

Research Paper

GSK-3 β as a target for protection against transient cerebral ischemia

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Abstract

Stroke remains the leading cause of death and disability worldwide. This fact highlights the need to search for potential drug targets that can reduce stroke-related brain damage. We showed recently that a glycogen synthase kinase-3 β (GSK-3 β) inhibitor attenuates tissue plasminogen activator-induced hemorrhagic transformation after permanent focal cerebral ischemia. Here, we examined whether GSK-3 β inhibition mitigates early ischemia-reperfusion stroke injury and investigated its potential mechanism of action. We used the rat middle cerebral artery occlusion (MCAO) model to mimic transient cerebral ischemia. At 3.5 h after MCAO, cerebral blood flow was restored, and rats were administered DMSO (vehicle, 1% in saline) or GSK-3 β inhibitor TWS119 (30 mg/kg) by intraperitoneal injection. Animals were sacrificed 24 h after MCAO. TWS119 treatment reduced neurologic deficits, brain edema, infarct volume, and blood-brain barrier permeability compared with those in the vehicle group. TWS119 treatment also increased the protein expression of β -catenin and zonula occludens-1 but decreased β -catenin phosphorylation while suppressing the expression of GSK-3 β . These results indicate that GSK-3 β inhibition protects the blood-brain barrier and attenuates early ischemia-reperfusion stroke injury. This protection may be related to early activation of the Wnt/ β -catenin signaling pathway.

Key words: blood-brain barrier; ischemic stroke; Wnt/ β -catenin signaling; TWS119

Introduction

Stroke is a leading cause of death and disability worldwide. Thus, identifying potential new drug targets is critical to developing therapies that will reduce stroke-related brain damage. It is known that early disruption of the blood-brain barrier (BBB) contributes to acute cerebral ischemia-reperfusion injury¹ In recent years, new strategies, such as delayed inhibition of VEGF signaling², and new drug candidates, such as a novel analog of ginkgolide B (XQ-1H)³ a novel adhesion molecule CEACAM1⁴,

orosomucoid⁵, and *lavandula officinalis* ethanolic extract⁶, have been tested for their potential to protect the BBB in ischemic stroke models. Because the results from these studies have been promising, increasing attention is turning toward the identification of potential drug targets to protect the BBB after ischemic stroke.

The Wnt/ β -catenin signaling pathway is involved in development of the BBB⁷, and its dysfunction could lead to BBB breakdown in

Alzheimer's disease⁸. Furthermore, activation of Wnt/ β -catenin signaling enhances neurogenesis and improves neurologic function after focal ischemic injury⁹. However, the role of the Wnt/ β -catenin signaling pathway in BBB breakdown and its effects on stroke outcomes are unknown.

Studies have shown that the serine-threonine kinase glycogen synthase kinase-3 β (GSK-3 β) is involved in the phosphorylation and degradation of β -catenin¹⁰, the key molecule of the Wnt/ β -catenin pathway¹¹. Therefore, inhibition of GSK-3 β may increase the level of β -catenin and further activate the Wnt/ β -catenin signaling pathway. TWS119, a 4,6-disubstituted pyrrolo-pyrimidine, is a potent inhibitor of GSK-3 β ¹². We have recently shown that TWS119 attenuates tissue plasminogen activator (tPA)-induced hemorrhagic transformation after permanent middle cerebral artery occlusion (MCAO)¹³.

In the present study, we used a rat model of transient MCAO to mimic the clinical scenario of acute ischemic stroke, and administered TWS119 to activate the Wnt/ β -catenin signaling pathway. We hypothesized that GSK-3 β inhibition would protect the BBB and reduce early ischemia-reperfusion stroke injury.

Materials and Methods

Animals

All protocols used in this study were approved by the Institutional Animal Care and Use Committee at Wuhan University. Adult male Sprague-Dawley rats weighing 250–280 g were purchased from Wuhan University Center for Animal Experiments and housed under standard conditions with a 12:12 h light/dark cycle. Food and water were provided to all animals ad libitum. The operators were blinded to the treatment status of the animals in all experiments.

