenone (MOL000322), sesamin (MOL001558), atropine (MOL009650), glyctein (MOL008400) and diosgenin (MOL000546).

Potential therapeutic targets of ZGP for ischemic stroke

We continued to investigate their potential therapeutic targets after clarifying the key compounds of ZGP. 1020 ischemic stroke-related targets were gathered from the TTD, OMIM, GeneCards, and DisGeNet databases after duplicates were removed. The overlapping targets of ZGP-related targets and ischemic stroke-related targets were regarded as potential therapeutic targets for ZGP anti-ischemic stroke. Screening drug component targets and ischemic stroke targets yielded 103 intersection targets, as depicted in Figure 3.

Construction of drug component target disease network

The disease-component-target network was set up in view of the strength of the 103 shared targets recognized as both potential ZGP targets and ischemic stroke targets. Figure 4 shows the 173 nodes and 720 edges in this interaction network. Quercetin, β-sitosterol, Stigmasterol, Kaempferol, and Isorhamnetin have been related with more than 15 genes among the compounds that can interact with targets related to ischemic stroke. In addition, more than ten components were linked to the genes encoding PTGS2, NR3C2, PTGS1, SCN5A, ADRB2, PPARG, DPP4, and F2. The comprehensive regulation features of multi-components and multi-targets were demonstrated by means of the disease-compound-target network.

PPI network construction

The PPI relationships of 103 target genes were obtained using the STRING platform to make clear the potential mechanism by which ZGP benefits ischemic stroke. The outcomes are depicted in Figure 5. The PPI network had 475 edges and 97 nodes. The "CytoNCA" function was used to analyze the topological properties and select the key targets whose DC, BC, and CC were higher than the average. Table 2 displays a total of 16 key targets that have been obtained.

Analysis of GO and KEGG enrichment

We inserted the aforementioned targets into the OmicShare evaluation platform for GO and KEGG enrichment analyses in order to gain a deeper comprehension of the achievable pharmacological activity of ZGP in the treatment of ischemic stroke. 3569 biological processes (BPs), 228 cellular components (CCs), and 350 molecular functions (MFs) were among the 4147 GO terms we obtained (Figure 6A). Figures 6B–D depict the top 20 BP, CC, and MF categories. The targets had the highest GO enrichment in the following biological process ontologies: response to an organic substance, cellular response to a chemical stimulus, and oxygen-containing compound. Membrane raft, membrane microdomain, membrane region, endomembrane system, and cytoplasmic part were among the most highly enriched cellular component ontologies. ZGP's synergistic effects covered binding of enzymes, identical protein, signaling receptors, and other molecular functions. 149 signaling pathways have been recognized via enrichment and screening of KEGG pathways ($p < 0.05$) (Figure 7). The KEGG pathway interaction network was established for the enrichment results of KEGG pathways (Figure 8A), and 21 key pathways related to ischemic stroke through using the "CytoNCA" function to analyze its topological

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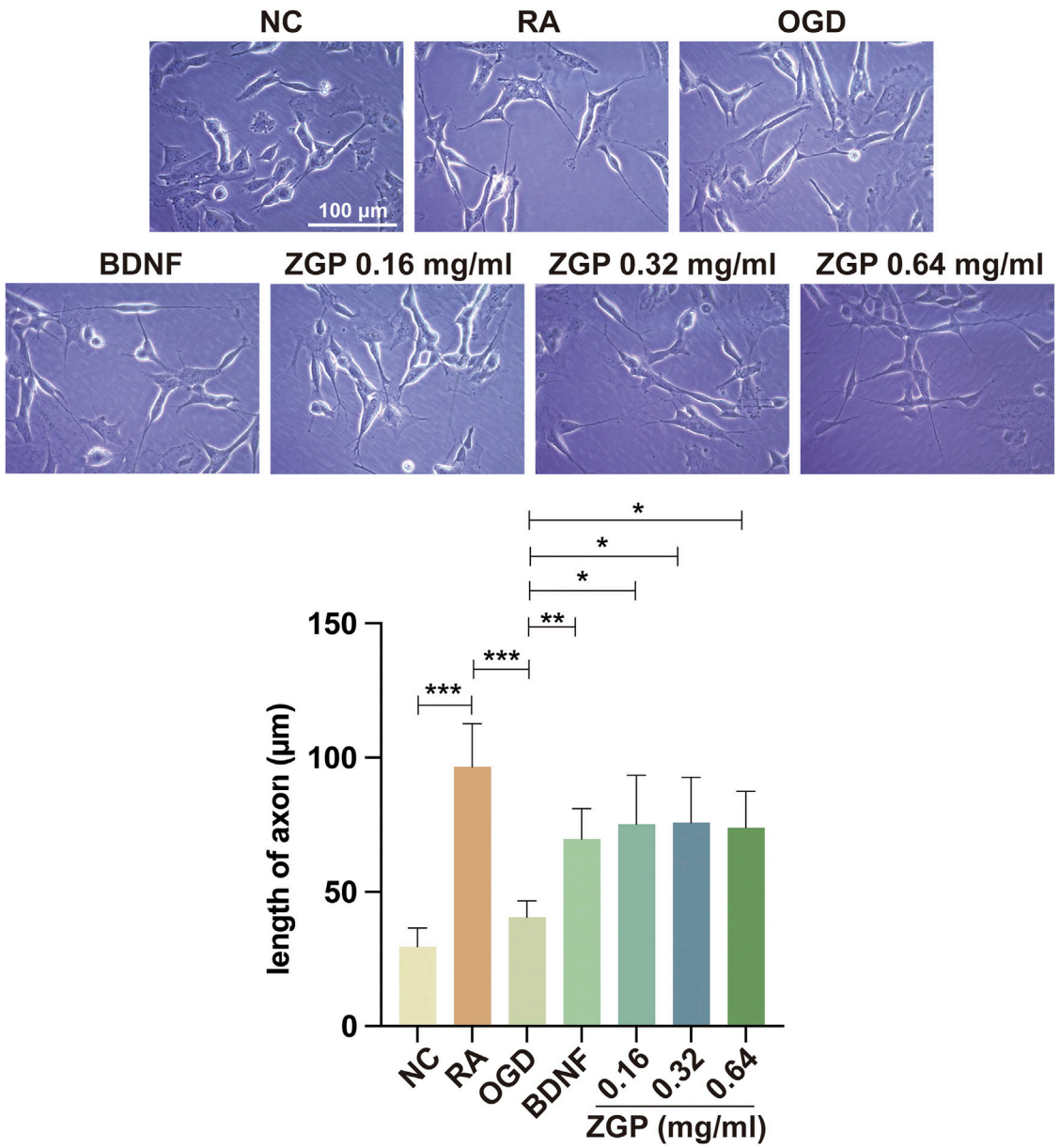

FIGURE 11
Effects of ZGP on neurite outgrowth. Illustrative microphotographs of morphological characteristics **(A)** and quantitative measurement outcomes of neurites length **(B)** of the normal control, differentiated SH-SY5Y cells between the OGD and ZGP cohorts 3 days after treatment; When compared to the OGD cohort, the cohorts with various concentrations of ZGP (0.16 mg/mL, 0.32 mg/mL, 0.64 mg/mL) had significantly longer neurites; each point represents mean ± SD (n = 3). ****$p < 0.0001$, compared with OGD cohort; scale bars: 100 μm.

properties (Figure 8B; Table 3). The main signaling pathway included MAPK, PI3K-Akt, Apoptosis, p53, Toll-like receptor, Jak-STAT, NF-kappa B, mTOR, and Wnt signaling pathway.

