Regular Article

Artesunate Provides Neuroprotection against Cerebral Ischemia–Reperfusion Injury via the TLR-4/NF-κB Pathway in Rats

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Inflammation has an important role in ischemia–reperfusion (I/R) injury. Artesunate (ART) has antimicrobial and anti-inflammatory pharmacological activities, and it is used for various types of serious malaria, including cerebral malaria. ART maintains a high concentration in the brain but little is known about the neuroprotective effect of ART against brain I/R injury. We studied the neuroprotection of ART against brain I/R injury and its underlying mechanism. In this study, rats were subjected to middle cerebral artery occlusion (MCAO) for 2 h. After 24 h of reperfusion, neurological deficits, cerebral water content, infarct volume, hematoyxlin–eosin (H&E)-staining, myeloperoxidase (MPO) activity, and proinflammatory cytokine levels were measured. Administration of 20, 40, 80, and 160 mg/kg ART intraperitoneally (i.p.) 10 min after MCAO significantly decreased brain water content and improved neurological deficits in a dose-dependent manner. An 80 mg/kg dosage was optimal. ART significantly reduced infarct volume, suppressed MPO activity and diminished the expressions of toll-like receptor (TLR)-4, MyD88, nuclear factor-κB (NF-κB), tumor necrosis factor (TNF)-α, and interleukin (IL)-6 in the area of the ischemic cortex. The neuroprotective action of ART against focal cerebral I/R injury might be due to the attenuation of inflammation through the TLR-4/NF-κB pathway.

Key words artemesunate; cerebral; ischemia–reperfusion; inflammation

INTRODUCTION

Stroke is a major cause of death and disability and ischemic stroke is the most widely recognized type of stroke. 1–3) Reperfusion of the blocked vessel is the standard treatment for patients diagnosed with ischemic stroke, but it may cause a cascade of secondary injuries. 4) The early inflammatory response to brain injury has great importance in the pathogenesis of ischemia–reperfusion (I/R) injury and this has produced enthusiasm for developing anti-inflammatory cures to combat I/R-caused damage. 5–7)

Artesunate (ART) is a water-soluble, semisynthetic compound derived from artemisinin (Fig. 1). It has positive effects on dermatitis, arthritis, allergic asthma, and other conditions. 8–10) ART has also been used as the standard treatment option for cerebral malaria and other serious types of malaria. 11) Clemmer et al. reported that ART was effective in saving mice with advanced cerebral malaria and it produced a rapid decrease in brain leukocytes. 12) The administration of ART at the beginning of reperfusion can relieve the myocardial injury associated with I/R. 13) Zhao et al. found that ART could be maintained at a high concentration in the brain. 14) Gugliandolo et al. found that ART exerted a neuroprotective impact in an experimental model of severe brain injury through its anti-inflammatory action. 15) Shao et al. reported that the influence of ART against cerebral I/R injury might be through increasing autophagy and the activity of mammalian target of rapamycin (mTOR). 16) Zhang et al. found that ART could mitigate cerebral I/R injury by enhancing neural stem cell proliferation. 17) However, the neuroprotective mechanism of ART on cerebral I/R injury remains poorly understood. The toll-like receptor 4 (TLR-4)/nuclear factor-κB (NF-κB) signaling pathway has a vital role in the inflammatory pathogenesis of cerebral I/R injury. 18,19) ART can attenuate TLR-4/NF-κB-related inflammatory reactions after injury to various organs. 20,21) Our research goal was to determine if the neuroprotection of ART on cerebral I/R injury is linked to the TLR-4/NF-κB signaling pathway.

RESULTS

Effects of ART on Neurological Function Deficit and Brain Edema The neuroprotective action of ART against combat I/R injury was explored by measuring the neurological score and the water content with, or absent from, the administration of ART. Figure 2A shows that the neurological scores in the groups treated with ART were significantly lower than scores in the vehicle group (p < 0.05), especially in ART treatment at a dosage of 80 and 160 mg/kg. The two dosage groups were not significantly different. Figure 2B shows that the ipsilateral hemisphere brain water content in the vehicle group was higher compared to the sham group (84.05 ± 0.89% vs. 78.42 ± 0.08%, p < 0.05), while the brain edema's condition in the 80 mg/kg ART group (80.52 ± 0.29%) and 160 mg/kg ART group (80.11 ± 0.17%) was relieved most significantly compared with the vehicle group. There was no difference

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between the 80 and 160 mg/kg ART group ($p > 0.05$). Hence, in the subsequent experiments, ART was administered at the optimal dosage of 80 mg/kg.