MCAO Model

Focal cerebral ischemia was produced by endovascular occlusion of the left middle cerebral artery (MCA) as described previously^{14–16}. Briefly, rats were anesthetized by intraperitoneal injection with pentobarbital sodium (Dainippon Sumitomo Pharma, Osaka, Japan). Body temperature was maintained at 36.5°C to 37.5°C throughout surgery. After a midline neck incision, the left common carotid artery (CCA) was isolated under a microscope and ligated with a 4-0 silk suture (Ethicon, Issy-Les-Moulineaux, France). The external and internal carotid arteries were temporarily ligated with a 4-0 silk suture. An arteriotomy was performed proximal to the bifurcation of the CCA. A silicone-coated nylon monofilament (40 mm long, 0.26 mm diameter,

Beijing Sunbio Biotech, China) was introduced through the arteriotomy and advanced into the internal carotid artery up to a distance of 18–20 mm to occlude the origin of the MCA. At 3.5 hours after this procedure, the rats were reanesthetized, the nylon monofilament was withdrawn to restore MCA blood flow, and TWS119 (30 mg/kg in 1% dimethyl sulfoxide, Selleck, Houston, TX, USA)^{17, 18} or an equal volume of vehicle (DMSO) was administered. After surgery, the rats were returned to their home cages with free access to food and water. A third group of sham control rats underwent the same surgical procedure but the monofilament was not inserted and they were administered only DMSO.

Neurologic Deficit Score

An investigator blinded to the experimental groups performed a neurologic examination of the rats at 24 h after MCAO using a modified version of the scoring system developed by Longa¹⁹: 0, no deficits; 1, difficulty in fully extending the contralateral forelimb; 2, unable to extend the contralateral forelimb; 3, mild circling to the contralateral side; 4, severe circling; 5, falling to the contralateral side. The higher the neurologic deficit score, the more severe the impairment of motor motion.

Brain Water Content

Brain water content was measured with the standard wet-dry ratio method²⁰. Rats were anesthetized and killed by decapitation at 24 h after MCAO. The brains were quickly removed and placed on a dry surface. A 4-mm-thick section of the frontal pole was dissected free before the brain was cut by a brain slicer (Beijing Sunny Instruments Co., Ltd., Beijing, China) and divided into the ipsilateral and contralateral hemispheres. The two hemisphere slices were packaged in preweighed aluminum foil and immediately weighed on an electronic balance to obtain the wet weight. The hemispheres were then dried for 24 h in an oven at 100°C and reweighed to obtain the dry weight. Brain water content was calculated as a percentage with the following formula: $(\text{wet weight} - \text{dry weight}) / \text{wet weight} \times 100\%$ ²¹

Brain Infarct Volume

Infarct volume after MCAO was determined by 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma, Santa Clara, CA) at 24 h after MCAO. Animals were euthanized, and the brains were quickly collected. Brain tissue was sliced into seven coronal sections (2-mm thick) and stained with a 2% solution of TTC at 37°C for 20 min, followed by fixation with 4% paraformaldehyde. TTC stains normal tissue red, but the infarct area remains a pale gray color. TTC-stained

sections were photographed, and Image-J image-processing software was used to calculate the infarct volume. The ratio of infarct volume = infarct area of the ipsilateral hemisphere/total area of the ipsilateral hemisphere $\times 100\%$ ²².

Evaluation of BBB Permeability

Evans blue (EB) dye (2%, 3 ml/kg, Sigma) was administered intravenously 3 h before the brain was collected. After being perfused with saline, each hemisphere was weighed and homogenized in 4 ml of 50% trichloroacetic acid solution. The homogenates were centrifuged at $10,000\times g$ for 30 min, and the supernatants were collected and diluted with ethanol (1:3). EB content was determined on a spectrophotometer (Epoch™&Take3™, Biotek, USA) at 620 nm and calculated from a standard curve of EB. EB extravasation was expressed as EB extravasation index (EBI): the ratio of absorbance intensity in the ischemic hemisphere to that in the nonischemic hemisphere²³.

Western Blotting

Based on our established protocol^{24, 25}, the total protein were prepared using Cytoplasmic and Nuclear Protein Extraction Kit (Feremants, Shanghai, China) and equal amounts of total protein were separated by Tris-glycine SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% skim milk and then incubated with the following primary antibodies overnight at 4°C: (1) rabbit polyclonal anti-GAPDH antibody (1:1000, Cell Signaling Technology, Danvers, MA, USA); (2) rabbit polyclonal anti- β -catenin antibody (1:5000, Abcam, Cambridge, MA, USA); (3) rabbit polyclonal anti-phosphorylated β -catenin (Ser 552) antibody (1:1000, Cell Signaling Technology, Cambridge, MA, USA); (4) mouse polyclonal anti-GSK-3 β antibody (1:1000, Cell Signaling Technology); (5) rabbit polyclonal anti-zonula occludens-1 (ZO-1) antibody (1:100, Santa Cruz Biotechnology, Dallas, TX, USA). After incubation with the secondary antibody (1:1000, Cell Signaling Technology) for 2 h, membranes were washed three times with phosphate-buffered saline (10 min each), and

the relative density of bands was analyzed on an Odyssey infrared scanner (LICOR Bioscience, Lincoln, NE, USA).