Molecular docking

Eleven active compounds (Quercetin, beta-sitosterol, stigmasterol, kaempferol, isorhamnetin, tetrahydroalstonine, kadsurenone, sesamin, atropine, glyctein and diosgenin) were obtained by network pharmacological analysis as the core active compounds of ZGP in the treatment of ischemic stroke. Take mTOR as the potential target. Through docking simulations, the results were shown in Figure 9A. The molecular-docking results suggested that the core active compounds of ZGP had good inter binding with mTOR. Detailed information about the optimal docking modes of the active compounds with mTOR were shown in Figure 9B.

ZGP increased the cell viability of differentiated SH-SY5Y cells after OGD/R

The relative cell viability was evaluated using the CCK-8 test. SH-SY5Y cells were induced to differentiate into mature neurons by ATRA.

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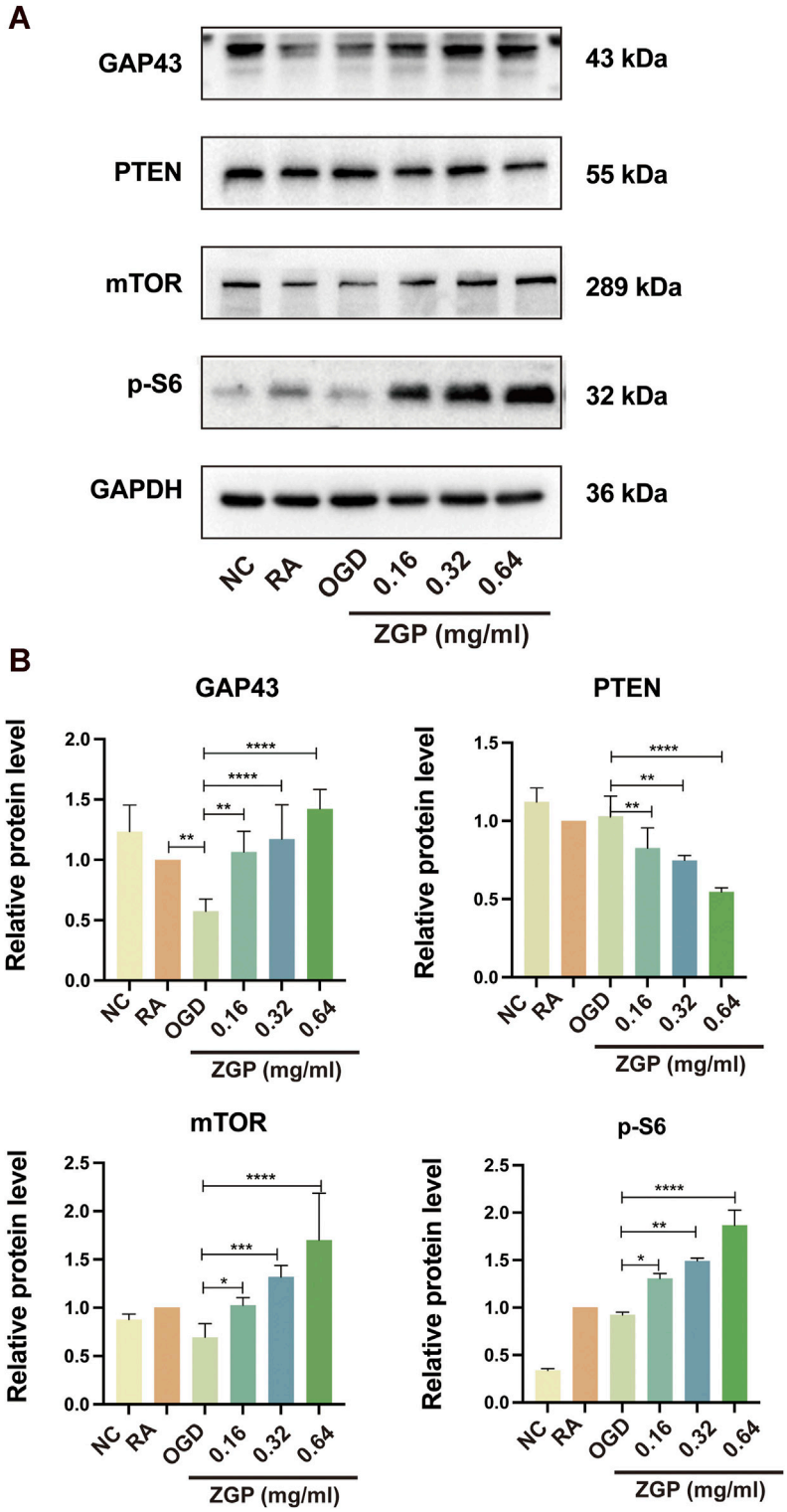

FIGURE 12
Effect of ZGP on GAP43, PTEN, mTOR, and p-S6 proteins expression. The differentiated SH-SY5Y cells were planted in 100-mm dishes and supplemented with various concentrations of ZGP (0.16 mg/mL, 0.32 mg/mL, 0.64 mg/mL) for 72 h; **(A)** the levels of GAP43, PTEN, mTOR, and p-S6 proteins expression were identified by Western blotting; **(B)** the expression of the GAP43, PTEN, mTOR, and p-S6 proteins in cells was quantified by normalizing to GAPDH; The data are shown as the means ± SD (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, compared with OGD cohort.

First, we evaluated ZGP's cytotoxic effects on differentiated SH-SY5Y cells. ZGP was applied to differentiated SH-SY5Y cells at varying concentrations (0.14–5.12 mg/mL) over 24 h; the highest nontoxic concentration was 1.28 mg/mL (Figure 10A). The concentration range at 0.16 mg/mL, 0.32 mg/mL, and 0.64 mg/mL, which demonstrated the best activity, was chosen for the future testing.

Differentiated SH-SY5Y cells were treated by OGD for 4 h. The cells were supplemented with ZGP at a concentration of 0.16 mg/mL, 0.32 mg/mL, and 0.64 mg/mL for 24 h. The ZGP cohorts had higher cell viability than the OGD cohort, and the difference between the ZGP cohorts (0.16 mg/mL, 0.32 mg/mL and 0.64 mg/mL) and the OGD cohort were statistically significant ($p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively) (Figure 10B). These results demonstrated that differentiated SH-SY5Y cells were protected from OGD/R damage by ZGP.

ZGP promoted neurite outgrowth of differentiated SH-SY5Y cells after OGD/R

The neurite alteration of neural cells was observed 3 days after treatment to determine the effect of ZGP on the neurite outgrowth of differentiated SH-SY5Y cells. Positive control was BDNF with 50 ng/mL, which is known to induce neurite outgrowth and promote synaptic plasticity. As depicted in Figure 11A, the cell morphology in the normal control group was irregular quadrilateral and elliptical, and the neurites were very short. In the ATRA induced group, the neurites of cells were much longer than those of immature cells, and the length of axons of some differentiated cells could reach 100–120 μm. The number of cells in the OGD cohort decreased, the cell body became flat, and the axons shortened compared with the ATRA induced group (Figure 11A). The OGD cohort's neurites were drastically shorter than those of the ATRA-induced group ($p < 0.0001$). As was to be expected, BDNF significantly improved the morphology of damaged cells and prolonged neurites growth. Meanwhile, the length of neurites was significantly longer after supplementation with various concentrations of ZGP (0.16, 0.32, 0.64 mg/mL) compared to that in the OGD cohort ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$) (Figure 11B). When compared to the OGD cohort, the impact of ZGP on neurite outgrowth was comparable to that of the positive control BDNF. The result suggested that ZGP reversed the damage to differentiated SH-SY5Y cells induced by OGD/R and promoted neurite or axon outgrowth.

