

Original Article

Xyloketal B alleviates cerebral infarction and neurologic deficits in a mouse stroke model by suppressing the ROS/TLR4/NF- κ B inflammatory signaling pathway

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Abstract

Xyloketal B (Xyl-B) is a novel marine compound isolated from mangrove fungus *Xylaria* sp. We previously demonstrated that pretreatment with Xyl-B exerted neuroprotective effects and attenuated hypoxic-ischemic brain injury in neonatal mice. In the present study we investigated the neuroprotective effects of pre- and post-treatment with Xyl-B in adult mice using a transient middle cerebral artery occlusion (tMCAO) model, and explored the underlying mechanisms. Adult male C57 mice were subjected to tMCAO surgery. For the pre-treatment, Xyl-B was given via multiple injections (12.5, 25, and 50 mg·kg⁻¹·d⁻¹, ip) 48 h, 24 h and 30 min before ischemia. For the post-treatment, a single dose of Xyl-B (50 mg/kg, ip) was injected at 0, 1 or 2 h after the onset of ischemia. The regional cerebral perfusion was monitored using a laser-Doppler flowmeter. TTC staining was performed to determine the brain infarction volume. We found that both pre-treatment with Xyl-B (50 mg/kg) and post-treatment with Xyl-B (50 mg/kg) significantly reduced the infarct volume, but had no significant hemodynamic effects. Treatment with Xyl-B also significantly alleviated the neurological deficits in tMCAO mice. Furthermore, treatment with Xyl-B significantly attenuated ROS overproduction in brain tissues; increased the MnSOD protein levels, suppressed TLR4, NF- κ B and iNOS protein levels; and downregulated the mRNA levels of proinflammatory cytokines, including IL-1 β , TNF- α , IL-6 and IFN- γ . Moreover, Xyl-B also protected blood-brain barrier integrity in tMCAO mice. In conclusion, Xyl-B administered within 2 h after the onset of stroke effectively protects against focal cerebral ischemia; the underlying mechanism may be related to suppressing the ROS/TLR4/NF- κ B inflammatory signaling pathway.

Keywords: xyloketal B; stroke; ischemic brain injury; middle cerebral artery occlusion; ROS; TLR4; NF- κ B

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Introduction

Stroke is one of the most common causes of death and disabilities worldwide^[1]. The mechanisms involved in the pathophysiology of strokes are not fully understood, and thus, there is a lack of effective pharmacological therapeutic drugs. The

only drug approved by the Food and Drug Administration (FDA) is tissue-plasminogen activator (tPA)^[2]. However, tPA displays certain severe side effects and has a very limited time window, which renders it applicable only in less than 10% of all stroke patients^[3]. Thus, there is an urgent need for new therapeutic drugs.

There is increasing evidence demonstrating that inflammation appears to play an important role in the pathogenesis of ischemic strokes and other forms of neurodegenerative brain injuries. Stroke patients who suffer from inflammation are closely associated with poorer clinical outcomes^[2]. Experimentally, there was a time-dependent recruitment and activation of inflammatory cells in focal cerebral ischemic strokes, while inhibiting the inflammatory response decreased the infarct volume and improved the neurological deficit^[4]. Toll-like receptors (TLRs) belong to the transmembrane pattern-recognition receptor family and play important roles in the induction and regulation of immune/inflammatory responses. Toll-like receptor 4 (TLR4), but not TLR3 or TLR9, plays a key role in ischemic strokes by activating nuclear factor kappa beta (NF- κ B) and, thus, inducing the excessive generation of inflammatory factors^[5]. The inhibition of the TLR4-NF- κ B signaling pathway protects the brain from neurodegeneration in MCAO rats^[6]. NF- κ B belongs to a family of transcription factors, consisting of five subunits (p50, RelA/p65, c-Rel, RelB and p52), that form homo- or hetero-dimers. The activation of TLR4 leads to the nuclear translocation of NF- κ B^[5] and regulates the transcription of genes related to inflammation, such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6). Moreover, following cerebral ischemia, NF- κ B rapidly increases in neurons and glial cells^[7]. Hence, the downregulation of the TLR4-NF- κ B signaling pathway could reduce the production of inflammatory factors and, thus, serve as a potential drug target for the treatment of strokes.

Xylometazoline B (Xyl-B) is a novel marine compound that is isolated from mangrove fungus^[8]. We first reported the protective effects of Xyl-B on an oxLDL (oxidatively modified low density lipoprotein)-induced endothelial injury^[9]. Furthermore, Xyl-B was shown to have multiple biomedical activities, including neuroprotective effects against toxicity, antioxidant effects on endothelial cells and zebrafish, anti-glioma effects through the inhibition of TRPM7 and the reduction of atherosclerosis plaque formation in apolipoprotein E-deficiency mice^[10-16]. Our previous study also demonstrated that Xyl-B protected against neonatal brain injuries induced by hypoxia-ischemia^[17]. In this study, we further used the tMCAO adult mice model to investigate the neuroprotective effects of Xyl-B in adult strokes and explored its potential mechanisms, including alterations in the TLR4/NF- κ B signaling pathway, inflammatory response and ROS generation.

Materials and methods

tMCAO (transient middle cerebral artery occlusion) mouse model
Adult male C57 mice, weighing 20-25 g, were used in this study. A transient middle cerebral artery occlusion (tMCAO)

was performed. First, the mice were anesthetized with sodium pentobarbital (Merck, New Zealand, America) at 60 mg/kg (ip). During the entire tMCAO surgery, the core body temperatures of the mice were maintained at 37°C using a heating pad (RWD Life Science, Shenzhen, China). The left common carotid artery and external and internal carotid arteries were separated, and then, a silicone-coated 6-0 suture (Doccol, USA) was gently inserted via the lumen of external and the internal carotid artery to embed into the cerebral artery for occluding the middle cerebral artery (MCA). The distance between the bifurcation of the internal and the external carotid arteries to the middle cerebral artery was 10 \pm 0.5 mm. After 2 h of occlusion, the suture was removed to allow reperfusion. Laser Doppler Flowmetry (PeriCam Psi-Zr, Sweden) was employed to verify that the occlusion was successful (reduction of at least 75% from the baseline readings). Sham-operated mice underwent the same procedure, except the suture was inserted along the internal carotid artery and then immediately withdrawn. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All animal handling and surgery procedures were in accordance with the ethical standards of the Institutional Animal Care and Use Committee at Sun Yat-sen University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Administration of Xyl-B

Xyl-B was dissolved in 15% propylene glycol saline (used as a vehicle control) to an injection concentration of 3 mg/mL, as used in our previous study^[15]. As shown in the schematic illustration of the experimental procedures in Figure 1, for the preventive treatments, multiple intraperitoneal injections of Xyl-B (12.5, 25 and 50 mg \cdot kg⁻¹ \cdot d⁻¹) were given at 48 h, 24 h and 30 min before the onset of the tMCAO, and the first injection was given 48 h before the onset of the tMCAO. To determine the therapeutic window of Xyl-B against a stroke, a single dose of Xyl-B (50 mg/kg, ip) was administered 0 h, 1 h and 2 h after the onset of ischemia (Figure 1).