Statistical Analysis

All data are expressed as mean \pm SD. SPSS for Windows 16.0 software package was used to analyze the data. Differences among groups were determined by one-way ANOVA followed by Tukey post-hoc tests. Student's *t*-test was used to compare the difference between two groups. Differences were considered significant at *P* values less than 0.05.

Results

Mortality Rates

Mortality was 0% (0/24) in the sham group, 12.5% (3/24) in the vehicle group, and 4.2% (1/24) in the TWS119 group. Mortality rate was not significantly different among the three groups (*P* > 0.05).

GSK-3 β Inhibition by TWS119 Improved Neurologic Function, Cerebral Edema, and Infarction Volume After Transient MCAO

Neurologic deficit, brain water content, and infarct volume were evaluated 24 h after MCAO. TWS119 significantly reduced the neurologic deficit scores compared with those in the vehicle group (*n*=24 rats/group, *P* < 0.05; Fig. 1.A). No neurologic deficit was observed in the sham group (data not shown). Similarly, brain water content was lower and infarct volume was smaller in the TWS119-treated group than in the vehicle-treated group (*n*=6 rats/group, both *P*<0.05; Figs. 1.B and 2), suggesting that GSK-3 β inhibition attenuates brain edema and infarct volume after transient MCAO. The sham group exhibited no cerebral edema or infarction.

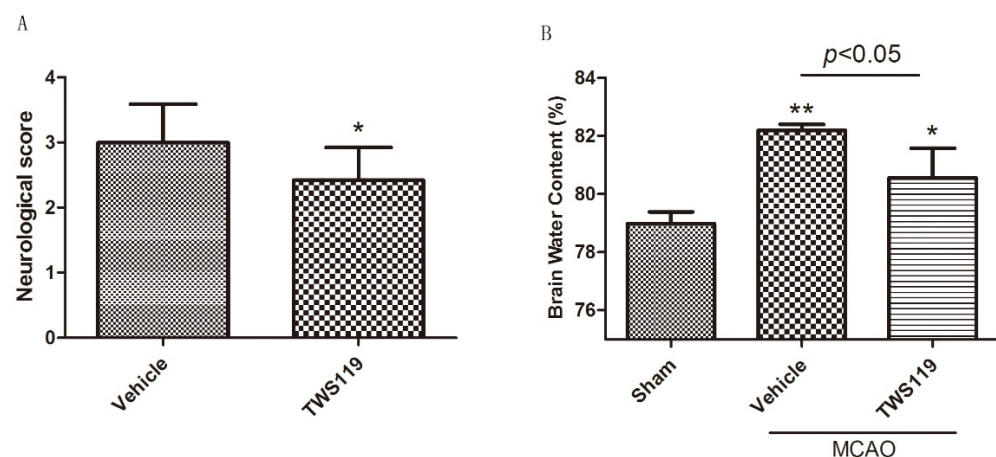


Figure 1. A. Neurologic deficit score at 24 h after transient middle cerebral artery occlusion (MCAO). Rats were treated with vehicle or TWS119 (*n*=24 rats/group). The data are expressed as means \pm SD. **P*<0.05 vs. vehicle group. **B.** Brain water content at 24 h after transient middle cerebral artery occlusion (MCAO). The data are expressed as means \pm SD. **P*<0.05, ***P*<0.01 vs. sham group.