ZGP regulated GAP43 and PTEN/mTOR/p-S6 signaling pathway proteins expression

Based on the results of GO and KEGG enrichment analysis, we selected the mTOR signal pathway protein and its negative regulatory factor PTEN protein for Western blot detection to verify the mechanism of ZGP promoting neurite growth. The expression of axon growth marker protein GAP43 in induced SH-SY5Y cells decreased after OGD/R ($p < 0.0001$), but gradually increased with the increase of ZGP concentration (Figure 12A). We observed statistically significant differences in 0.16 mg/mL ($p < 0.01$), 0.32 mg/mL ($p < 0.0001$) and 0.64 mg/mL cohort ($p < 0.0001$) compared to that in OGD cohort (Figure 12B). The expression of mTOR and p-S6 also had similar changes with GAP43 in various concentrations of ZGP (0.16 mg/mL, 0.32 mg/mL, 0.64 mg/mL) cohorts, which increased dramatically compared with the OGD cohort ($p < 0.05$–0.0001). Compared to the OGD cohort, PTEN protein expression was significantly downregulated in the ZGP cohorts at various concentrations (0.16 mg/mL, 0.32 mg/mL, 0.64 mg/mL) ($p < 0.01$, $p < 0.01$, $p < 0.0001$). These findings suggest that ZGP increased the expression of the axon-related protein GAP43 and promoted the growth of neuronal axons. ZGP's mechanism may involve increasing mTOR and p-S6 protein expression while decreasing that of the negative regulator PTEN protein.

Discussion

The incidence of ischemic strokes in China has increased by 86.0% since 1990, with 3.94 million new cases annually (Ma et al., 2021). Researchers have demonstrated that ZGP can significantly improve the neurological function of patients or mice who have suffered an ischemic stroke in clinical trials and basic experiments (Li et al., 2019; Liu et al., 2022). The total effective rate of ZGP for stroke patients was 92.0% (Chen et al., 2015). Using network pharmacology methods and a series of experiments, we determined the efficacy and molecular mechanisms of ZGP on ischemic stroke.

There were 86 active ingredients and 207 compound-related targets verified in ZGP in this study, and 1020 targets related to ischemic stroke were confirmed in the database. A total of 107 of these targets were related to ischemic strokes and ZGP. Additionally, 11 core active compounds (Quercetin, beta sitosterol, stigmasterol, kaempferol, isorhamnetin, tetrahydroalstone, kadsurenone, sesamin, atropine, glyctein and diosgenin) were obtained by network pharmacological analysis. Quercetin exerts neuroprotection for acute ischemic stroke by improving neurological function score (NFS), reducing infarct volume, and protecting blood brain barrier (BBB) integrity (Guo et al., 2022). There are several mechanisms by which quercetin protects the brain from damage, namely antioxidant, anti-inflammatory, anti-apoptosis, and the ability to resist calcium overload (Wang et al., 2020). Moreover, Quercetin might upregulate expression of GAP43, promote neurite outgrowth and regeneration of DRGs neurons and PC12 cells (Chen et al., 2015; Katebi et al., 2019), enhance the proliferation and migration of Schwann cells, improve locomotor function recovery, axonal regeneration and energy metabolism after spinal cord injury (SCI) (Wang et al., 2020). As naturally occurring sterol compounds, beta-sitosterol and stigmasterol play an important in cholesterol homeostasis, antioxidation activity, anti-inflammation activity, and nervous system development (Gao et al., 2021). There is evidence that stigmasterol protects against ischemic and reperfusion injury through its actions on AMPK/mTOR and JNK signaling pathways, which reduce oxidative stress and inactivate autophagy (Sun et al., 2019). The genes involved in the formation of neurites and synaptic transmission could be upregulated by stigmastero (Haque et al., 2018). It has been demonstrated that Stigmasterol modulated both pre-and post-synaptic events after ischemic and reperfusion, especially by attenuating GluN2B-mediated excitotoxicity and oxidative stress, and inducing mitophagy (Haque et al., 2021). β-sitosterol and stigmastero were also found to have neurite outgrowth-promoting activity in PC12 cells, which was induced by enhancing NGF and neurofilament expression (Koga et al., 2020). In addition to its anti-inflammatory and antioxidant properties, a flavonoid known as kaempferol has antibacterial and antiviral

properties as well (Silva Dos Santos et al., 2021). Kaempferol has been found to exhibit neuroprotective properties during cerebral ischemia. It can prevent cell death, oxidative stress, mitochondrial malfunction, and apoptosis that are caused by oxygen-glucose deprivation (OGD) (Wang et al., 2020). Kaempferol improved neurological impairments in cerebral ischemia reperfusion rats by reducing neuroinflammation and blood brain barrier dysfunction; the NF-B pathway was involved in this process (Li et al., 2019). Through increasing the levels of brain-derived neurotrophic factor (BDNF), kaempferol is also crucial for memory, neuronal plasticity, and the development of new neural networks (Yan et al., 2019). Isorhamnolicin has the effect of promoting neurite outgrowth and may drive PC12 cells to differentiate into neural cells (Xu et al., 2012). It was discovered that administering isorhamnetin to experimental ischemic mice decreased infarct volume and caspase-3 activity, reduced cerebral edema, protected blood-brain barrier, and accelerated the recovery of neurological function (Zhao et al., 2016). Sesamin is a lignan that has the ability to delay aging, resist oxidation and apoptosis, regulate oxidative stress, and diminish inflammatory response (Rao et al., 2018). Due to its extensive spectrum of pharmacological effects and therapeutic qualities, diosgenin, a well-known steroidal sapogenin, has been utilized for the treatment of neurological illnesses such cerebrovascular disease, Parkinson's disease, Alzheimer's disease, and brain damage (Cai et al., 2020). Enhanced expression of nerve growth factor (NGF) was related to greater neurite outgrowth, repaired damaged axons, restored ultrastructural alterations, and neuronal regeneration in a diabetic mice model (Kang et al., 2011). According to these studies, ZGP's primary ingredients are useful for treating ischemic stroke since they not only protect the brain from damage but also encourage nerve regeneration and repair. The active ingredient-target network diagram demonstrates ZGP's multiple constituents and multiple targets. PPI system analysis identified a total of 16 critical targets genes, with AKT1, JUN, IL6, and CASP3 placing at the top of the list. Serine/threonine protein kinase known as AKT1 is involved in a variety of physiological and pathological processes, including cell differentiation, apoptosis, inflammation, and metabolism following ischemia (Zhao et al., 2016; Samakova et al., 2019). Activated AKT1 triggers a series of signal cascade reactions, which can reduce the death of brain cells, promote the growth of neural cells and vascular endothelial cells, enhance the regeneration and repair of nerve tissue and vessels, and improve neural function after cerebral ischemia (Huang et al., 2021; Wang et al., 2021). Researches show that the expression of Jun gene is related to nerve regeneration, participate in the processes of neurovascular remodeling and recovery after cerebral ischemia (Murata et al., 2012). Whether in peripheral nerve tissue or central nerve tissue, the expression of Jun gene is induced after nerve injured. The higher the expression level and the longer the expression duration of Jun gene, the stronger the ability of nerve regeneration (Jessen and Mirsky, 2016). L6, an vital inflammatory cytokine, has many biological functions, which include regulating immune responses and inflammation (Lasek-Bal et al., 2019). After cerebral ischemia, the damaged brain tissue produces immune response and activates inflammatory cells. IL-6 is secreted to participate in secondary brain injury, increases infarct size and worsens clinical outcomes (Zhang et al., 2019). A key member of the Caspase family, Caspase-3 is the "molecular switch" that controls apoptosis in cells (Asadi et al., 2022). Caspase-3 protein expression significantly increased in the ischemic area of the brain, according to studies. The recovery of neural function and the survival of neural and vascular cells can both be enchanced by inhibiting Caspase-3 protein expression (Wesley et al., 2021).