Hemodynamics of the regional cerebral perfusion

The regional cerebral perfusion was monitored using a laser-Doppler flowmeter (PeriCamPsi-Zr, Sweden). After removing the scalp, a flowmeter probe was placed on the dura and kept away from the visible cerebral vessels. The steady-state baseline values were recorded before the tMCAO. The regional cerebral perfusion was monitored continuously until a steady state was achieved after the ischemia or reperfusion. The data were normalized to the baseline values and expressed as percentages of the baseline values.

TTC staining

TTC (2, 3, 5-triphenyltetrazolium chloride, Sigma) staining was performed as described in a previous study to determine the brain infarction volume^[17]. Briefly, the mice were rapidly sacrificed after 24 h of ischemia. The brains were isolated, frozen at -20°C for 30 min and cut into 2.0-mm-thick slices.

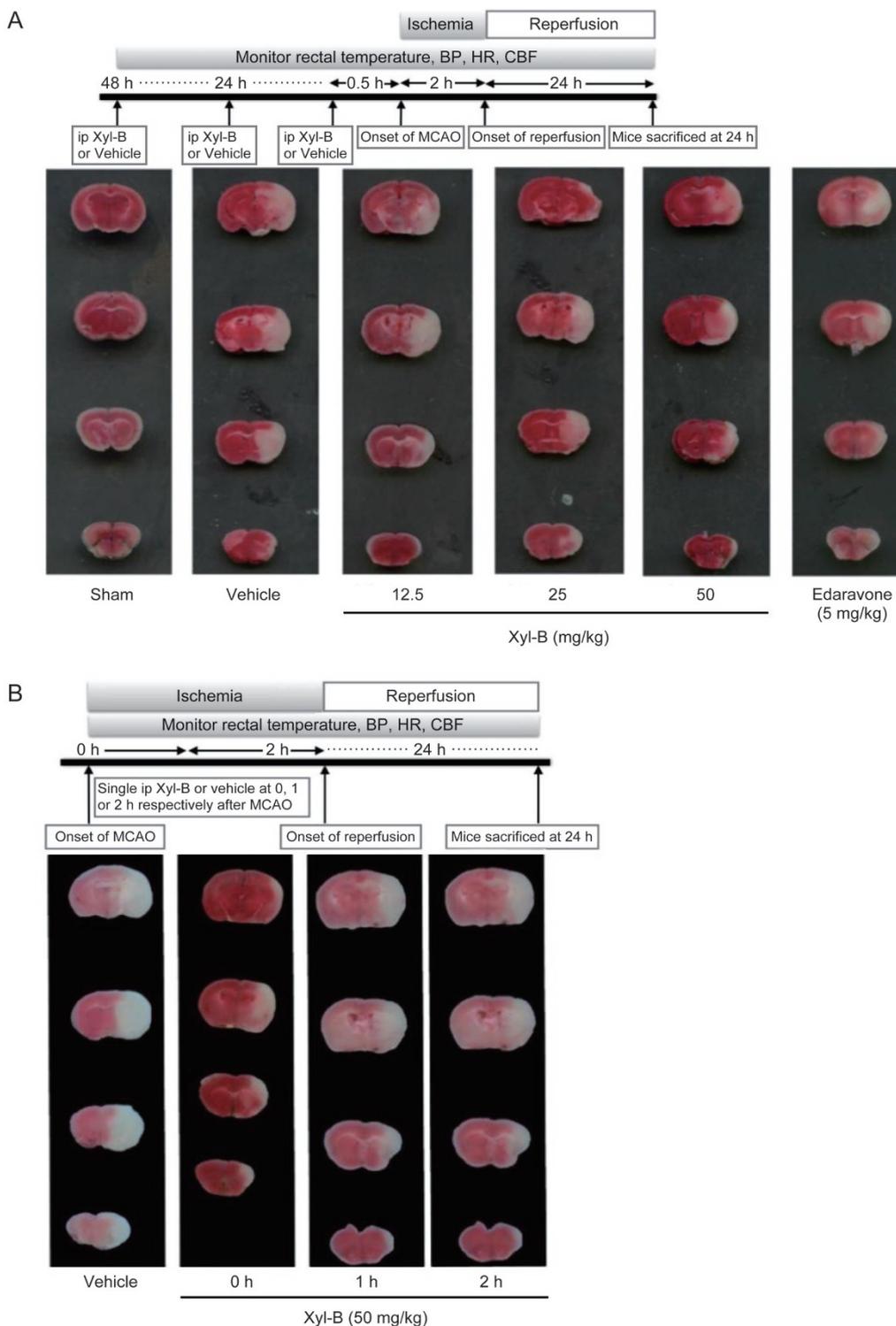


Figure 1. Xyl-B reduced infarct volume in the tMCAO mice. A schematic overview of the experimental procedure is shown in the upper panel. TTC staining was performed at 24 h after ischemia/reperfusion. (A) Pretreatment with multiple intraperitoneal injections of Xyl-B (12.5, 25 and 50 mg/kg) was given at 48 h, 24 h and 30 min before the onset of tMCAO. (B) A single intraperitoneal injection of Xyl-B at 50 mg/kg was given at 0 h, 1 h and 2 h after the onset of tMCAO. $n=7$ mice/group.

Coronal brain slices were stained with 2% TTC at 37°C for 10 min and then fixed in 4% paraformaldehyde for 6 h. Images of the stained slices were taken using a scanner. The infarct area

in each slice was measured using the software Image-Pro Plus 6.0 (Media Cybernetics, USA). The volumes of the infarctions were calculated using the following formula: Corrected infarct

volume (%)=[contralateral hemisphere volume-(ipsilateral hemisphere volume-infarct volume)]/contralateral hemisphere volume × 100%, as described in our previous study^[17].

Neurological deficiency assessment

The neurological status was assessed using a double-blind method according to the following 4-tiered grading system: 0 score, no observable deficits; 1 score, torso flexion to the right; 2 score, spontaneous circling to the right; 3 score, leaning/falling to the right; and 4 score, no spontaneous movement^[18,19].