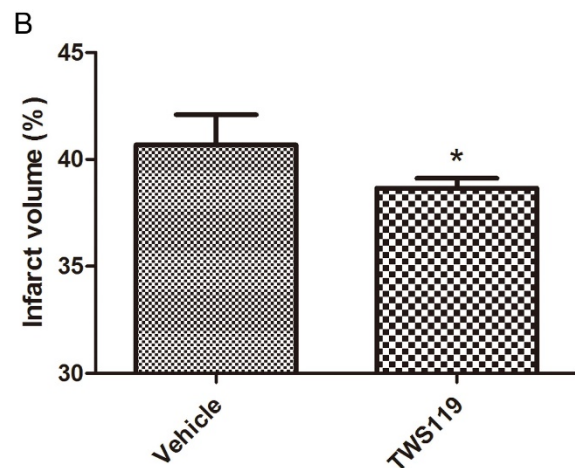
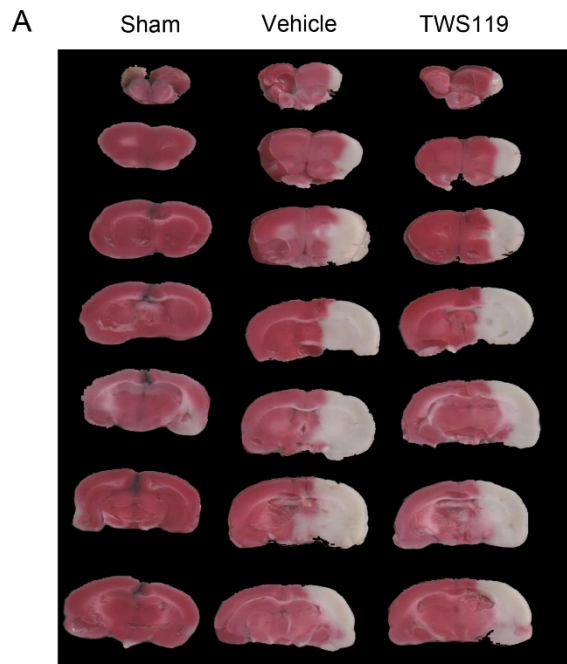


Figure 2. Infarct volume at 24 h after transient middle cerebral artery occlusion (MCAO). Rats were treated with vehicle or TWS119 (n=6 rats/group). **A.** Representative TTC-stained, 2-mm coronal brain sections. **B.** Quantification of the infarct volume in groups that underwent MCAO and treatment with vehicle or TWS119. Infarct volume was calculated as a percentage as follows: infarct area of the ipsilateral hemisphere/total area of the ipsilateral hemisphere \times 100%. No infarction was detected in the sham group. The results are expressed as means \pm SD. * $P < 0.05$ vs. vehicle group.

GSK-3 β Inhibition by TWS119 Decreased BBB Permeability After Transient MCAO

In our study, EB dye leakage was concentrated mainly in the regions of ischemic hemisphere, and BBB leakage was shown with the EBI. We found that EBI of the TWS119-treated group was significantly lower than that of the vehicle-treated group (n=6 rats/group, $P < 0.05$; Fig. 3), indicating that GSK-3 β inhibition protects the BBB after transient MCAO.

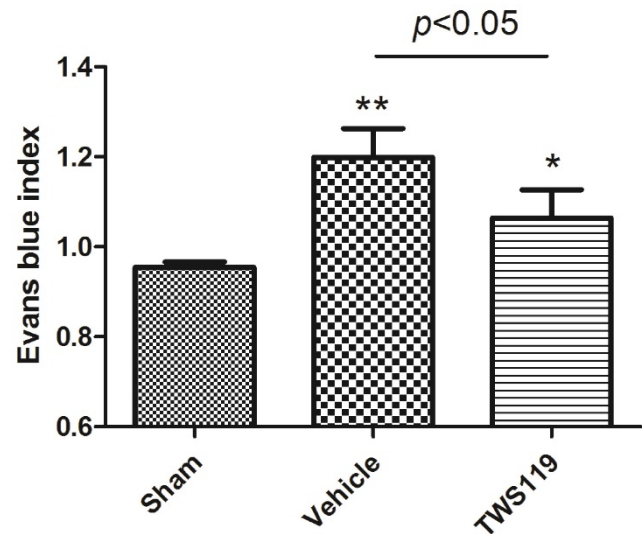


Figure 3. Blood-brain barrier permeability as measured by Evans blue leakage at 24 h after transient middle cerebral artery occlusion (MCAO). Rats were treated with vehicle or TWS119 (n=6 rats/group). The Evans blue index (ratio of absorbance intensity in the ischemic hemisphere to that in the nonischemic hemisphere) was used to evaluate blood-brain barrier permeability. The results are expressed as means \pm SD. * $P < 0.05$, ** $P < 0.01$ vs. sham group.