**Effects of ART on Infarction and Morphology** No infarction was found in the sham group. However, in the vehicle group, there was massive infarction in areas of the striatum and lateral cortex (Figs. 3A, B). In contrast to the vehicle group, cerebral infarct volumes were decreased after treatment with ART ($p < 0.05$). We used Hematoxylin–eosin (H&E) staining to study the morphological variation and found H&E-staining of injured cerebral hemispheres (Figs. 3C, D). No morphological change was noted for the cortex neurons in the rats with sham treatment, despite the fact that a large number of atrophic neurons were detected in the vehicle group, which were signs of contracted cytoplasm and nuclear pyknosis. ART exposure resulted in a significant decrease in the number of injured neurons and a significant increase in the number of normal neurons ($p < 0.05$).

**Impacts of ART on Neutrophil Infiltration of the Brain Tissues** The neutrophil influx in the ischemic cerebral cortex was studied using a myeloperoxidase (MPO) activity assay (Fig. 4A). Compared to the sham group, the MPO activity of the vehicle group was significantly higher. The ART (at dosage of 80 mg/kg) treated group, had lower MPO activity compared to the vehicle group after suffering I/R injury.

**Impacts of ART on Proinflammatory Cytokine Expression Activated by Reperfusion** The expression of proinflammatory cytokines tumor necrosis factor-α (TNF-α) combined with interleukin-6 (IL-6) activated by reperfusion was also measured. The vehicle group exhibited higher TNF-α content than the sham group ($p < 0.05$) (Fig. 4B). However, the increased expression of TNF-α was downregulated by ART treatment ($p < 0.05$). The changes of IL-6 content were similar to those of TNF-α (Fig. 4C).

**Impacts of ART on Expression of TLR-4, MyD88, NF-κB p65, and TNF-α** Western blot examination was conducted to assay the protein expressions of TLR-4, MyD88, NF-κB p65 and TNF-α (Fig. 5). The TLR-4 level of the vehicle group was increased relative to the sham group and was downregulated by ART treatment ($p < 0.05$). The expression levels of MyD88, NF-κB p65, and TNF-α, were all similar to that of TLR-4 ($p < 0.05$).

**DISCUSSION**

We showed that ART improved neurological outcomes and decreased brain water content as well as infarct volume in rats that suffered focal cerebral I/R injury. We also demonstrated that ART can suppress MPO activity and significantly reduce...
the expressions of TLR-4, MyD88, NF-κB, TNF-α, and IL-6. These findings suggest that the protective effects of ART on neurons might be partially attributed to its anti-inflammatory activity through the TLR-4/NF-κB pathway.

ART improves neurological outcomes and reduces brain water content as well as the volume of infarct subsequent to I/R injury. This suggests a protective effect of ART in the battle against brain I/R injury. ART is a water-soluble semi-synthetic compound derived from artemisinin, which has been widely used to treat various types of malaria, including cerebral malaria.11) Zhao et al. reported that a high concentration of ART could be maintained in the brain with very slight neurotoxicity.14) ART used in combination with valacyclovir demonstrated good efficacy in mice with herpes simplex virus encephalitis.22) ART has also shown the ability to maintain the wholeness of blood–brain barrier and improve neurological outcomes after subarachnoid hemorrhage. 23) Previous studies have shown that ART can alleviate I/R injury after a stroke.16,17) These findings are consistent with our results.

Shao et al. used two ART groups (30 and 60 mg/kg) and both showed neuroprotective effects.16) Zhang et al. used three ART groups (50, 150, and 250 mg/kg) and showed that 150 mg/kg was the optimal ART dosage.17) We designed four ART groups (20, 40, 80, and 160 mg/kg) and all showed neuroprotective effects vs. the vehicle group. Among them, 80 mg/kg was the optimal ART dosage for the rats. These results are not contradictory and they indicate that ART has a protective effect on cerebral I/R injury in a wide dose range. With reference to the practice guide for dose conversion between animals and humans, the 80 mg/kg dose for rats converts to a 12.9 mg/kg corresponding dose for humans, which is higher than the current recommended dosage for human malaria.24,25) Intravenous
(i.v.) artesunate is safe in humans including infants, children, and pregnant women and the only formal contraindication to i.v. artemisinins is an allergy to i.v. artemisinins. However, it is unknown if a high-dose of ART will produce side effects in humans. Animal model studies can provide useful references for future human application but further research is needed. Shao et al. reported that the protectiveness of artesunate against ischemic cerebral infarction is mediated by increased autophagy. Zhang et al. found that ART could alleviate I/R injury by enhancing neural stem cell proliferation. However, the neuroprotective mechanism of ART for brain I/R injury remains unclear. Here, we have provided new evidence about the mechanism.