Western blotting experiment

Western blotting was carried out as described in our previous studies^[9]. The cytoplasmic and nuclear extracts were carried out according to the manual of the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo, #78833). Sample protein (50 µg) from the ipsilateral hemisphere was separated on 8% or 12% SDS-PAGE gels, which were subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. Then, the membrane was immunoprobed with a TLR4 primary antibody (Santa Cruz; 1:200; sc-10741), NF-κB (p65) primary antibody (CST; 1:1000; #3031P), iNOS primary antibody (Abcam; 1:500; ab129372) and Lamin B (Santa Cruz; 1:500; sc-6216). The blots were incubated with an HRP-conjugated secondary antibody, and the signals were developed using an enhanced chemiluminescence substrate. The images of the chemiluminescence were captured with an imaging system (Bio-Rad, Hercules, and CA). The optical densities were normalized to those of β-actin, and the fold difference for each target protein was calculated as the ratio of the target protein expression/β-actin expression (ImageJ 1.42q software, U.S. National Institutes of Health).

DHE staining

The brains were cut into 8-µm-thick sections and placed on a glass slide; the tissues were processed and imaged in parallel. For this assay, the sections were dried for 30 min at 37°C, washed 3×5 min with PBS and then incubated with DHE (0.5 µmol/L) for 30 min at room temperature in the dark. The sections were washed thrice with ice-cold PBS, and then fluorescent oxyethidium was detected (excitation/emission at 485 nm/590 nm). All sections were analyzed using a confocal system (Olympus FV500-IX81). The fluorescence intensity was normalized to the control samples without the addition of DHE.

Immunohistochemistry

For the immunohistochemistry, mice were perfused via a cardiac puncture with 4% PFA, and then, the brains were collected and sequentially cut into 8 µm thick sections. The sections were heated in a tissue-drying oven for 45 min at 60°C, blocked with endogenous peroxidases for 15 min at room temperature, and washed 3×5 min with PBS. The sections were incubated for 30 min at 37°C with 5% BSA. The sections were covered with the NF-κB antibody, which was diluted in the blocking buffer by 1:200, overnight at 4°C. Then, the sections

were washed 3×5 min with PBS. The sections were covered with the secondary antibody for 30 min at 37°C and washed 3×5 min with PBS. The sections were stained with DAB for 30 s and counterstained with hematoxylin. Images were scanned using a confocal system (Olympus FV500-IX81) and analyzed by Image-Pro Plus 6.

Quantitative real-time PCR

The quantitative real-time PCR experiment was conducted as described previously^[20]. Briefly, the total RNA from the ischemic region was isolated using the TRIzol reagent (Invitrogen). The RNA concentration was determined by a spectrophotometer (NanoDrop 2000, Thermo, Wilmington, DE, USA). In total, 1 µg of total RNA was reverse transcribed and used for the SYBR Green-based real-time RT-PCR as previously described^[20]. The samples were run in triplicate with RNA preparations from three to five independent experiments. The QuantiTect primers are as follows: IL-1β, F/5'-AAATGCCACCTTTTGACAGTG-3', R/5'-GAGTGATACTGCCTGCCTGA-3'; IL-6, F/5'-GACAAAGC-CAGAGTCCTTCAGA-3', R/5'-GAGCATTGGAAATTGGGG-TAGG-3'; TNF-α, F/5'-TGCCTCAGCCTCTTCTCATT-3', R/5'-GGGCTTGCTACTCGAGTTTT-3'; IFN-γ, F/5'-GTATT-GCCAAGTTTGAGGTCAAC-3', R/5'-GCTTCCTGAGGCTG-GATTC-3'; and α-Tubulin, F/5'-TCTAACCCGTTGCTAT-CATGC-3', R/5'-GCCATGTTCCAGGCAGTAG-3'. The real-time PCR reactions were carried out on the IQ5 Real-time PCR Detection System (Bio-Rad, USA) in a volume of 20 µL, containing 10 µL FastStart Universal SYBR green master (Roche, USA) and 0.3 µm of each primer of the tested target genes. The reaction profile consisted of 40 cycles (qPCR condition: 95 °C for 15 s, 60 °C for 45 s) after the initial 10-min incubation at 95 °C. A dissociation stage was performed at the end of the reaction, consisting of 50 cycles of 10 s with the temperature increasing 0.5 °C/cycle to demonstrate the specificity of the amplification. The mRNA expression analysis was performed in triplicate for each sample. The fold change in the mRNA expression of each gene was determined by the 2^{-ΔΔCt} method using α-Tubulin mRNA as an internal control.

Evans blue extravasation

The Evans blue extravasation measurement was carried out according to our previous description^[18]. Briefly, 2 h after the onset of the ischemia, 2% Evans blue (4 mL/kg, Sigma) in normal saline was injected into the tail vein of the mouse. The ipsilateral and contralateral hemispheres were separated and homogenized in 1 mL of formamide at 37°C for 24 h. Then, the samples were centrifuged at 14000 rpm for 30 min. The Evans blue concentration in the supernatant was quantitatively determined by measuring the 630 nm absorbance.

Statistical analysis

All data are expressed as the mean±SEM. Unpaired Student's *t*-test was used to compare 2 groups. A one-way analysis of variance (ANOVA), followed by a Bonferroni multiple comparison post hoc test with a 95% CI, was used for 3 or more

groups. The statistical significance was evaluated by the analysis of variance using Prism 4 software (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Xyl-B reduced the infarct volume after focal ischemia

First, we determined the effect of the pretreatment with Xyl-B on brain infarction using TTC staining. As shown in Figure 1A, multiple intraperitoneal injections of different doses of Xyl-B (12.5, 25 and 50 mg/kg) were given 48 h, 24 h and 30 min before the ischemia. Pretreatment with multiple injections of Xyl-B significantly reduced the infarct volume in the tMCAO mice in a dose-dependent manner. In the sham group, the brain slices with TTC staining showed a uniform red color, indicating normal tissues. The infarct area in the brain slices from the tMCAO group showed a white color. Compared with the relative infarct volume of $26.2\% \pm 1.1\%$ in the vehicle-treated tMCAO group (Vehicle), the relative infarct volumes in the Xyl-B pretreatment groups were $24.5\% \pm 1.6\%$, $20.1\% \pm 1.1\%$ and $18.2\% \pm 1.9\%$ at the 3 different doses of Xyl-B (12.5, 25 and 50 mg/kg, respectively). The reduction in the infarct volume in the tMCAO groups that were pretreated with Xyl-B at 25 and 50 mg/kg/day was significantly different from that in the corresponding vehicle group (Table 1). Edaravone, which is a novel free-radical scavenger that prevents transient hypoxic-ischemic brain injury^[19], was used as a positive treatment control. The pretreatment with edaravone also significantly reduced the relative infarct volume in the tMCAO mice compared with that in the vehicle group.

