ORIGINAL RESEARCH

Protective Effect of Erythromycin Pre-adaptation on Focal Cerebral Ischemia in Rats and its Changes in TNF-α and nNOS

Danqing Gou, MD; Rimiao Yang, MD; Weicheng Lu, MD; Shangchen Han, MM; Mingbin Si, MM; Guangyu Li, MD

ABSTRACT

Context • Ischemic stroke accounts for 85% of all types of stroke. Ischemic preconditioning can provide protection against cerebral ischemic injury. Erythromycin can induce ischemic preconditioning in brain tissue.

Objective • The study intended to investigate the protective effects of erythromycin preconditioning on infarct volume after focal cerebral ischemia in rats and on the expression of tumor necrosis factor-alpha (TNF-α) and neuronal nitric oxide synthases (nNOS) in rat-brain tissue.

Design • The research team performed an animal study.

Setting • The study took place in the Department of Neurosurgery at the First Hospital of China Medical University in Shenyang, China.

Animals • The animals were 60 healthy male Wistar rats, aged 6 to 8 weeks and weighing 270 to 300 g.

Intervention • The research team randomly divided the rats into a control group in simple randomization and intervention groups preconditioning them according to their body weights using different concentrations of erythromycin—5, 20, 35, 50, and 65 mg/kg, with 10 rats in each group. The team induced focal cerebral ischemia and reperfusion using a modified, longa-wire embolization method. The control group, also 10 rats, received an injection intramuscularly of normal saline.

Outcome Measures • The research team: (1) calculated the volume of cerebral infarction using triphenyltetrazolium chloride (TTC) staining with image analysis software and (2) investigated the effects of erythromycin preconditioning on the expression of TNF-α and nNOS mRNA and protein in the rat-brain tissue using real-time polymerase chain reaction (PCR) and Western blot.

Results • Erythromycin preconditioning reduced the volume of cerebral infarction after induction of cerebral ischemia, showing a U-shaped, dose-response relationship, and the cerebral infarction volume significantly decreased in the 20-, 35-, and 50-mg/kg erythromycin preconditioning groups ($P < .05$). Erythromycin preconditioning at 20-, 35-, and 50-mg/kg significantly down-regulated the mRNA and protein expression of TNF-α in the rat-brain tissue ($P < .05$), with the 35-mg/kg erythromycin preconditioning group having the most significant downregulation. Erythromycin preconditioning at 20-, 35-, and 50-mg/kg upregulated the mRNA and protein expression of nNOS in the rat-brain tissue ($P < .05$), with the 35-mg/kg erythromycin preconditioning group having the most significant upregulation of the mRNA and protein of nNOS.

Conclusions • Erythromycin preconditioning had a protective effect against focal cerebral ischemia in rats, and the best protective effect occurred for the 35-mg/kg preconditioning. The reason may be related to the fact that erythromycin preconditioning significantly upregulated nNOS and downregulated TNF-α in the brain tissue (*Altern Ther Health Med.* 2023;29(3):212-217).

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Improved living standards have caused cerebrovascular disease to become one of the diseases most threatening to human health. It has a rapid onset and a high mortality rate, and even if a patient survives, severe neurological damage can occur. Ischemic stroke is a brain dysfunction that a blockage of an artery in the brain and a sudden interruption of blood flow causes.[1]

The consequences not only seriously affect patients' quality of life but also impose a heavy financial burden on their families and society.[2] Statistically, stroke has become the second leading cause of death and major disabling disease

worldwide and is characterized by high morbidity, recurrence, and mortality.[3,4] Ischemic stroke accounts for 85% of all types of stroke.[5]

Ischemic preconditioning (IP)

The discovery of the phenomenon of ischemic tolerance was derived from clinical practice, and in fact, external stimuli activate the endogenous protective mechanism of the body and provide resistance to the next severe injury. Ischemic preconditioning (IP) refers to the significant tolerance of the body's tissues to prolonged ischemic injury after a person has experienced a nonfatal ischemic injury and is an important protective mechanism of the body.[6]