Our results demonstrated that ART can reduce MPO activity and significantly inhibit TNF-α and IL-6 expression. This indicates that the protective effect of ART is related to inhibition of inflammation. Focal cerebral I/R injury is a complicated medical condition involving the activation associated with secondary inflammatory and neurodegenerative cascades. Anti-inflammatory effect is one of the main actions of ART. Li et al. reported that the protective effect of ART against type I diabetes in mice is largely attributed to its action in inducing protective T-cells that can produce IL-4 and regulatory T cells. Renal ischemia/reperfusion-mediated remote lung inflammation is inhibited by ART. ART treated mice are protected from cerebral malaria by inhibiting T cell infiltration. These results are consistent with the results of this study, MPO plays an important role in animal cerebral ischemia-reperfusion injury. Kim et al. reported that compared to wild-type rats, MPO knockout rats had significantly improved neurological function and significantly increased cell proliferation after ischemic stroke. Yu et al. showed that the MPO inhibitor N-acetyl lysyltyrosylesteine amide (KYC) preserved neuronal function and helped the brain recover from injury after stroke. Therefore, MPO-mediated neuroinflammation could be a critical therapeutic target for reducing ischemic brain injury. We found that ART can reduce MPO activity after I/R injury. Other studies have shown that MPO is highly expressed in multiple inflammatory cells, including neutrophils, activated microglia, monocytes/macrophage, as well as astrocytes and neurons. Gugliandolo et al. found that the expression of astrocytes and microglia increased significantly in mice with traumatic brain injuries and ART treatment can significantly reduce their increase. The protective effect of ART is also related to inflammatory responses of microglial cells. Therefore, the protective effect of ART on brain IR damage may involve the participation of astrocytes and microglia. In our next study, we will determine whether the protective effect of ART on brain IR damage involves the participation of astrocytes and microglia, and how they are involved.

We found that ART can decrease the expression levels of TLR-4, MyD88, NF-κB p65, and TNF-α protein. The toll-like receptors play a major mediator role when organ ischemic injury occurs and are critical to the original responsive actuation of the immune cells. Lai et al. showed that ART alleviates both the hepatic fibrosis and inflammation which might be attributed to inhibition of TLR-4/NF-κB signaling pathway. ART attenuates glomerular mesangial cell injury induced by high glucose in rats through inhibition of the TLR-4/NF-κB/NLRP3 inflammasome pathway. ART attenuates lipopolysaccharide-stimulated proinflammatory responses through down-regulation of TLR-4 and MyD88 expression and NF-κB activation in microglial cells. Cerebral ischemia can acti-
vate NF-κB in neurons and astrocytes and promote ischemic injury.\textsuperscript{42–44} We previously found that the inhibition of NF-κB activity was associated with the reduced release of TNF-α and IL-6 in brain tissue after I/R injury.\textsuperscript{45} ART attenuates TLR-4-related inflammatory reactions after I/R injury to various organs. Our data indicate that the protection of ART against cerebral I/R injury is related to the TLR-4/NF-κB pathway.

In summary, the results of this study show that ART can improve conditions subsequent to focal cerebral ischemia. This is partly due to its ability to combat inflammation and is correlated with the TLR-4/NF-κB pathway.

\textbf{MATERIALS AND METHODS}

\textbf{Chemicals} ART was purchased from Xinrui Biological Technology Co. (Shaanxi province, China). The purity of ART (98\%) was determined by HPLC as indicated by the assay requirements of ART in the Chinese Pharmacopoeia. ART (Fig. 1) was then dissolved in phosphate buffered saline (PBS) containing 1\% dimethyl sulfoxide (DMSO) for administration to rats. We also arranged the test of MPO kit that had been purchased from the Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Enzyme-linked immunosorbent assay (ELISA) kits to detect the levels of TNF-α, IL-6 in brain tissue after I/R injury.\textsuperscript{45} ART attenuates TLR-4-related inflammatory reactions after I/R injury to various organs. Our data indicate that the protection of ART against cerebral I/R injury is related to the TLR-4/NF-κB pathway.