Next, a single intraperitoneal injection of Xyl-B or vehicle at 50 mg/kg was given at 0, 1 or 2 h after the onset of the ischemia to detect its therapeutic window against a stroke. As shown in Figure 1B and summarized in table 2, a single dose of Xyl-B at 0, 1, or 2 h after the ischemia significantly reduced the infarct volume in the tMCAO mice, and the best therapeutic effect was displayed immediately after the tMCAO insult (0 h). Accordingly, the following experiments included the administration of a single dose of 50 mg/kg Xyl-B at 0 h after the onset of the ischemia (Xyl-B treatment). As shown in Figure 2 and table 3, after 2 h of occlusion and reperfusion, the Xyl-B treatment tended to increase the blood flow in the infarct area but did not reach a statistically significant difference ($P > 0.05$ vs vehicle-treated tMCAO group, $n = 6$). The blood flow in the contralateral hemisphere remains unchanged during and after the tMCAO ($P > 0.05$, vs before tMCAO, $n = 6$).

Xyl-B improved the neurological outcome

We assessed the effects of the Xyl-B treatment on the ischemia-induced neurological deficits using a 4-tiered scoring system^[19]. Although this neurological deficiency assessment is variable, it can indicate the successful establishment of the MCAO model^[18]. As shown in table 4, the Xyl-B treatment significantly improved the neurological outcome of the tMCAO mice compared with that of the vehicle group ($P < 0.05$ vs Vehicle controls, $n = 7$ mice/group). Consistently, the tMCAO

Table 1. Effects of Xyl-B pretreatment through multiple intraperitoneal injection on infarction volume of tMCAO mice. Data are expressed in mean \pm SEM. $n = 7$ mice/group. ** $P < 0.01$, vs vehicle controls.

Group	Relative infarction volume (%)
Vehicle	$26.2\% \pm 1.1\%$
Xyl-B (12.5 mg/kg, ip)	$24.5\% \pm 1.6\%$
Xyl-B (25 mg/kg)	$20.1\% \pm 1.1\%^{**}$
Xyl-B (50 mg/kg)	$18.2\% \pm 1.9\%^{**}$
Edaravone (5 mg/kg)	$18.1\% \pm 2.2\%^{**}$

Table 2. Effects of Xyl-B (50 mg/kg) post-treatment after ischemia through single intraperitoneal injection on infarction volume of tMCAO mice. Data are expressed in mean \pm SEM. ** $P < 0.01$, vs vehicle controls, $n = 7$ mice/group.

Group	Relative infarction volume (%)
Vehicle	$26.1\% \pm 0.9\%$
Xyl-B (0 h of ischemia)	$10.9\% \pm 1.2\%^{**}$
Xyl-B (1 h of ischemia)	$16.4\% \pm 1.5\%^{**}$
Xyl-B (2 h of ischemia)	$19.6\% \pm 1.3\%^{**}$

Table 3. Normalized regional cerebral perfusion (% of baseline) in different groups of mice in relation to the MCAO for 2 h with a single intraperitoneal of vehicle or Xyl-B (50 mg/kg) at the onset of ischemia. Data are expressed in mean \pm SEM. $n = 6$.

Group	Before MCAO	After MCAO	After reperfusion
Vehicle	100 ± 2.5	35.5 ± 3.8	77.8 ± 16.3
Xyl-B	100 ± 2.0	32.8 ± 5.9	83.3 ± 10.7
tMCAO contralateral	100 ± 3.5	98.4 ± 6.8	100.8 ± 6.7

Table 4. Effects of Xyl-B and edaravone on neurological deficiency assessed by a 4-tiered grading system.

Animal No	Vehicle	Xyl-B (50 mg/kg)	Edaravone (5 mg/kg)
#1	3	1	1
#2	3	1	1
#3	2	2	2
#4	2	2	2
#5	3	2	2
#6	3	1	1
#7	2	2	1

Xyl-B treatment significantly improved neurological outcome of tMCAO mice compared with the vehicle group ($P < 0.05$ vs vehicle controls, $n = 7$ mice/group). Consistently, tMCAO mice treated with edaravone also displayed significant neuroprotective effects compared with vehicle control group ($P < 0.05$ vs vehicle controls, $n = 7$ mice/group).

mice that were treated with edaravone also displayed significant neuroprotective effects compared with the vehicle control