Liu et al indicated that research on the protective effects and mechanisms of ischemic and hypoxic preadaptation in neurological diseases has become an important research topic.[7] Kitagawa et al first proposed the concept of cerebral ischemic preconditioning (IPC), and those researchers used rats to establish a global, cerebral ischemia model.[8]

Working with mice with transient global cerebral ischemia, other researchers examined the mechanism by which ischemia reperfusion can induce ischemic tolerance in experimental mice.[7] Reperfusion is the paradoxical exacerbation of cellular dysfunction and death following restoration of blood flow to previously ischemic tissues. Those researchers' intent was to learn how to protect brain tissue and prevent damage that a later, longer period of global cerebral ischemia could produce.

Because cerebral ischemia preconditioning can protect the brain for a long time and a wide range, it has greater potential practical value and clinical significance. Liu et al found that early intervention can reduce the occurrence of more than 80% of secondary cerebral infarctions.[9] However, research hasn't clarified the specific mechanism.

Although clinicians have applied ischemic preconditioning to some extent in cardiac surgery,[10-12] its promotion has been limited based on invasive operations. In neurosurgery, clinicians have made attempts to apply the preconditioning protective mechanisms, but the procedures are still not clear.[13] Because ischemic preconditioning is a noxious stress process, its clinical application is greatly limited.

Many classical pathways and factors play an important role in the regulation of preconditioning, including the mammalian target of rapamycin (mTOR)[14] and protein kinase B (AKT)[15] pathways and tumor necrosis factor-alpha (TNF-a),[16] hypoxia-inducible factor 1-alpha (HIF-1α),[17] adenosine, *N*-methyl-D-aspartate (NMDA) receptor,[18] and nitric oxide (NO).

TNF-α and neuronal nitric oxide synthases (nNOS)

Two studies have examined the effects of TNF-α and nNOS.[19,20] Both of these are involved in brain damage and protection.

TNF-α. TNF-α is a pleiotropic cytokine with a wide range of biological functions and is mainly related to inflammation and immune response. In 1985, Shalaby named the TNF produced by macrophages TNF-α. It can increase the permeability of vascular endothelial cells and induce the expression of cell-adhesion factors. The increase in TNF-a secretion or synthesis in the early stage of cerebral ischemia is the main cause of cerebral infarction.[21]

TNF-α levels increase early in cerebral ischemia and correlate with the degree of neurological deficits.[22] Vila et al found that patients with primary cerebral infarction whose condition worsened within 48 hours of it had significantly higher TNF-α levels in their blood and cerebrospinal fluid compared with those without aggravation.[23]

Neuronal nitric oxide synthases (nNOS). Interest in the role of nNOS in hypoxic preconditioning has increased, with Qin et al finding that nNOS can play an important role in chemokine (C-C motif) ligand 2 (CCL 2)-induced preconditioning.[24] So far, understanding of the mechanism of nNOS in preconditioning is still unclear. Gonzalez-Zulueta et al indicate that preconditioning can cause increased expression of nNOS, which in turn can activate the P21/rat sarcoma (Ras) pathway.[25]

Erythromycin Preconditioning

Two studies using global cerebral ischemia models found that erythromycin can induce ischemic preconditioning in brain tissue.[26,27] Pharmacological preconditioning is based on the theory of ischemic preconditioning, using drugs that simulate ischemic preconditioning. The drugs can provide protection against cerebral ischemic injury, providing a good clinical value.

Erythromycin is an antibiotic commonly used in clinical practice. Recently, Brambrink AM and Koerner IP et al found that erythromycin can simulate the ischemic effect by interfering with the energy metabolism of mitochondria and can induce the effect of preconditioning.[26,27] However, no studies have clearly determined whether it can effectively act in brain tissue through a simulated, ischemic preconditioning effect and make brain tissue tolerant to ischemia and hypoxia, a protective effect, reducing cerebral infarction and brain edema.