GSK-3 β Inhibition by TWS119 Activated the Wnt/ β -Catenin Signaling Pathway and Increased the Expression of ZO-1 After Transient MCAO

Western blot quantification showed that the total protein expression of β -catenin was upregulated, whereas phosphorylated β -catenin (p- β -catenin) and GSK-3 β expression were downregulated in the TWS119-treated group compared with that in the vehicle-treated group (n=6 rats/group, $P < 0.05$; Fig. 4). And the ratio of p- β -catenin to the total β -catenin was reduced significantly after TWS119 treatment (n=6 rats/group, $P < 0.05$; Fig. 4). Additionally, the expression of ZO-1 was significantly higher in the TWS119-treated group than in the vehicle-treated group (n=6 rats/group, $P < 0.05$; Fig. 4). These data indicate that GSK-3 β inhibition by TWS119 might activate the Wnt/ β -catenin signaling pathway, which is associated with increased ZO-1 expression and attenuated BBB disruption.

Discussion

In this study, GSK-3 β inhibition by TWS119 reduced neurologic deficit score, brain edema, infarct volume, and BBB disruption. This protection was associated with an early activation of Wnt/ β -catenin signaling and an increase in ZO-1 expression. Together, these findings suggest that GSK-3 β inhibition by TWS119 attenuates early ischemia-reperfusion injury and protects the BBB.

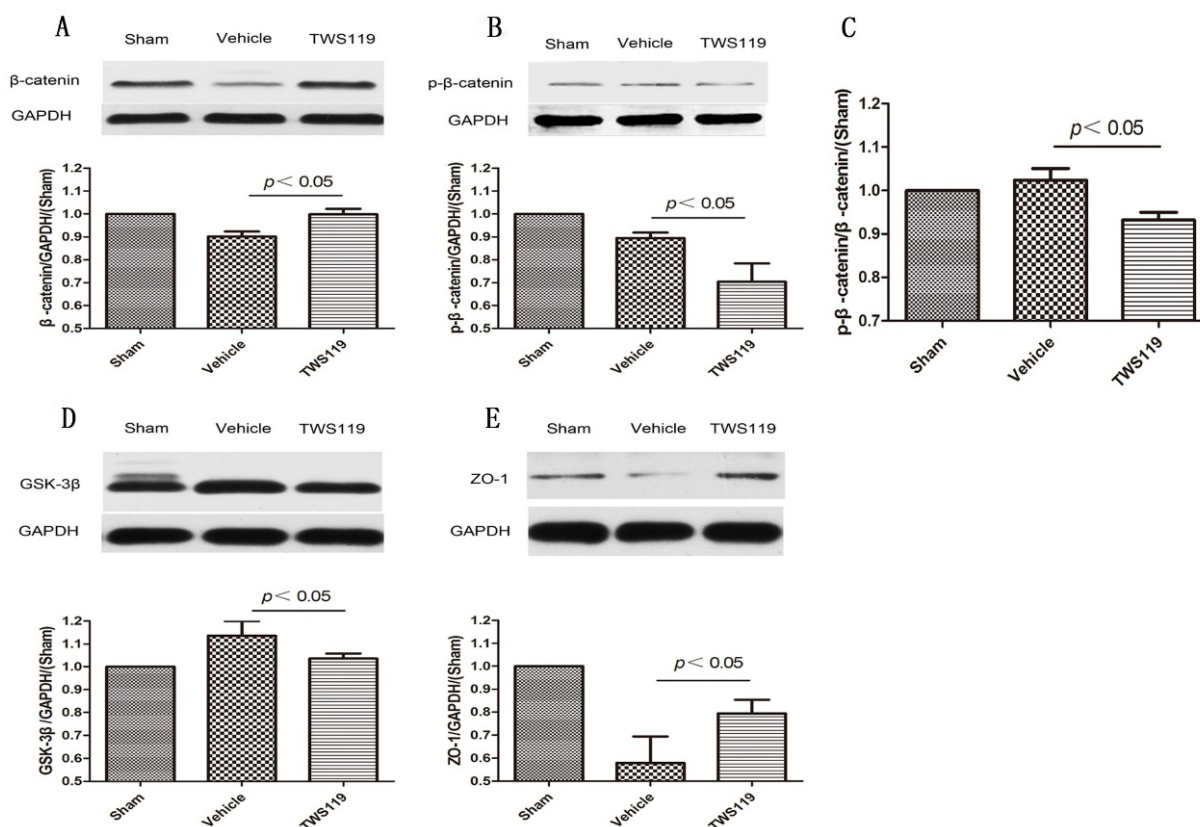


Figure 4. Western blot analysis of proteins in the Wnt/ β -catenin signaling pathway and tight junctions of the blood-brain barrier 24 h after transient MCAO (n=6 rats/group). Representative Western blots and densitometric analysis of (A) total β -catenin, (B) phosphorylated β -catenin, (C) The ratio of p- β -catenin to the total β -catenin, (D) GSK-3 β and (E) zonula occludens-1 (ZO-1). GAPDH was used as a loading control. The expression of β -catenin and ZO-1 increased in the TWS119-treated group compared to that in the vehicle-treated group. TWS119 treatment reduced the expression of phosphorylated β -catenin and GSK-3 β . Data are representative of at least three independent experiments. The results are expressed as means \pm SD.