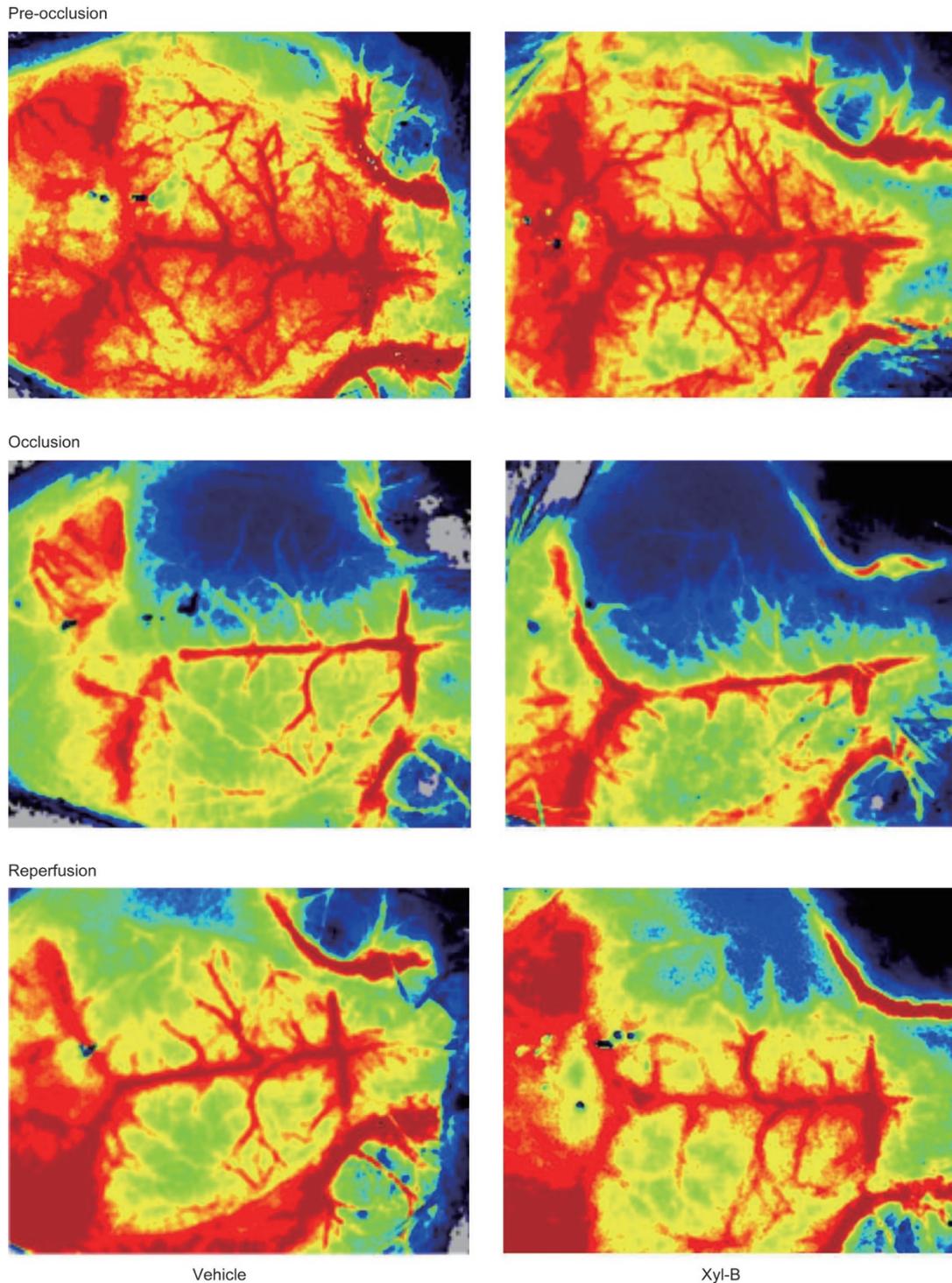


Figure 2. Effects of Xyl-B treatment on brain hemodynamics. Representative images of the hemodynamics are shown ($n=6$ mice/group).

group ($P<0.05$ vs Vehicle controls, $n=7$ mice/group). There was no significant difference in the neurological outcome between the Xyl-B and edaravone groups ($P>0.05$).

Xyl-B decreased the ROS level and increased the SOD2 level
Reactive oxygen species (ROS) and reactive nitrogen species

(RNS), such as nitric oxide (NO), play a key role in the pathophysiological response of the brain after an ischemia injury^[20]. Here, we detected the total ROS levels by DHE staining. As shown in Figure 3A, the total ROS level in the brain tissues from the Xyl-B-treated tMCAO mice decreased compared with that in brain tissues from vehicle-treated tMCAO mice.

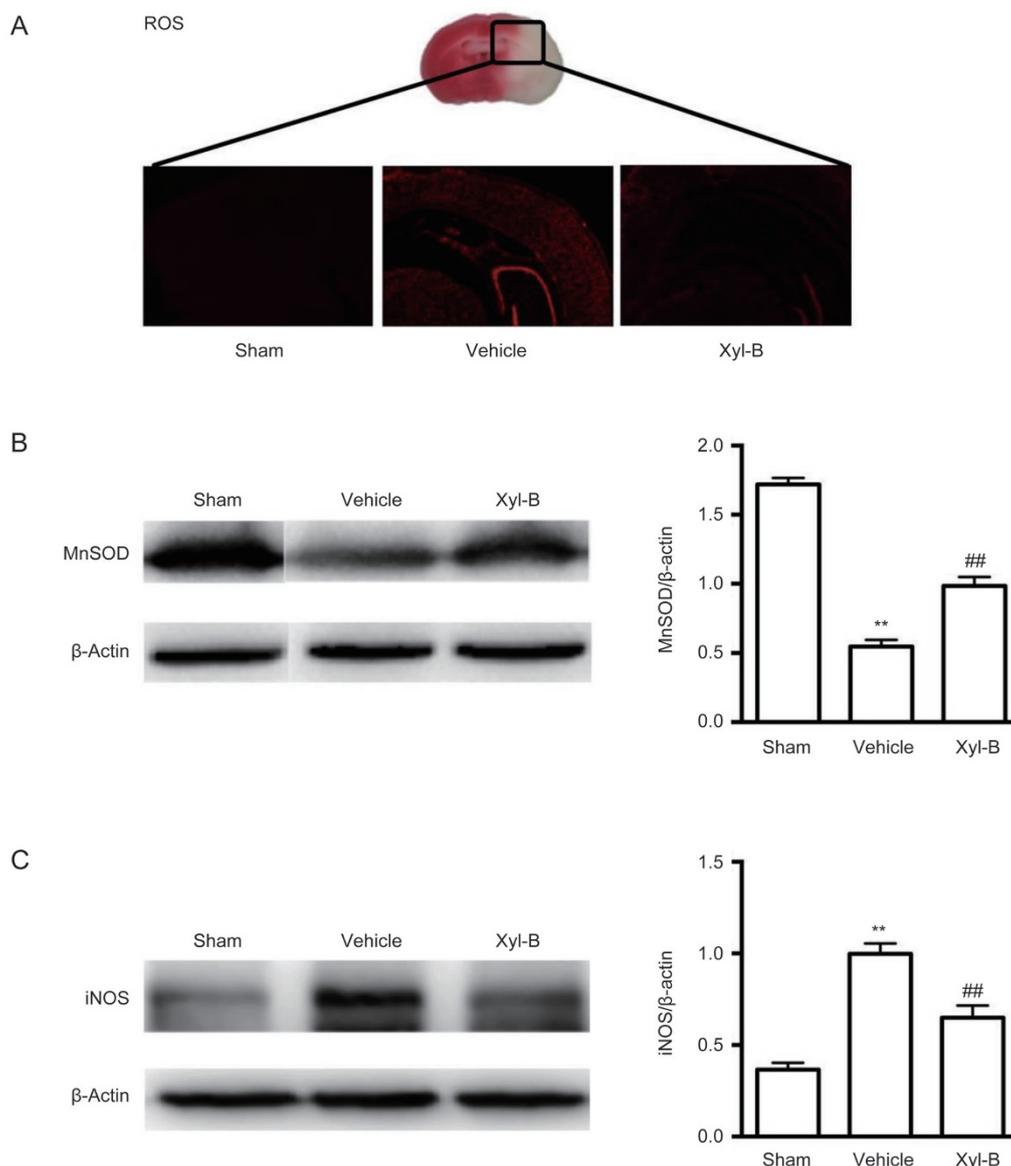


Figure 3. Xyl-B treatment reduced ROS generation and iNOS protein expression and increased MnSOD protein expression. (A) Representative images of brain tissues stained with DHE dye ($\times 40$), a ROS indicator. Xyl-B treatment refers to a single dose of 50 mg/kg Xyl-B given immediately after acute ischemia. The fluorescence intensity of DHE in the ischemic brain with Xyl-B (50 mg/kg) treatment immediately after tMCAO decreased compared with that in the vehicle group ($n=6$ mice /group). Western blot analyses of MnSOD (B) and iNOS (C) were performed in the brain tissues from the sham-operated mice, vehicle-treated mice and Xyl-B-treated tMCAO mice. ** $P<0.01$ vs sham-operated controls. ## $P<0.01$ vs vehicle controls, $n=6$ mice/group.