Current Study

The role of TNF-α and nNOS during erythromycin preconditioning isn't clear. The present study intended to investigate the protective effects of erythromycin on infarct volume after focal cerebral ischemia in rats and on the expression of tumor necrosis factor-alpha (TNF-α) and neuronal nitric oxide synthases (nNOS) in rat-brain tissue.

METHODS

Animals

The research team performed an animal study. The study took place in the Department of Neurosurgery at the First Hospital of China Medical University in Shenyang, China. The Animal Department of China Medical University provided 60 healthy Wistar male rats, aged 6 to 8 weeks and

weighing 270 to 300 g. The team housed the rats in a single cage. Temperature: 20-26°C. Humidity: 40-70%. Food and water are changed twice a week. them, what the temperature was, and any other conditions under which you kept them.

Procedures

Materials. Sangon Biotech (Shanghai, China) synthesized the primers for TNF-α and nNOS. The research team purchased: (1) TRIzol reagent from Gibco PRL (Shanghai, China); (2) triphenyltetrazolium chloride (TTC) red dye from Shanghai Reagent Factory III (Shanghai, China); and (3) TNF-α (#52B83) and nNOS (#A-11) antibodies from Santa Cruz (Shanghai, China).

Intervention. The research team randomly divided the rats into six groups with 10 rats in each group in simple randomization. One group was the control group, and the other five groups were intervention groups.

At 12 h before induction of ischemia, the control group received an injection intramuscularly of normal saline. The team preconditioned the intervention groups according to their body weights. The intervention groups received an injection intramuscularly of different concentrations of erythromycin—5, 20, 35, 50, and 65 mg/kg, respectively.

Induction of focal cerebral ischemia. Based on Longa et al's procedures,[28] the research team induced right focal cerebral ischemia and reperfusion in each rat using a modified, longa-wire embolization method, inserting a suture plug into the right internal carotid artery. The animals showed the following signs upon awakening after a successful induction: (1) inward flexion of the left forelimb when lifting the tail; (2) circling on the left side of the collar when crawling; (3) leaning to the right when standing. The team selected any rat with one of the three signs.

Staining with TTC. The research team: (1) 1 day after induction of ischemia, rapidly decapitated their brains after anesthesia; (2) harvested and collected the cortex in the penumbra region of the right ischemic cerebral hemisphere—7 to 11 mm from the tip of the olfactory bulb, sagittal fissure to the upper third of the lateral fissure—in an EP tube and stored the tissue in a -70°C refrigerator; (3) sectioned the whole brains every 2 mm from anterior to posterior, cutting six sections; (4) placed the brain slices in 1% TTC saline and incubated them at 37°C in the dark for 30 min; and (5) after complete color development—red in the normal brain tissue and white in the injured tissue, fixed them in a formaldehyde solution.

Measurement of brain tissue infarct volume. The research team: (1) measured brain tissue infarct volume using image analysis software (imagJ, Java, US) to calculate the cerebral infarction area and each layer area, using the formula $V = \Sigma (A1 + A2)\ t/2$; where "t" is the slice thickness, and A1 and A2 represent the rostral and the caudal infarct size of the cut block, respectively, and (2) calculated the rat-brain volume using the same method to find the percentage of infarct volume to rat-brain volume.

Messenger RNA (mRNA) expression of TNF-α and nNOS. The research team: (1) determined the expression using SYBR green stain (Takara, Japan) with quantitative PCR (qPCR); (2) extracted the RNA from the tissues using Trizol and an RNA extraction kit (Takara, Japan); (3) used real-time PCR to perform reverse transcription of RNA with a reverse transcription kit from Promega (Takara, Japan) and synthesized complementary DNA (cDNA); (4) used fluorescent real-time qPCR with a Rotor-Gene 3000 fluorescent qPCR instrument (Takara, Japan), with the fluorescent dye-labeled primers.