\textbf{Rat Model of Transient Focal Cerebral Ischemia} The transient cerebral ischemia in rats was activated by middle cerebral artery occlusion (MC AO).\textsuperscript{46,47} The rats were intraperitoneally injected with chloral hydrate (350 mg/kg) for anesthesia and we monitored and maintained their body temperature at 36.5–37.5 °C. The carotid arteries on the left were exposed and isolated. Then the internal carotid arteries were inserted into a nylon filament whose diameter ranged from 0.24 to 0.28 mm, with a purpose of closing the origin of the cerebral artery in the middle so that we could achieve occlusion of the cerebral artery in the middle. After 2 h of ischemia, we withdrew the nylon filament to accomplish reperfusion. We excluded two types of the rats from this study. One type of rat exhibited no neurological deficits, which meant their neurological score was equal to or greater than 1, but they later exhibited signs of hemorrhage. Among 83 rats, 5 were eliminated. The sham group underwent the same procedures except without occlusion of the common carotid artery.

\textbf{Grouping and Drug Intervention} \textbf{Experiment 1: Drug concentration screening} We divided 36 rats randomly into 6 groups. These were the sham group, vehicle group and ART groups respectively with dosages of 20, 40, 80, and 160 mg/kg. The rats in the treatment group were administered intraperitoneally with ART at 10 min after MCAO and those in the vehicle group were administered with 1 mL/kg PBS containing 1\% DMSO.

\textbf{Experiment 2: Studying the mechanism of the ART neuroprotective effect} A total of 42 rats were randomly divided into 3 groups including a sham group, vehicle group, and the ART treatment group. Ten minutes after MCAO, the rats were injected either with ART at a dosage of 80 mg/kg or 1 mL/kg PBS containing 1\% DMSO.

\textbf{Scoring of Neurologic Deficit Condition} A neurological test was performed after reperfusion for 24 h in accordance with the procedure described by Zhang \textit{et al.}\textsuperscript{48,49} A researcher with no prior knowledge of the experimental groups conducted a relevant neurological test. There were 5 point levels for the neurological findings: Level 0—normal neurological condition; Level 1—INability to fully expand right forepaw; Level 2—circling to right; Level 3—falling to right; and Level 4—inability to walk spontaneously accompanied by depressed level of consciousness.

\textbf{Measurement of Brain Edema} We measured the brain edema conditions in all 6 groups of Experiment 1. The detection method was described previously.\textsuperscript{47} We calculated brain water content with the formula: (wet weight – dry weight)/wet weight × 100\%.

\textbf{Measurement of Brain Infarct Volume} Analyses were made with samples from Experiment 2. The rats were decapitated at 24 h after reperfusion, and then their brains were stored at −20 °C for 15 min. Brain coronal sections 2 mm thick were stained using 1% TTC at 37 °C for 20 min and then fixed in 4% paraformaldehyde. Images of the staining were taken by a Canon Ixus 950 IS digital camera and quantified using ImageJ (ver 1.37c, NIH). To minimize the impact of brain edema on the hemisphere lesion volume percentage, we performed calculations using the following formula: half-brain lesion volume percentage (% HLV) = [{(whole infarct volume – (left side of the brain volume – right side of the brain volume))/ right side of the brain volume} × 100%]\textsuperscript{50,51} An examiner who did not know the group divisions conducted the calculations on infarct volume.

\textbf{Histological Measurement} The samples used for TTC staining were also used for H&E staining. After fixation with 4% paraformaldehyde, the brains were embedded in paraffin. We cut five micrometers of the coronal segments for H&E staining and subsequent measurement.

\textbf{MPO Assay} We analyzed the other samples from Experiment 2. We measured MPO activity to assess neutrophil accumulation level in the ischemia cerebral cortex using manufacturer directions. The brain samples taken 24 h after reperfusion were homogenized in cool normal saline (brain tissue: normal saline = 1 : 10) and MPO activity was measured with a spectrophotometer set at 460 nm.\textsuperscript{52}

\textbf{ELISA of Cytokines} We used samples obtained 24 h after the reperfusion to prepare brain tissue homogenate. We used ELISA kits to detect the levels of TNF-α and IL-6.

\textbf{Western Blot Analysis} We performed Western blotting as previously reported.\textsuperscript{45} We chose TLR4 antigen (diffused to 1 : 500, Cell Signaling Technology, Danvers, MA, U.S.A.), MyD88 antigen (diffused to 1 : 500, Cell Signaling Technology), NF-κB p65 antigen (diffused to 1 : 500, Cell Signaling Technology), TNF-α antigen (diffused to 1 : 500, Cell Signaling Technology), β-actin (diffused to 1 : 1000, Santa Cruz, Dallas, TX, U.S.A.), and Histone H3 (diffused to 1 : 1000, Santa Cruz, Dallas, TX, U.S.A.). We used samples obtained 24 h after the reperfusion to prepare brain tissue homogenate. We used ELISA kits to detect the levels of TNF-α and IL-6.

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