To investigate whether Xyl-B affects endogenous antioxidant enzymes, we examined the expression of manganese superoxide dismutase (MnSOD or SOD2). As shown in Figure 3B, the Xyl-B treatment markedly enhanced the protein levels of MnSOD in the brain tissues that were reduced by the tMCAO.

Excessive NO in a brain with an ischemia injury is mainly produced by inducible NO synthase (iNOS)^[20]. The western blotting results showed that the Xyl-B treatment significantly reduced the expression of the iNOS protein in the ischemic brains compared with that in the brains of the vehicle-treated tMCAO group (Figure 3C).

Xyl-B reduced brain inflammatory cytokine mRNA levels

The inflammatory response plays a key role in the pathogenesis of ischemic stroke^[21]. Inflammatory cytokines, including IL-1 β , TNF- α , IL-6 and IFN- γ , were detected by quantitative real-time RT-PCR. As shown in Figure 4, these proinflammatory cytokines increased significantly in the vehicle-treated tMCAO mice compared with those in the sham-operated mice. By contrast, the Xyl-B treatment significantly reduced the mRNA expression of IL-1 β , TNF- α , IL-6 and IFN- γ .

Xyl-B protected the blood-brain barrier (BBB)

To evaluate the effects of Xyl-B on BBB permeability after an

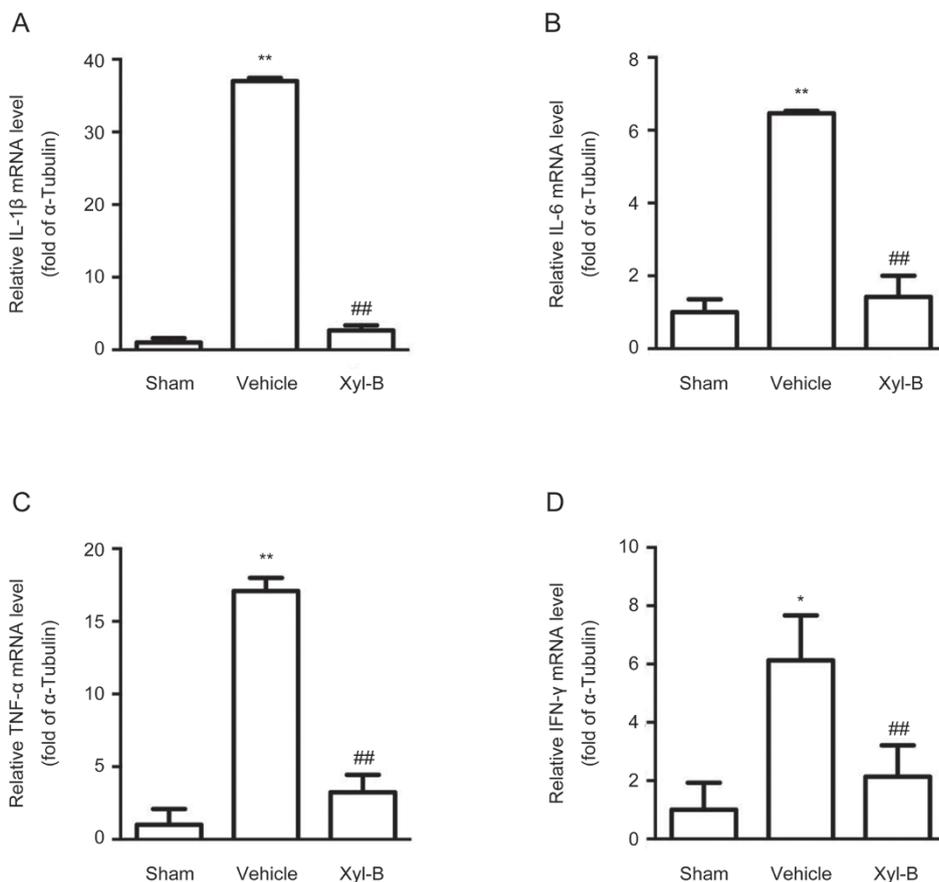


Figure 4. Inflammatory cytokine mRNA levels in the brain were reduced in the Xyl-B-treated mice. Quantitative real-time RT-PCR was performed to analyze the cytokine mRNA levels, including IL-1 β (A), IL-6 (B), TNF- α (C), and IFN- γ (D), in the brain tissues from the sham-operated mice, vehicle-treated mice and Xyl-B-treated tMCAO mice. ** $P < 0.01$ vs sham-operated controls. ## $P < 0.01$ vs vehicle controls. $n = 6$ mice/group.

ischemic injury, BBB leakage was measured by Evans blue extravasation. As shown in Figure 5, the Evans blue extravasation index in the ipsilateral/contralateral hemisphere was significantly increased in the tMCAO group compared with that in the sham group, indicating a BBB disruption, while it was significantly decreased by the Xyl-B and edaravone treatments, indicating that Xyl-B protected BBB integrity after an ischemic injury.

Xyl-B suppressed the expression of TLR4 and the activation of NF- κ B

The TLR4-NF- κ B signaling pathway is enhanced in the brain after the tMCAO and, consequently, leads to inflammatory responses. As shown in Figure 6, the western blotting data confirmed that the protein expression of TLR4 and NF- κ B significantly increased after ischemia. The Xyl-B treatment significantly decreased the ischemia-induced elevation in the expression of TLR4 and NF- κ B in the ipsilateral hemisphere of the tMCAO mice compared with that in the corresponding vehicle group (Figure 6A & B). Consistent with the Western blot data, the immunohistochemistry staining of the mouse brain preparations showed that Xyl-B greatly attenuated the elevation of NF- κ B protein expression that was induced by

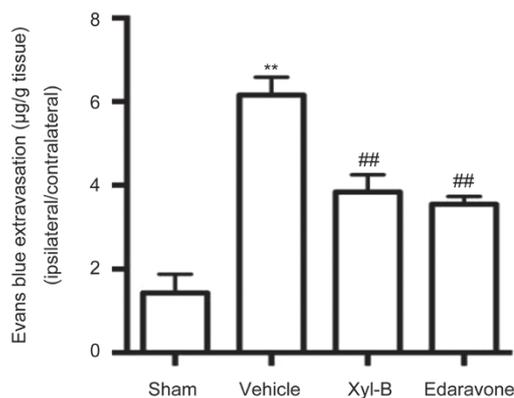


Figure 5. Evans blue extravasation was attenuated in the Xyl-B-treated tMCAO mice. Evans blue extravasation was used to determine BBB disruption in the brains of the sham-operated mice, vehicle-treated mice and Xyl-B-treated tMCAO mice. Evans blue extravasation index of the ipsilateral/contralateral hemisphere is shown in the bar graph. ** $P < 0.01$ vs sham-operated controls. ## $P < 0.01$ vs vehicle controls. $n = 6$ mice/group.