Table 1. Primer Sequences and Reaction Conditions

Primer	Primer sequence 5 '-3'
nNOS	Forward: GTCTCCTCTGACTTCAACAGCG Reverse: ACCACCCTGTTGCTGTAGCCAA
TNF-α	Forward: CTCTTCTGCCTGCTGCACTTTG Reverse: ATGGGCTACAGGCTTGTCACTC
GAPDH	Forward: ACACGCATGTCTGGAAAGGCAC Reverse: CTCTGTGGCATAGAGGATGGTC

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; nNOS, neuronal nitric oxide synthases; TNF-α, tumor necrosis factor-alpha.

Table 1 shows the primer sequences. The research team counted the number of amplification cycles of target genes in each sample and calculated the mRNA expression levels of target genes relative to the internal reference gene β-actin using the 2-ΔΔCt method.

TNF-α and nNOS protein expression. The research team: (1) created a Western blot to determine the TNF-α and nNOS contents using chemiluminescence and an enhanced chemiluminescence (ECL) kit (Beyotime, Beijing, China); (2) used β-actin as an internal reference; (3) scanned the Western-blot results with the software Chemi Imager 5500 V2.03 (Tanon, Shanghai, China), and (4) quantitatively analyzed them with an image analysis system (imagJ, Java, US).

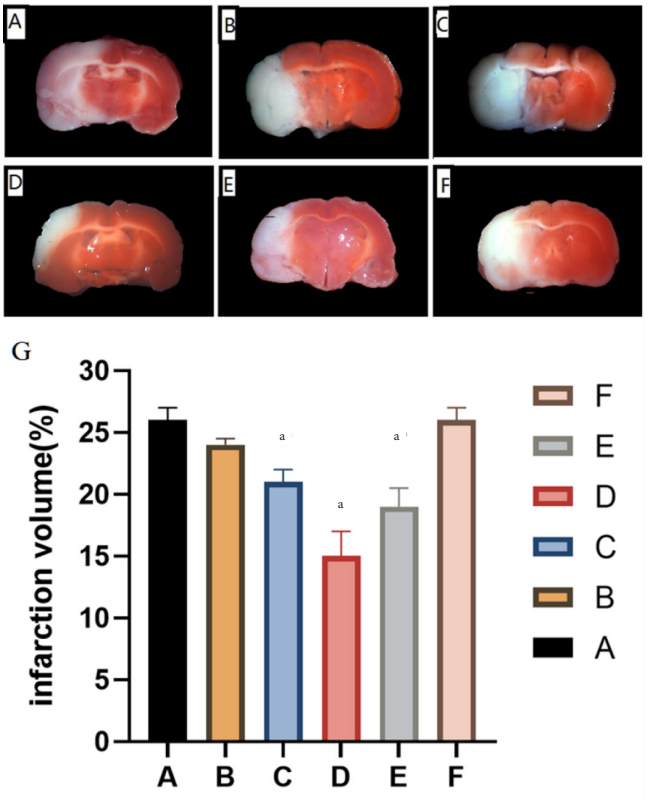
Outcome Measures

The research team: (1) calculated the volume of cerebral infarction using triphenyltetrazolium chloride (TTC) staining with image analysis software and (2) investigated the effects of erythromycin preconditioning on the expression of TNF-α and nNOS mRNA and protein in the rat-brain tissue using real-time polymerase chain reaction (PCR) and Western blot.

Statistical Analysis

The research team statistically analyzed the data using Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA). The team: (1) expressed the obtained data as means ± standard deviations (SDs), (2) tested comparisons among multiple groups using one-way analysis of variance (ANOVA); and (3) tested comparisons between two groups using the least significant difference (LSD) test when variances were homogeneous and the Dunnett's T3 test when variances were heterogeneous. $P < .05$ indicated a statistically significant result.