To gain a deeper comprehension of the target genes' interaction and action pathways, GO and KEGG pathway analyses were utilized. The following biological processes (BPs) were found to be strongly related with target genes through GO analysis: response to an organic substance, cellular response to a chemical stimulus, and oxygen-containing compound. Membrane raft, membrane microdomain, membrane region, endomembrane system, and cytoplasmic part were the most highly enriched CCs ontologies. Enzyme binding, identical protein binding, signaling receptor binding, and various other molecular functions were included in the enriched MF ontologies. The MAPK, PI3K-Akt, apoptosis, p53, Toll-like receptor, Jak-STAT, NF-kappa B, mTOR, and Wnt signaling pathways were the primary focus of the KEGG pathway analysis. The results shown that ZGP may act on multiple signal pathways.

The mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways play a significant role in the activation of a number of critical signal transduction pathways in response to stress (Zheng et al., 2020; Liu et al., 2021). They not only participate in the regulation of apoptosis and inflammatory response after cerebral ischemia, but also mediates the proliferation, differentiation, and growth of neurons (Kong et al., 2016; Jayaraj et al., 2019). PI3K/Akt signal pathway is the most effective anti apoptotic pathway after the activation by granulocyte and macrophage stimulator, p-Akt can inhibit the expression of downstream apoptotic factors NF- κ B, regulates cell metabolism, proliferation, apoptosis and migration (Zhu et al., 2018). The JAK/STAT signaling pathway plays a major role in the process of apoptosis following ischemia reperfusion injury, which is abnormally activated to accelerate neuronal apoptosis and aggravate brain injury (Wu et al., 2018).

mTOR, p53, and Wnt signaling pathways are all associated with nerve regeneration and repair after ischemic brain injury. mTOR is a key signal pathway regulating the intrinsic growth ability of neurite outgrowth and axonal regeneration (Park et al., 2008; Bei et al., 2016; Liu et al., 2017; Zhang et al., 2022). The phosphorylation expression level of its downstream protein p-S6 can indirectly characterize the activity of mTOR ((Ma et al., 2021; Li et al., 2022). The ability to regenerate after ischemic brain injury is closely related to the enhancement of mTOR/p-S6 activity, which increases the expression of growth associated protein-43 (GAP-43) (Li et al., 2022). According to another research, the PI3K/Akt signaling pathway is also essential for axonal regeneration, which is accomplished via activating mTOR. (Wang et al., 2019). Meanwhile, phosphatase and tensin homology (PTEN) is an important molecular target to inhibit the internal growth ability of neurons (Liu et al., 2010). It steadily increases in expression as the nervous system develops and matures, suppresses the mTOR pathway of cellular internal growth, and dramatically lowers the capacity of mature axons to sprout and regenerate (Hausott et al., 2022). Inhibiting PTEN expression to reactivate mTOR pathway can promote significant sprouting, regeneration and functional recovery of optic nerve axons and CST axons after cerebral ischemia (He and Jin, 2016). The role of tumor suppressor p53 in regulating neurite outgrowth and axonal regeneration has been confirmed over the past decade (Di Giovanni and Rathore, 2012). As a highly conserved signal pathway in

multicellular eukaryotes, Wnt protein family is crucial to neuronal differentiation, neuronal survival, axonal regeneration after ischemic stroke and other aspects at the early stage of nervous system development (Garcia et al., 2018).

From the above results, we can see that ZGP not only has anti-apoptosis and neuroprotective effects on ischemic stroke, but also can promote neurite outgrowth and axonal regeneration after cerebral ischemia. Our *in vitro* experiments in this study demonstrated that ZGP protected ischemic neurons by increasing the cell viability of differentiated SH-SY5Y cells following OGD/R. Several other investigations have revealed that the mechanism behind ZGP's protective impact on ischemic stroke is related to inhibit oxidative stress and inflammatory reaction and regulate PI3K/Akt pathway (Liu et al., 2017; Liu et al., 2021; Liu et al., 2022). However, the effect and mechanism of ZGP on neurite growth and regeneration have not been experimentally verified. In current research, we targeted on the effect of ZGP on neurite growth and regeneration in hypoxic-ischemic neurons, and verified its mechanism. As we expected, ZGP reversed the damage to differentiated SH-SY5Y cells induced by OGD/R and promoted neurite and axon outgrowth obviously. Our previous research results and those of other scholars confirmed that PTEN/mTOR are the key signal pathway to control neurite and axon remodeling after nerve injury (Park et al., 2008; Liu et al., 2017). We verified that ZGP promotes neurite growth and regeneration through the PTEN/mTOR signaling pathway. The fact that ZGP increased GAP43, mTOR, and p-S6 expression and decreased PTEN expression indicates that the PTEN/mTOR signal pathway was connected to ZGP's role in promoting neurite regeneration and outgrowth.

In conclusion, this research is the first time to use multiple network models to study the mechanism of ZGP on ischemic stroke, and provide new insights for the role of ZGP in ischemic stroke treatment. Network analysis of ZGP identified 86 active ingredients and 107 compound-related targets correlated with ischemic stroke. Additionally, 11 core active compounds were obtained, such as Quercetin, beta sitosterol, and stigmasterol. Most of the compounds have been proved to have pharmacological activities. Pathway enrichment demonstrated that ZGP may exert neuroprotective effect through MAPK, PI3K-Akt signaling pathway, and exert promoting neurite outgrowth and axonal regeneration effect via mTOR, p53 and Wnt signaling pathway. *In vitro* experiment, ZGP treatment greatly boosted the survivability of ischemic neurons and enhanced their capacity for neurite outgrowth. Western blot assays shown that the pro-neurite outgrowth effect of ZGP on ischemic stroke may be relate to PTEN/mTOR signal pathway. All of the findings offered fresh explanations for the molecular basis of ZGP and served as a guide for its use in clinical settings. However, due to the limited database of traditional Chinese medicine and many network prediction results, the experimental verification is not comprehensive. Future research will still need to evaluate the therapeutic effects of ZGP *in vivo* and its potential mechanisms for ischemic stroke treatment.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Author contributions

LL and YL performed the major research, analysed the data, and drafted the manuscript. YZ and CL participated in network pharmacology analysis. JZ participated in language editing and manuscript revision. DW and XY contributed to literature review and cell culture. MW provided the conception and design of the study. WL provided the conception and design of the study, participated in the drafting and revision, obtained funding and supervised the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Mechanical thrombectomy with intra-arterial alteplase provided better functional outcomes for AIS-LVO: a meta-analysis

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Background: Several clinical trials have shown that intra-arterial thrombolysis using alteplase during mechanical thrombectomy (MT) has a better outcome than MT alone in ischemic stroke management. We performed the current meta-analysis to estimate the efficacy and safety of MT with intra-arterial alteplase therapy.

Methods: The MEDLINE, Embase, Cochrane Library, and ClinicalTrials.gov databases were searched up to Mar. 2022 to identify the clinical trials that compared MT alone versus MT with intra-arterial alteplase therapy. STATA 16.0 was used for statistical analysis. The odds ratios (ORs) and 95% confidence intervals (95%CIs) were calculated with a random effect model.

Results: Seven studies involving 1,083 participants were included. The primary outcomes were better functional outcomes, defined as a modified Rankin Scale (mRS) score between 0 and 2 at 90 days, and successful recanalization, defined as a modified thrombolysis in cerebral infarction (mTICI) score ≥ 2b. Compared to MT alone, MT with intra-arterial alteplase did not lead to higher mTICI scores (OR 1.58, 95%CI 0.94 to 2.67, p=0.085, I^2 =16.8%) but did lead to better mRS (OR 1.37, 95%CI 1.01 to 1.86, p=0.044). There was no increase in mortality or bleeding events in the overall or subgroup analyses.

Conclusion: MT with intra-arterial alteplase did not improve the recanalization rate but provided better functional outcomes. The intervention did not increase adverse effects in any subgroup at the same time.

Clinical trial registration: http://inplasy.com, identifier INPLASY202240027.