tMCAO (Figure 6C). In the basal state, NF- κ B is stabilized in the cytoplasm, and the distribution of NF- κ B from the cyto-

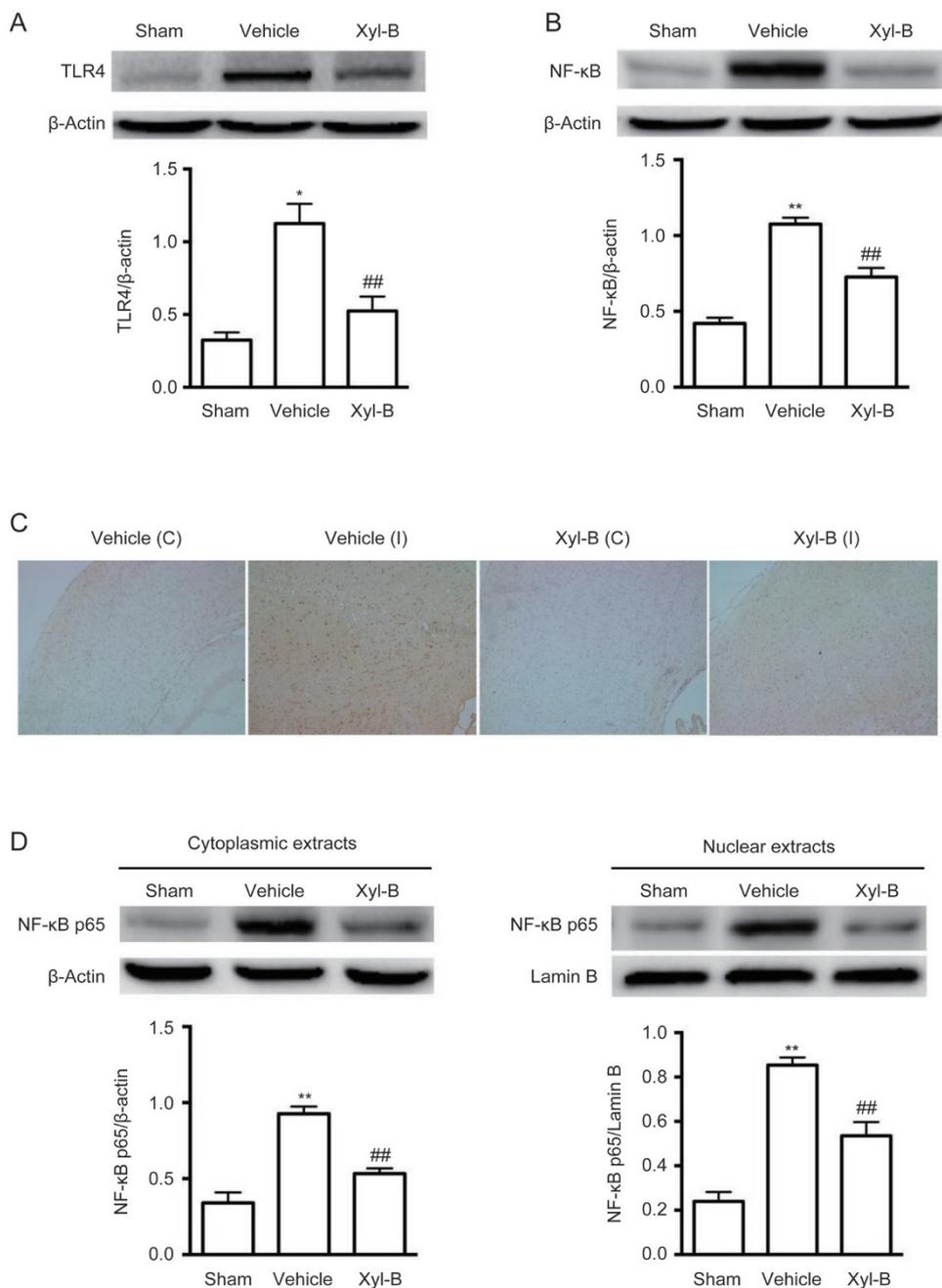


Figure 6. Xyl-B treatment reduced the protein expression levels of TLR4 and NF-κB and repressed the activation of NF-κB. Western blot analysis of TLR4 shows that the protein expression levels of TLR4 (A) and NF-κB (B) were reduced in the Xyl-B-treated mice vs those in the vehicle controls. (C) Representative immunohistochemistry staining images ($\times 40$) demonstrate that the expression of NF-κB is reduced in the Xyl-B-treated mice vs that in the vehicle controls. "C" and "I" represent the contralateral hemisphere and ipsilateral hemisphere, respectively. (D) Western blot analysis was used to determine the expression of NF-κB p65 in the cytoplasmic and nuclear fractions isolated from the ipsilateral hemisphere of the sham-operated mice, vehicle-treated mice and Xyl-B-treated tMCAO mice. * $P < 0.01$ vs sham-operated controls. ## $P < 0.01$ vs vehicle controls. $n = 6$ mice/group.

plasm to the nucleus indicates the activation of NF-κB upon stimulation. The present data demonstrated that the nuclear translocation of NF-κB p65 was enhanced after ischemia, which was significantly reduced by the Xyl-B treatment (Figure 6D).

Discussion

The main findings of this study are as follows: (1) Multiple administrations of Xyl-B at 12.5, 25 or 50 mg/kg before ischemia significantly reduces brain infarct volume in a dose-dependent manner; (2) a single dose of Xyl-B at 50 mg/kg

given after the onset of ischemia significantly reduces brain infarct volume, and the benefit is still significant at 2 h of ischemia; (3) a single dose of Xyl-B given immediately after ischemia (Xyl-B treatment) markedly improves neurological outcomes and protects the integrity of the BBB but does not cause a significant hemodynamic change after ischemia; (4) Xyl-B treatment dramatically reduced ROS overproduction and increased the reduction in MnSOD induced by acute ischemia; (5) the Xyl-B treatment significantly decreased the mRNA levels of proinflammatory cytokines, such as IL-1 β , TNF- α , IL-6 and IFN- γ , after ischemic brain injury; and (6) Xyl-B treatment attenuates inflammatory protein expression levels, including TLR4, NF- κ B and iNOS, after ischemia.