Ischemic stroke remains a significant health problem worldwide²⁶. Previous studies have shown that thioredoxin²⁷, anfibatide²⁸, and inhibition of CD36-mediated inflammation²⁹ mitigate ischemic stroke injury. Recently, Luo et al.³⁰ demonstrated that interleukin-33 ameliorates ischemic brain injury by promoting Th2 response and suppressing Th17 response. Nicotinamide mononucleotide adenylyltransferase 1 is another molecule that has exhibited potential neuroprotective effects *in vitro* and *in vivo* models of cerebral ischemia³¹. We have recently shown that GSK-3 β inhibitor TWS119 with reported dose and treatment regimen¹⁷ attenuates tPA-induced hemorrhagic transformation after permanent focal cerebral ischemia¹⁸. Here, we showed that TWS119 reduced early ischemia-reperfusion injury and protected the BBB.

Although many efforts have been made to attenuate ischemic stroke injury, there is no effective therapy that can be used clinically to protect the BBB. The BBB is a key factor that affects acute stroke outcome¹² and should be targeted for protection. The European Cooperative Acute Stroke Study (ECASS)-3 has shown that rtPA alteplase has a safe therapeutic

window of 3–4.5 h after the onset of stroke symptoms³². Therefore, in our study, we administered TWS119 at 3.5 h after MCAO and found that it reduced the permeability of the BBB. This finding suggests that TWS119 has a protective effect on the BBB after ischemic stroke, and GSK-3 β could be a potential target for brain protection, which was consistent with other studies^{33,34}.

Previous studies have demonstrated the role of the Wnt/ β -catenin signaling pathway in the brain's vascular development and BBB formation³⁵. Dickkopf-1, a negative modulator of the Wnt pathway, is involved in the development of ischemic neuronal death³⁶. Growing evidence indicates that Wnt signaling protects the BBB in several brain diseases³⁷. However, the relationship between Wnt/ β -catenin signaling and BBB integrity in acute ischemic stroke is still unclear.

To elucidate the underlying mechanisms of action of TWS119, we examined the expression of key proteins in the Wnt/ β -catenin pathway as well as tight junction protein ZO-1 at 24 h after MCAO. While suppressing the expression of GSK-3 β , TWS119 inhibited phosphorylation of β -catenin and increased

its total protein expression³⁸. β -catenin is a key component for activating the Wnt/ β -catenin pathway³⁹, and phosphorylation at Ser552 has been shown to induce beta-catenin accumulation in the nucleus and increases its transcriptional activity. However, other studies found that the Ser-552 phosphorylation site did not seem important for modulation of β -catenin transcriptional activity^{40,41}. In our study, we found that β -catenin was upregulated, whereas GSK-3 β and p- β -catenin were downregulated in the TWS119-treated group compared with levels in the vehicle-treated group, indicating that TWS119 may activate the Wnt/ β -catenin pathway by inhibiting GSK-3 β expression, reducing p- β -catenin, and enhancing β -catenin expression. The tight junction protein ZO-1 participates in the maintenance of BBB integrity and is the major transmembranal protein of tight junctions in the BBB⁴². ZO-1 controls angiogenesis and barrier formation⁴³. However, whether Wnt/ β -catenin signaling can regulate ZO-1 expression after BBB disruption in ischemic stroke is unknown. Our results showed that in the TWS119-treated group, a reduction in BBB permeability was associated with activation of Wnt/ β -catenin signaling and upregulation of ZO-1 expression. The sequence of the signaling may need additional research in both in vitro and in vivo models of the BBB.

In summary, our study showed that GSK-3 β inhibition with TWS119 protected the BBB and attenuated early ischemia-reperfusion stroke injury. This protection may be related to an early activation of the Wnt/ β -catenin signaling pathway and an increase in ZO-1 expression. Thus, the activation of Wnt/ β -catenin signaling has potential to protect the BBB after ischemic stroke. However, the specific mechanism needs additional research.

Acknowledgments

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Competing Interests

The authors have declared that no competing interest exists.

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