KEYWORDS

intra-arterial thrombolysis, alteplase, mechanical thrombectomy, acute ischemic stroke, functional outcomes after acute stroke

Introduction

Acute ischemic stroke (AIS) is the leading cause of mortality and disability worldwide. AIS caused by large vessel obstruction (AIS-LVO) has a worse prognosis. Current evidence-based treatment options include intravenous thrombolysis (IVT) and mechanical thrombectomy (MT); however, these approaches still have several limitations (Ospel et al., 2020).

Intravenous alteplase has been showen substantially improved the outcomes of AIS patients and had become the first-line therapy for patients having AIS (Hacke et al., 2008). But the narrow time window of 4.5 h and several contradictions limited the recanalization efficacy of IVT for AIS-LVO (Bhatia et al., 2010).

MT has become the standard care for AIS with definitive efficacy and good safety (Powers et al., 2019). Intra-arterial thrombolysis (IAT) emerged initially in the PROACT trials (Prolyse in Acute Cerebral Thromboembolism) (del Zoppo et al., 1998). The Multicenter Randomized Clinical trial of Endovascular treatment for Acute ischemic stroke in the Netherlands (MR CLEAN) proved that intra-arterial therapy is effective and safe for AIS that is caused by proximal intracranial occlusion of the anterior circulation within 6 h after stroke onset (Berkhemer et al., 2015). The Interventional Management of Stroke (IMS) trials yielded promising outcomes in phase I and II studies but the phase III randomized clinical trial (RCT) came out with negative results (Investigators IS, 2004; Investigators IIT, 2007; Broderick et al., 2013). However, the role of IAT evolved from a primary therapy to adjunct or rescue therapy to mechanical thrombolysis.

It is necessary to evaluate the efficacy and safety of IVT, MT, IAT, and the combination of these therapies. A previous network meta-analysis comparing MT alone, IAT alone, MT + IVT, and IAT + IVT concluded that MT + IVT seemed to be the most effective strategy without increasing adverse effects (Hui et al., 2020). The efficacy of MT + IAT remained unclear. Another meta-analysis based on observational studies evaluating all modalities of MT and all categories of thrombolytics supported the potential role of IAT as an adjunct to MT (Chen et al., 2021).

Thrombolytic pharmaceuticals include urokinase, recombinant tissue plasminogen activator (rtPA, also named alteplase), and glycoprotein IIb/IIIa inhibitors, among which alteplase is the most well studied. Alteplase was first introduced in IVT and showed substantial improvement in outcomes.

Previous data comparing MT with intra-arterial alteplase and MT alone were mainly derived from observational studies. The two most commonly used efficacy outcomes were functional outcomes assessed by modified Rankin Scale (mRS) and recanalization assessed by modified Thrombolysis In Cerebral Infarction (mTICI) scale. The mRS scores were similar between the two groups or better in MT + IA tPA group. Recanalization showed heterogeneity in different studies. Heiferman et al. (2017) reported that MT with IA-tPA had a lower rate of mTICI = 2b but a higher rate of mTICI = 3. Anadani et al. (2019) also found a higher complete recanalization rate, while other studies showed no significant difference between the two groups. Consistently, these studies did not find an increase in adverse effects. Recently, a randomized controlled trial published the results of intra-arterial alteplase following successful MT (Renú et al., 2022). It concluded that intra-arterial alteplase as an adjunct therapy to MT resulted in a greater likelihood of excellent neurological outcomes at 90 days. We performed the current meta-analysis to estimate the efficacy and safety of MT with intra-arterial alteplase. We performed further subgroup analysis to investigate the potential value in specific patients.

Methods

Before the project started, we designed the protocol following the PRISMA guidelines (Page et al., 2021). We have submitted our study protocol to the INPLASY register (No. INPLASY202240027).

Eligibility and exclusion criteria

Eligibility Criteria: (i) participants: patients with AIS-LVO, (ii) intervention: MT with intra-arterial alteplase, (iii) Control: MT alone or MT with placebo, and (iv) outcomes: efficacy outcomes including the mRS and mTICI; safety outcomes including hemorrhage transformation and mortality. Included studies were not requested to have all the outcome data.

Exclusion Criteria: (i) study type: case reports or case series and (ii) active control (i.e., that is known to be an effective treatment as opposed to a placebo).

Search strategy and information sources

Two independent investigators (XYY and ZLW) systematically searched the MEDLINE, EMBASE, Cochrane Library, and ClinicalTrial.gov databases, up to Mar. 2022 to identify relevant studies. "Alteplase", "recombinant tissue plasminogen activator", "mechanical thrombectomy" and "stroke" were used as search keywords. The detailed search strategies are presented in the Supplementary Table S1.

Study selection and data collection

Two reviewers independently screened and evaluated all study records from the database search according to the eligibility criteria listed above. A third reviewer who did not participate in the process of data collection was consulted to resolve disagreements. The two reviewers extracted the following data using a standardized form: baseline information, inclusion and exclusion criteria, efficacy and safety outcome results, and conclusions.

Risk of bias

Two reviewers assessed the risk of bias using the methodological index for nonrandomized studies (MINORS) tool. Disagreements between the two reviewers were resolved by consulting a third reviewer, Each study was checked with the 12-item MINORS scale to obtain a total score that represents the quality of the study. The RCT was assessed with Cochrane Collaboration risk of bias tool, which included the following domain: selection bias, performance bias, detection bias, attrition bias, reporting bias, and other potential biases. Each domain was classified as "low," "high" or "unclear."

Statistical analysis

We used STATA 16.0 for data analysis. Statistical heterogeneity was estimated via the I^2 statistic. All analyses used a random effect model. Heterogeneity was classified as low heterogeneity ($I^2 < 30\%$),moderate heterogeneity ($30\% < I^2 < 50\%$),substantial heterogeneity (I^2 of 50% or more). Odds ratios (ORs) and 95% confidence interval (95% CI) were used for dichotomous variables and were presented with a Forest plot. All statistical tests were 2-tailed and significance was set at $p < 0.05$. Sensitivity analysis was used to explore the stability of the pooled results.

Outcome of interest

Efficacy outcomes included functional outcomes assessed at 3 months by the and modified Rankin Scale (mRS) and recanalization assessed by the modified Thrombolysis In Cerebral Infarction (mTICI) scale. The good functional outcome was set as mRS 0 to 2. The successful recanalization was defined as mTICI≥2b.

Safety outcomes were assessed by determining the rate of adverse effects, including mortality, symptomatic intracerebral hemorrhage (sICH), parenchymal hemorrhage type 2 (PH-2), and any hemorrhage.

Subgroup analysis

We performed subgroup analysis according to the baseline characteristics such as age, NIHSS score, and the timing of intra-arterial alteplase administration. We set two subgroup marks: (i) age above or below 70 years old and (ii) IAT as adjunct or rescue therapy to MT.

Results

Baseline characteristics

We identified 2,136 references from the database searches. A total of 529 duplicates were removed. Irrelevant records were excluded after screening. Eligible articles were further assessed and seven studies (Lin et al., 2009; Heiferman et al., 2017; Yi et al., 2018; Anadani et al., 2019; Zaidi et al., 2019, 2021; Renú et al., 2022) were included in our final analysis (Figure 1). A total of 1,083 participants were pooled. The characteristics of each included study are listed in Table 1.

Efficacy outcomes

We combined data for the outcome of recanalization using OR with random effects model. Compared to MT alone, MT with intra-arterial alteplase did not show higher recanalization rate (OR 1.58, 95%CI 0.94–2.67, $p = 0.085$, $I^2 = 16.8\%$) but yielded better functional outcome of mRS 0 to 2 (OR 1.37 95%CI 1.01–1.86, $p = 0.044$, $I^2 = 0.0\%$) (Figure 2).

Subgroup analysis did not yield any positive result. The Forrest plot of each subgroup was presented in Supplementary Figure S1.