Pharmacological therapies for acute ischemic strokes require further development. Tissue plasminogen activator (tPA) is the only approved drug for the treatment of acute ischemic strokes, but it has the limitation of a short therapeutic window and some adverse side effects^[22]. The marine compound Xyl-B has a unique chemical structure and was isolated from mangrove fungus *Xylaria* sp^[8]. It was found that Xyl-B protected PC-12 cells against oxygen glucose deprivation (OGD) and 1-methyl-4-phenylpyridinium (MPP+)-induced damage^[10, 11]. Recently, we found that pretreatment with Xyl-B reduced brain damage in neonatal mouse hypoxic-ischemic injury^[17]. However, the effects of Xyl-B on adult ischemic brain injuries had not been previously investigated. In this study, we found that both pretreatment and post-treatment with Xyl-B after the onset of ischemia exerted significant protective and therapeutic effects on tMCAO-induced stroke mice. Our findings provide important evidence regarding the potential application of Xyl-B in stroke therapy with a therapeutic window of up to 2 h after the onset of ischemia with single-dose administration. Our previous study demonstrated that Xyl-B increased endothelial NO generation in human umbilical vein endothelial cells^[9], indicating that Xyl-B may influence hemodynamics via vasodilation. In this study, Xyl-B did not significantly change brain hemodynamics in the tMCAO mice, suggesting that Xyl-B may provide protective effects through other mechanisms rather than through improving the blood supply in the ischemic brain.

Excessive ROS generation and the increased expression or activity of iNOS under ischemic conditions in stroke may result in an increase in NO production and, subsequently, an increase in peroxynitrite formation^[23]. Our previous studies suggested that Xyl-B inhibited ROS or RNS generation through multiple approaches, such as the suppression of NADPH oxidase and the activation of endogenous antioxidants^[9, 10, 13]. Thus, the protective effects of Xyl-B against a stroke might be through inhibiting ischemia-induced oxidative stress. In this study, we found that the Xyl-B treatment attenuated ROS generation in the ischemic brains of the tMCAO mice and reduced the iNOS protein expression, suggesting that it would decrease peroxynitrite formation. Edaravone, a novel free-radical scavenger, was used to treat the tMCAO mice in parallel with Xyl-B, and it reduced brain infarction in tMCAO mice. Thus, our results suggested that treatment with Xyl-B can suppress

excessive ROS generation after cerebral ischemia. Our previous studies have demonstrated that Xyl-B can increase some endogenous antioxidant enzymes and proteins, such as GSH, HO-1 and Bcl-2^[9, 13]. Here, we found that Xyl-B treatment significantly enhanced the reduction of MnSOD after ischemia. MnSOD is one of the major endogenous antioxidant enzymes and is considered an effective therapeutic target for the treatment of cerebral ischemia^[24]. MnSOD mimics have been shown to be neuroprotective after acute ischemia^[25]. Altogether, the findings of this study suggest that the suppression of excessive ROS generation and the elevation of endogenous antioxidants by Xyl-B was involved in its therapeutic effects against acute cerebral ischemia.

Multiple lines of evidence, including experimental and clinical studies, indicate that an acute and prolonged inflammatory response plays a key role in ischemic brain injury^[26-28]. Cytokines, such as IL-1 β , TNF- α , IL-6 and IFN- γ , have been demonstrated to increase 24 h after ischemia in a tMCAO model, and the reduction in these cytokines was beneficial for the therapy of ischemic strokes^[18]. Consistently, this study also found that IL-1 β , TNF- α , IL-6 and IFN- γ mRNA expression increased after ischemia in tMCAO stroke mice, while expression was reduced by Xyl-B treatment, indicating that Xyl-B attenuated inflammatory responses both during and after acute ischemic brain injury. However, ischemia and ischemia-associated inflammation lead to higher BBB permeability and disruption^[29]. The inhibition of cytokines, such as TNF- α , preserved BBB integrity after ischemia insult^[30]. Xyl-B treatment significantly reduced ischemia-induced BBB disruption as indicated by the Evans blue extravasation assay, suggesting that Xyl-B may maintain BBB integrity by reducing the generation of inflammatory cytokines.

It has been revealed that the TLR4-NF- κ B signaling pathway is critical for activating secondary inflammation in strokes^[5, 6, 31]. TLR4 can signal through adaptor proteins, such as myeloid differentiation primary-response protein 88 (MyD88), to activate NF- κ B, in turn resulting in the elevation of inflammatory cytokines^[31]. Animals deficient in TLR4 show significantly reduced infarct sizes in experimental stroke models^[32]. Consistently, we confirmed that ischemia induced TLR4 expression and the nuclear translocation of NF- κ B, whereas Xyl-B markedly attenuated the activation of TLR4 and NF- κ B signaling after ischemia. Similarly, Xyl-B also significantly reduced the downstream genes of NF- κ B, TNF- α and IL-1 β after ischemic injury. Our present findings are the first to demonstrate that Xyl-B attenuates the activation of the TLR4-NF- κ B signaling pathway and inflammatory responses under diseased conditions.

The activation of NF- κ B and the excessive generation of inflammatory cytokines is also closely related to increased iNOS expression^[33], while the inhibition of iNOS expression could reduce ischemic brain injury^[34]. Xyl-B treatment can decrease the expression of iNOS and inflammatory cytokines; thus, the suppression of iNOS-mediated inflammatory responses may be involved in the therapeutic benefit of Xyl-B. Lipopolysaccharide (LPS) is a stimulator of the TLR4-NF- κ B inflammatory signaling pathway^[35], and it was found

that TRPM7 is involved in LPS-induced ROS generation in endothelial cells and increased inflammation in aldosterone-infused mice^[36]. Because Xyl-B also blocks TRPM7 currents^[14], it is very possible that Xyl-B may attenuate ischemia-induced inflammatory responses by inhibiting TRPM7. However, future studies are needed to investigate these potential mechanisms.

In summary, a single injection of 50 mg/kg of the marine compound Xyl-B within 2 h after tMCAO significantly reduces the infarct volume without affecting cerebral blood flow. This study also demonstrated that Xyl-B offers protection against focal ischemic strokes by reducing excessive ROS production, increasing the antioxidant MnSOD levels and inhibiting TLR4-NF- κ B-mediated inflammatory reactions. The findings of this study indicate that Xyl-B is a potential new drug candidate for the treatment of strokes.

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Author contribution

Guan-lei WANG and Wen-liang CHEN designed the research study; Ni PAN, Liu-yi LU, Jian-wen CHEN and Li-yan ZHAO performed the research study; Mei LI and Guo-hao WANG analyzed the data; Fang-yun SUN, Hong-shuo SUN, Ji-yan PANG, Jian-ding CHENG, Jie LIU, and Yong-yuan GUAN revised the manuscript; Wen-liang CHEN and Guan-lei WANG wrote the paper; Hong-shuo SUN and Xue-jun WEN helped with polishing the language of the manuscript.

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