Figure 1. The TTC Staining Results After Focal Ischemic Infarction in the Rat Brain. The figure shows representative images for each group (n = 10). The white part represents the tissues with cerebral infarction, and the red part represents normal brain tissues without infarction. Figure 1A shows the control group; Figure 1B shows the 5-mg/kg erythromycin group; Figure 1C shows the 20-mg/kg erythromycin group; Figure 1D shows the 35-mg/kg erythromycin group; Figure 1E shows the 50-mg/kg erythromycin group; and Figure 1F shows the 65-mg/kg erythromycin group. Figure 1G shows the semiquantitative analysis of the relative infarct volume in each group—cerebral infarction volume/whole brain volume percentage (%)



[a]$P < .05$, indicating that the relative infarct volume for the 20-mg/kg, 35-mg/kg, and 50-mg/kg erythromycin groups was significantly lower than that of the control group

Abbreviations: TTC, triphenyltetrazolium chloride.

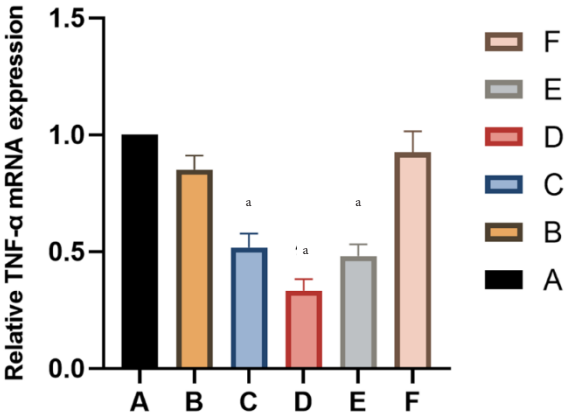
RESULTS

Cerebral Infarction Volume

Figure 1 shows the focal cerebral ischemia in the rats after TTC staining. The white part represents the tissues with cerebral infarction, and the red part represents normal brain tissues without infarction. The preconditioning showed a U-shaped, dose-response relationship.

The groups that received 20 mg/kg, 35 mg/kg, and of erythromycin had a cerebral infarction volume that was significantly lower than that of the control group ($P < .05$). Compared with the control group, the infarct volume in the 20 mg/kg group was about 5.02% lower, in the 35 mg/kg group was about 10.07% lower, and in the 50 mg/kg group was about 5.55% lower. These results suggest that preconditioning with 35 mg/kg of erythromycin had a better protective effect than did preconditioning with 20 mg/kg or 50 mg/kg mg/kg.

Figure 2. Relative mRNA Expression of TNF-α. Figure 2A shows the control group; Figure 2B shows the 5-mg/kg erythromycin group; Figure 2C shows the 20-mg/kg erythromycin group; Figure 2D shows the 35-mg/kg erythromycin group; Figure 2E shows the 50-mg/kg erythromycin group; and Figure 2F shows the 65-mg/kg erythromycin group



[a]$P < .05$, indicating that the relative mRNA expression of TNF-α for the 20-mg/kg, 35-mg/kg, and 50-mg/kg erythromycin groups was significantly lower than that of the control group

Abbreviations: mRNA, messenger RNA; TNF-α, tumor necrosis factor-alpha.

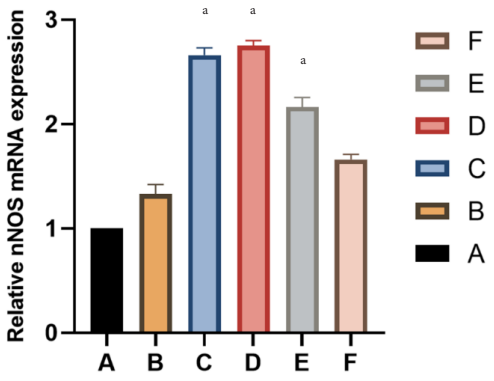
mRNA Expression of TNF-α

Figure 2 shows the mRNA expression of TNF-α in brain tissues preconditioned with erythromycin. Compared with the control group, the mRNA expression of TNF-α in brain tissue was significantly downregulated in the erythromycin groups receiving 20, 35, or 50 mg/kg of erythromycin ($P < .05$). No significant difference existed in the mRNA expression of TNF-α in the brain tissue of the erythromycin groups receiving 5 or 65 mg/kg compared to that of the control group. The decreased mRNA expression of TNF-α in the brain may be related to the reduction in cerebral infarct size from erythromycin preconditioning.