Safety outcomes

Among the four analyzed indicators of adverse effects, we did not observe significant differences between the two groups (Figure 3). The administration of intra-arterial alteplase during MT did not increase the risk of mortality or hemorrhage. The results were as follows: mortality rate (OR 0.70, 95%CI 0.49–1.01, $p = 0.055$, $I^2 = 0.0\%$), sICH (OR 0.71, 95%CI 0.21–2.38, $p = 0.584$, $I^2 = 23.7\%$), PH-2 (OR 0.78, 95%CI 0.34–1.17, $p = 0.550$, $I^2 = 0.4\%$), and any hemorrhage (OR 1.00, 95%CI 0.65–1.53, $p = 0.998$, $I^2 = 0.0\%$). Subgroup analysis also revealed negative results (Table 2). The analysis of sICH patients aged>70 years old showed a higher level of heterogeneity ($I^2 = 55.3\%$). We performed a sensitivity analysis and the results are shown in Supplementary Figure S2.

Risk of bias

We included three retrospective studies, three prospective studies, and one RCT in this meta-analysis. The risk of bias in observational studies is listed in Table 3. The total points ranged from 17 to 20. The RCT (Renú et al., 2022) was categorized as "low risk of bias" for each domain.

Discussion

We pooled 1,083 participants from seven studies in our meta-analysis to estimate the safety and efficacy of intra-arterial alteplase during MT. The results suggested that compared to MT alone, MT with intra-arterial alteplase led to better functional outcomes but did not improve recanalization. Further more, there was no increase in adverse effects. Overall, intra-arterial alteplase showed good efficacy and safety outcomes.

The efficacy outcomes were assessed by widely used tools, the mRS was used to assess functional outcome and the mTICI was used to assess recanalization. The MT with intra-arterial alteplase group did not show a higher successful recanalization rate but did show a higher rate of good functional outcomes. This phenomenon might be explained by the limitations of the mTICI scale. The reperfusion rate is typically calculated by the operator at the end of the procedure, which could be influenced by experience. Patients might benefit from intra-arterial thrombolysis owing to the better reperfusion that is not reflected in mTICI score. The most recent TICI (expanded TICI, eTICI) was published in 2019. The expanded TICI scale divided reperfusion extent into 7 grades. It provided cut-off points by demonstrating excellent reliability for distinguishing eTICI 2b50 and 2b67. The efficacy of eTICI was examined using a large multinational dataset (Liebeskind et al., 2019). Renú et al. (2022) utilized the recent iteration to obtain a more objective reperfusion assessment.

The most important safety concern about the addition of intra-arterial alteplase to MT might be the potential hemorrhagic transformation, especially intracerebral hemorrhage. The hemorrhagic risk was estimated by the rate of hemorrhage events such as sICH, PH-2, and any hemorrhage in most studies. Our results did not show any significant difference in these hemorrhagic indices, nor did the subgroup analysis. The results are somewhat unexpected but not unreasonable. Thrombolytic agents truly have a direct effect on hemorrhage tendency, but the mechanisms of ICH secondary to intra-arterial revascularization therapies in AIS are complex. The blood–brain barrier damage triggered by ischemia is another pivotal process (Mokin et al., 2012). The risk of hemorrhage and the benefit of timely reperfusion need equilibrium. Another meta-analysis comparing MT with adjunctive intra-arterial thrombolysis also obtained similar results that sICH rates were not increased (Diprose et al., 2021). However, the included studies used different classification systems of sICH that might cause heterogeneity and bias. The results also showed no difference in mortality between the two groups. MT with IAT tended to decrease mortality compared to MT alone.

Our study is the first meta-analysis that focused on the combination of specific intra-arterial thrombolytic alteplase and MT

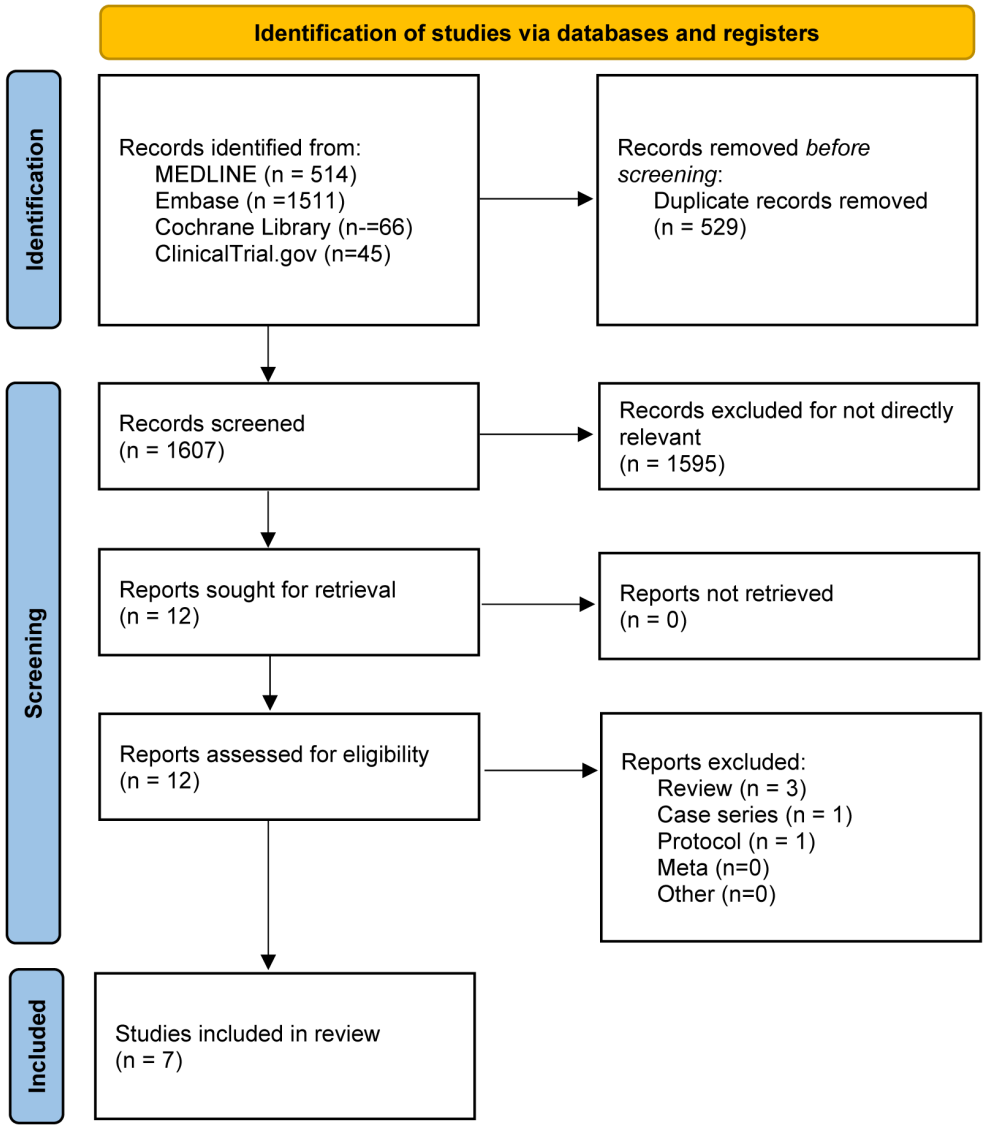

FIGURE 1
The study search, selection, and inclusion process.

by incorporating the data from the most recent RCT. The results and conclusions are consistent with the outcomes of the RCT and most previous studies. The level of heterogeneity was low in the majority of outcomes analyzed herein. The subgroup analysis of sICH in age>70 showed substantial heterogeneity. But only two studies, Zaidi et al. (2019) and Renú et al. (2022), were included in this subgroup. Zaidi et al. (2019) was a retrospective study while Renú et al. (2022) was the most updated RCT, which might cause heterogeneity.

The CHOICE trial (Chemical Optimization of Cerebral Embolectomy) was the first RCT to publish the outcomes of intra-arterial alteplase following successful MT. The RCT differs from the previous observational studies because it used successful recanalization as an indicator of eligibility for intra-arterial alteplase administration. It also used the most updated expanded Thrombolysis In Cerebral Infarction (eTICI) index to assess recanalization. Therefore, the RCT could not be included in the analysis of recanalization herein. Another regretful thing is that CHOICE trial was terminated early and did not reach its expected recruitment target due to the COVID-19 pandemic. The most updated RCT had a relatively small sample size and thus weighted less heavily in this meta-analysis.

Previous RCTs about intra-arterial therapy, such as PROACT and MR CLEAN, are not included in our meta-analysis due to different study designs. A more recent observational study of MR CLEAN Registry analyzed the participants receiving IA thrombolytics following EVT and showed neutral results (Collette et al., 2023). This study got similar rate of favorable outcome (defined as mRS 0–2) between the groups with or withour IA thrombolytics and found less reperfusion rate in patients treated with IA thrombolytics, which are inverse to our results. But the neutral results about sICH are consistent with us.

There are some other limitations in our meta-analysis. A certain percentage of participants received IVT, the impact of which could not be ignored, but we could not perform a subgroup analysis of these

TABLE 1 Baseline characteristics of included studies.

Trials	Study type	Country/ Center	Period	Timing of IAT to MT	Group	No. of participants	Age [Mean (SD)/ Median (IQR), years]	Sex [Male, *n* (%)]	NIHSS [Mean (SD)/ Median (IQR), years]
Lin et al. (2009)	Retrospective	USA	1998 to 2008	Before	MT + IAT	40	68 (13)	19 (47.5%)	17 (6–25)
					MT alone	18	71 (13)	10 (55.6%)	18 (10–29)
Heiferman et al. (2017)	Prospective	USA	Jan 2015 to Mar 2016	Adjunct	MT + IAT	28	67 (56–74)	10 (36%)	20 (15–25)
					MT alone	12	69 (63–78)	5 (42%)	18 (14–22)
Anadani et al. (2019)	Retrospective	USA	Nov 2014 to Jan 2018	Rescue	MT + IAT	419	66.1 (15)	34 (50.7%)	15.2 (7.6)
					MT alone	67	68.2 (14.2)	205 (48.9%)	15.9 (7.4)
Yi et al. (2018)	Retrospective	China	2015 to 2017	Adjunct	MT + IAT	37	66 (13)	17 (45.9%)	18 (11–23)
					MT alone	56	65 (11)	30 (53.6%)	18 (10–28)
Zaidi et al. (2019)	Retrospective	24 sites in the USA	Mar 2012 to Feb 2013	Rescue	MT + IAT	37	70.7 (15.4)	21 (56.8%)	17.5 (14–22)
					MT alone	44	69.1 (17.8)	22 (50%)	19 (13–21)
Zaidi et al. (2021)	Prospective	55 centers in the USA	Aug 2014 to Jun 2016	Rescue	MT + IAT	129	68 (15.2)	69 (53.5%)	17.0 ± 5.5
					MT alone	83	65.9 (15.3)	53 (63.9%)	17.6 ± 5.7
Renú et al. (2022)	RCT	7 centers in Spain	Dec 2018 to May 2021	Adjunct	MT + IAT	61	73 (71–76)	33 (54%)	14 (8–20)
					MT + placebo	52	73 (69–77)	28 (54%)	14 (10–20)

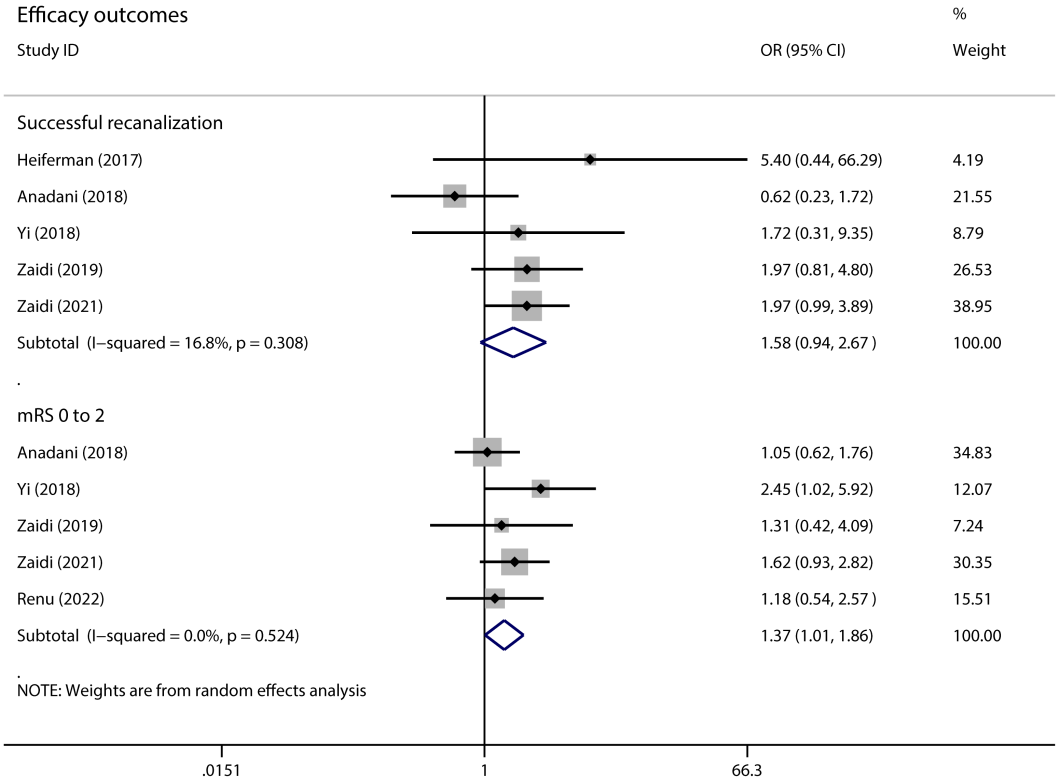

FIGURE 2
The pooled OR and 95%CI of efficacy outcomes. The diamond indicates the estimated OR and 95%CI and the square indicated the weight of each study.