mRNA expression of nNOS

Figure 3 shows the mRNA expression of nNOS in brain tissue preconditioned with erythromycin. Compared with the control group, the mRNA expression of nNOS in brain tissue was significantly upregulated in the erythromycin

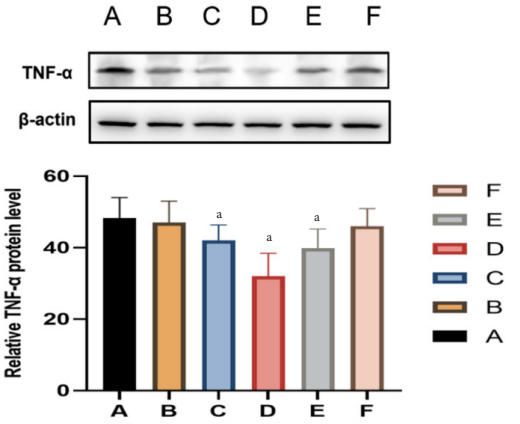
Figure 3. Relative mRNA Expression of nNOS. Figure 3A shows the control group; Figure 3B shows the 5-mg/kg erythromycin group; Figure 3C shows the 20-mg/kg erythromycin group; Figure 3D shows the 35-mg/kg erythromycin group; Figure 3E shows the 50-mg/kg erythromycin group; and Figure 3F shows the 65-mg/kg erythromycin group



[a]$P < .05$, indicating that the relative mRNA expression of nNos for the 20-mg/kg, 35-mg/kg, and 50-mg/kg erythromycin groups was significantly higher than that of the control group

Abbreviations: mRNA, messenger RNA; nNos, neuronal nitric oxide synthases.

Figure 4. The Relative Protein Expression of TNF-α Using Western Blot. The figure shows the expression for each group: Figure 4A—the control group; Figure 4B—the 5-mg/kg erythromycin group; Figure 4C—the 20-mg/kg erythromycin group; Figure 4D—the 35-mg/kg erythromycin group; Figure 4E—the 50-mg/kg erythromycin group; and Figure 4F—the 65-mg/kg erythromycin group. The graphic at the bottom shows the semi-quantitative analysis.



[a]$P < .05$, indicating that the relative protein expression of TNF-α for the 20-mg/kg, 35-mg/kg, and 50-mg/kg erythromycin groups was significantly lower than that of the control group

Abbreviations: TNF-α, tumor necrosis factor-alpha.

groups receiving 20, 35, or 50 mg/kg ($P < .05$). No significant difference existed in the mRNA expression of nNOS in the brain tissue of the erythromycin groups receiving 5 mg/kg or 65 mg/kg compared to that of the control group. These results suggest that preconditioning with erythromycin can reduce cerebral infarct size, which may be related to upregulation of nNOS mRNA expression in the brain.