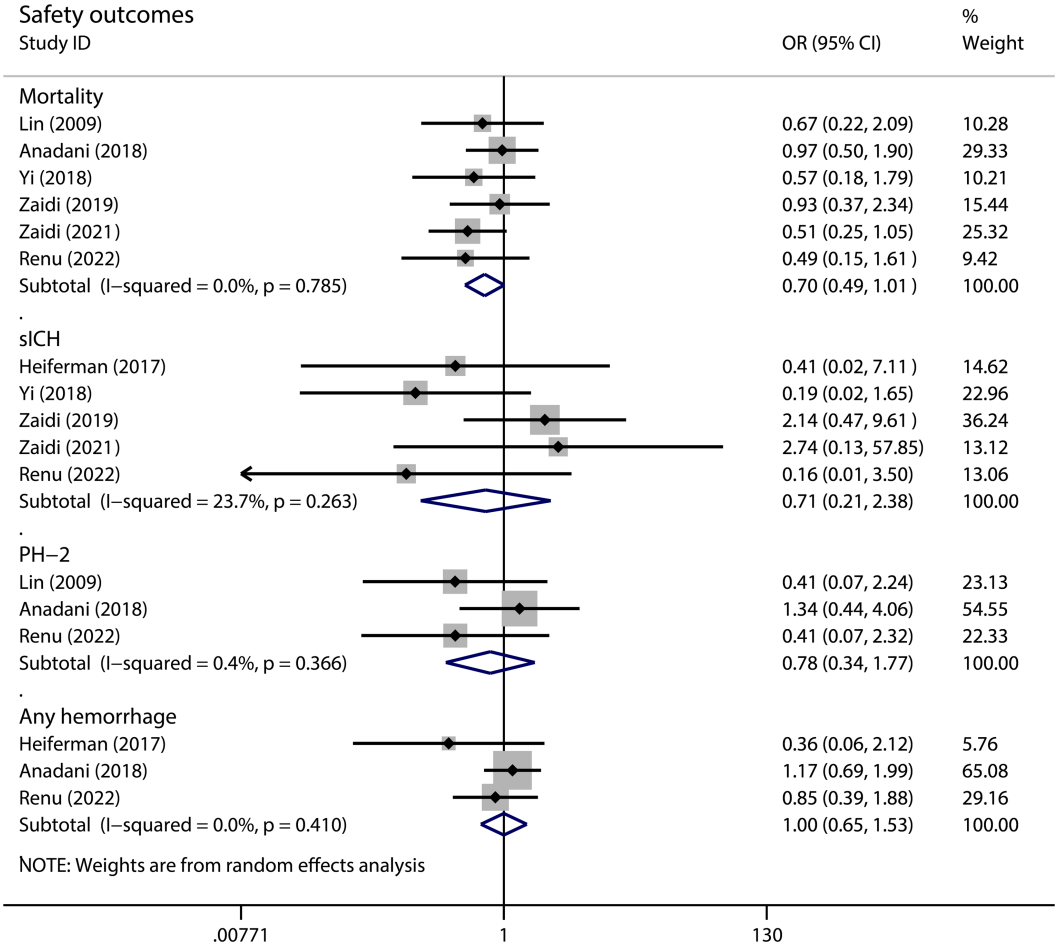

FIGURE 3
The pooled OR and 95%CI of safety outcomes. The diamond indicates the estimated OR and 95%CI and the square indicated the weight of each study.

TABLE 2 The results of subgroup analysis.

	Efficacy outcomes				Safety outcomes			
	Good functional outcome		Recanalization		Mortality		sICH	
	OR (95%CI)	*p* value	OR (95%CI)	*p* value	OR (95%CI)	*p* value	OR (95%CI)	*p* value
Age								
Age>70	1.221 (0.642, 2.320)	0.543	NA	NA	0.728 (0.351, 1.511)	0.394	0.840 (0.072, 9.768)	0.889
Age<70	1.466 (0.942, 2.282)	0.090	1.457 (0.693, 3.063)	0.322	0.692 (0.455, 1.053)	0.085	0.448 (0.101, 1.996)	0.292
IAT as adjunct or rescue therapy to MT								
Adjunct	1.650 (0.808, 3.368)	0.169	2.458 (0.603, 10.014)	0.209	0.532 (0.234, 1.209)	0.132	0.229 (0.051, 1.019)	0.053
Rescue	1.284 (0.896, 1.840)	0.173	1.436 (0.725, 2.844)	0.299	0.762 (0.493, 1.176)	0.219	2.242 (0.582, 8.639)	0.241

subsets due to the lack of data. The occlusion location of vessels and the door-to-needle time or onset-to-recanalization time might also influence the outcomes, but fewer studies have reported detailed data on the specific patients' prognoses.

IAT had potential efficacy as adjunctive therapy to MT and could not be discarded. The evaluation of other currently used thrombolytics such as urokinase and tirofiban is also necessary. But present data about intra-arterial thrombolysis for AIS management

TABLE 3 A summary table for risk of bias item assessed by MINORS scale of each study.

Item	Lin et al. (2009)	Heiferman et al. (2017)	Anadani et al. (2019)	Yi et al. (2018)	Zaidi et al. (2019)	Zaidi et al. (2021)
1. A clearly stated aim	2	2	2	2	2	2
2. Inclusion of consecutive patients	2	2	2	2	2	2
3. Prospective collection of data	0	2	2	0	0	2
4. Endpoints appropriate to the aim of the study	2	2	2	2	2	2
5. Unbiased assessment of the study endpoint	1	0	0	0	0	0
6. Follow-up period appropriate to the aim of the study	2	2	2	2	2	2
7. Loss to follow up less than 5%	2	2	2	2	1	1
8. Prospective calculation of the study size	0	0	0	0	0	0
9. An adequate control group	2	2	2	2	2	2
10. Contemporary groups	2	2	2	2	2	2
11. Baseline equivalence of groups	2	2	2	2	2	2
12. Adequate statistical analysis	2	2	2	2	2	2
13. Total points	19	20	20	18	17	19

The items are scored 0 (not reported), 1 (reported but inadequate), or 2 (reported and adequate). The global ideal scores are 16 for non-comparative studies and 24 for comparative studies.

were still mainly derived from observational studies. More RCTs are needed to obtain higher-quality evidence for the administration of intra-arterial thrombolysis. We are looking forward to the results from ongoing research, for example, the TECNO trial (NCT05499832) aiming at assessing safety and efficacy of intra-arterial Tenecteplase for noncomplete reperfusion of intracranial occlusions.

Conclusion

Compared to MT alone, MT with intra-arterial alteplase did not improve the recanalization rate but provided better functional outcomes. The intervention did not increase hemorrhage or mortality risk. Thus, MT with intra-arterial alteplase could be a potential therapy for AIS-LVO.

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 authors.

Author contributions

ZhW was the principal investigator. ZC designed the study protocol. XY, ZiW, and HC searched the databases, screened the studies, and analyzed the data. YQ, HT, and GC revised the manuscript and polished the language. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2023.1137543/full#supplementary-material

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Glossary

AIS	Acute ischemic stroke
CHOICE	Chemical Optimization of Cerebral Embolectomy
IAT	Intra-arterial thrombolysis
IMS	Interventional Management of Stroke
IVT	Intravenous thrombolysis
LVO	Large vessel occlusion
MINORS	Methodological index for non-randomized studies
MR CLEAN	Multicenter Randomized Clinical trial of Endovascular treatment for Acute ischemic stroke in the Netherlands
mRS	modified Rankin Scale
mTICI	modified Thrombolysis In Cerebral Infarction
MT	Mechanical thrombectomy
NIHSS	National Institute of Health stroke scale
PH-2	Parenchymal hemorrhage type 2
PROACT	Pyrolyse in Acute Cerebral Thromboembolism
RCT	Randomized clinical trial
rtPA	Recombinant tissue plasminogen activator
sICH	Symptomatic intracerebral hemorrhage

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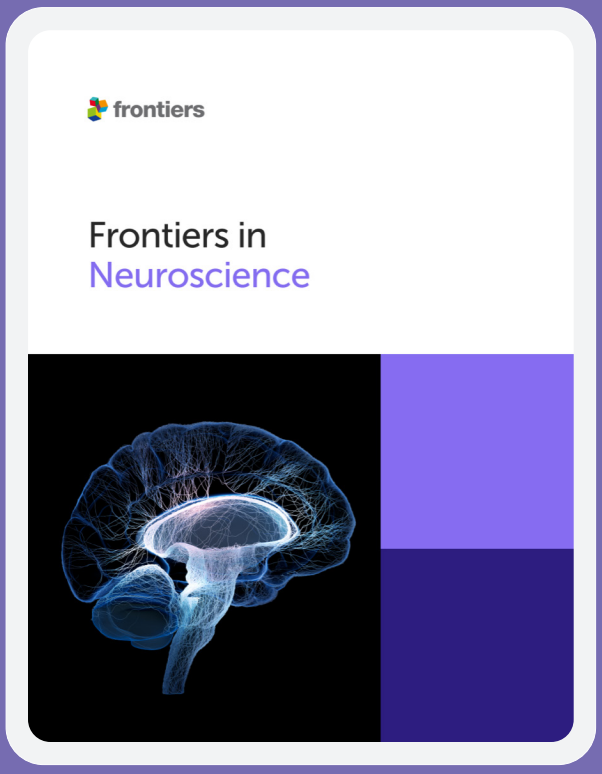

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