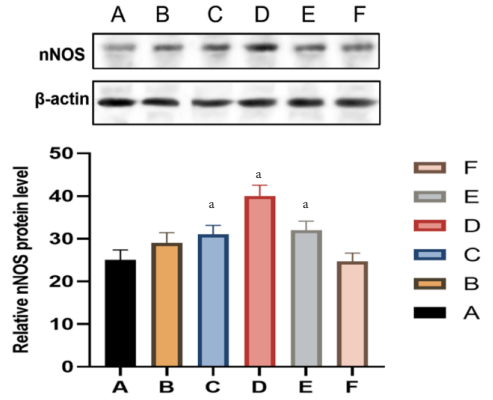
TNF-α Protein Expression

Figure 4 shows the protein expression using Western blot and the semiquantitative analysis of the TNF-α in the brain tissues of the intervention groups. Compared with the control group, the protein expression of TNF-α in the brain tissue of the groups receiving 20, 35, or 50 mg/kg of erythromycin was significantly downregulated ($P < .05$). The protein expression of TNF-α in the brain tissue of the groups receiving 5 or 65 mg/kg of erythromycin wasn't significantly different from that of the control group. This suggests that the pharmacological reduction of the cerebral infarction area from erythromycin may be related to its downregulation of protein expression of TNF-α in the brain.

nNOS Protein Expression

Figure 5 shows the protein expression using Western blot and the semiquantitative analysis of nNOS in brain tissues preconditioned with erythromycin. Compared with the control group, the protein expression of nNOS in brain

Figure 5. The Relative Expression of nNOS Protein Using Western Blot. The figure shows the expression for each group: Figure 5A—the control group; Figure 5B—the 5-mg/kg erythromycin group; Figure 5C—the 20-mg/kg erythromycin group; Figure 5D—the 35-mg/kg erythromycin group; Figure 5E—the 50-mg/kg erythromycin group; and Figure 5F—the 65-mg/kg erythromycin group. The graphic at the bottom shows the semi-quantitative analysis.



[a]$P < .05$, indicating that the relative protein expression of nNOS protein for the 20-mg/kg, 35-mg/kg, and 50-mg/kg erythromycin groups was significantly higher than that of the control group

Abbreviations: nNos, neuronal nitric oxide synthases.

tissue was significantly upregulated in the groups receiving 20, 35, or 50 mg/kg of erythromycin ($P < .05$). The protein expression of nNOS in brain tissue of the groups receiving 5 or 65 mg/kg of erythromycin wasn't significantly different from that of the control group. This suggests that the reduction in the cerebral infarction area from preconditioning with erythromycin may be related to its upregulation of protein expression of nNOS in brain.

DISCUSSION

The current study confirmed that erythromycin preconditioning can have an important protective effect on focal cerebral ischemic injury in rats, and the 35 mg/kg erythromycin preconditioning dose was the optimal erythromycin preconditioning dose. Moreover, the mechanism of erythromycin preconditioning protection was closely related to TNF-α and nNOS. The specific mechanism of action needs to be confirmed by further in-depth study.

Further study of preconditioning phenomenon can be helpful to elucidate the endogenous protective mechanism of the body, prolong the treatment time of ischemic cerebrovascular disease, support the efforts of clinical practice, and protect the body's function. It has attractive prospects for clinical application.

The phenomenon of preconditioning also may have broad application in neurosurgery. The protective effects of preconditioning can significantly enhance a patient's tolerance to surgery, reduce surgical complications, and improve the patient's prognosis, which can produce significant social and economic benefits. In the past, the application of preconditioning in clinical practice was limited mainly because didn't understand the cause of preconditioning's effects. The current study of the effects and mechanisms of erythromycin, a safe and commonly used antibiotic, in inducing preconditioning can help clinicians apply the preconditioning effect in neurosurgical clinical work.

CONCLUSIONS

Erythromycin preconditioning had a protective effect against focal cerebral ischemia in rats, and the best protective effect occurred for the 35 mg/kg preconditioning. Erythromycin preconditioning significantly upregulated the mRNA and protein expression of nNOS and decreased the mRNA and protein expression of TNF-α in brain tissue, which may be related to the mechanism of the cerebral protective effect of erythromycin preconditioning.

DATA AVAILABILITY

The datasets that the research team used and analyzed during the current study are available from the corresponding author upon reasonable request.

AUTHORS' DISCLOSURE STATEMENT

The authors declare that they have no conflicts of interest related to the study.

AUTHOR CONTRIBUTIONS

Danqing Gou and Rimiao Yang contributed equally to this work.

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