

# Pentoxifylline exerts anti-inflammatory effects on cerebral ischemia reperfusion-induced injury in a rat model via the p38 mitogen-activated protein kinase signaling pathway

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**Abstract.** Pentoxifylline exhibits complex functions with extensive pharmacological effects and is used therapeutically due to its therapeutic effects and rapid metabolism in the body, with no cumulative effects and few side effects. The present study investigated the effects of pentoxifylline on cerebral ischemia reperfusion-induced injury (IRI) through suppression of inflammation in rats. Hematoxylin and eosin staining was performed to evaluate the number of neurocytes, and ELISAs were applied to measure tumor necrosis factor- $\alpha$ , interleukin-6, malondialdehyde and superoxide dismutase activities. Treatment with pentoxifylline significantly recovered the cerebral ischemia reperfusion-induced neurological deficit score and cerebral infarct volume in rats. In addition, pentoxifylline treatment significantly reversed the cerebral ischemia reperfusion-induced interleukin-6, tumor necrosis factor- $\alpha$ , malondialdehyde and superoxide dismutase levels *in vivo*. Furthermore, pentoxifylline significantly inhibited cyclooxygenase-2 and inducible nitric oxide synthase mRNA and protein expression in cerebral IRI mice. Treatment with pentoxifylline also significantly suppressed the expression of cleaved caspase-3 and p38 mitogen-activated protein kinase (MAPK) protein in cerebral IRI mice. These results indicate that the protective effects of pentoxifylline on cerebral IRI may occur via the p38 MAPK signaling pathway.

## Introduction

Cerebrovascular disease poses a substantial threat to human health. Annually, ~1.5 million new patients are diagnosed in China and the most common type is ischemic stroke, namely cerebral ischemia (1). In recent years, incidence trends have shifted towards younger individuals, and the majority of

patients experience nerve function defect following the attack, which greatly affects the quality of life of patients and is associated with economic and mental burdens to society and the family and friends of patients (2). However, at present, no effective treatment exists.

Cerebral ischemia reperfusion-induced injury (IRI) is a highly complex pathological process that involves a series of cellular and molecular events (3). It is divided into the following three stages: Acute stage, which primarily manifests as metabolic disorders and excitatory toxicity; sub-acute stage, where the major pathological changes are inflammation and apoptosis; and chronic stage, which primarily consists of repair and regeneration (4). The sub-acute and chronic stages are also collectively termed the 'late stage', as the boundary between the two stages is difficult to determine (5).

A previous study demonstrated that inflammation has an important role in cerebral IRI (6). The early accumulation of neutrophil granulocytes at ischemic regions has been verified by histopathology and biochemical methods (7). The most frequently used reliability index to evaluate the degree of neutrophil granulocyte infiltration in tissue is myeloperoxidase (8), which is an enzyme that catalyzes peroxide reduction and is an essential constituent of the oxygen-dependent sterilization system of neutrophil granulocytes (9).

Pentoxifylline is a type of alkaloid that is formed by introducing a hexanone group to theobromine extracted from cocoa beans (10). It is a derivative of methylxanthine and non-selective phosphodiesterase inhibitors (11). Pentoxifylline has been reported to increase the deformability of red blood cells, improve the hemorheology of leukocytes, inhibit the adhesion and activation of neutrophil granulocytes, expand capillaries, reduce blood viscosity, increase the oxygen partial pressure of tissue and eliminate free radicals (12,13). Therefore, the present study was performed to investigate the protective effects of pentoxifylline on cerebral IRI and the potential underlying mechanisms.

## Materials and methods

**Animals and treatments.** Male Sprague-Dawley rats weighing 260-300 g (n=36; 1.5 years old) were obtained from the Experimental Animal Centre of Zhangqiu People's Hospital (Jinan, China) and were allowed free access to laboratory chow and tap water in day-night quarters at 25°C with

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50-60% humidity and a 12-h light/dark cycle. The experiment was approved by the Committee on Animal Experiments of Zhangqiu People's Hospital (14). All rats were randomly divided into the following three experimental groups (n=12 per group): Sham, sham-operated rats pretreated intraperitoneally with normal saline for 3 days; cerebral IRI model, cerebral IRI model rats pretreated intraperitoneally with normal saline for 3 days; and pentoxifylline treatment group, cerebral IRI model rats pretreated daily with 46.7 mg/kg pentoxifylline (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) intraperitoneally for 3 days. The chemical structure of pentoxifylline (3,7-dimethyl-1-(5-oxohexyl)-3,7-dihydro-1H-purine-2,6-dione) is presented in Fig. 1.

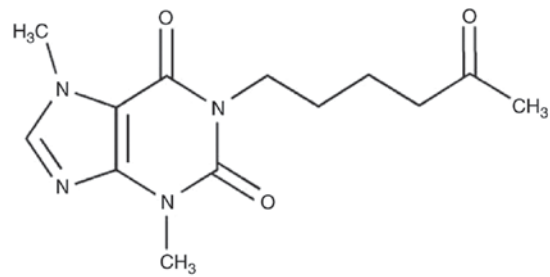


Figure 1. The chemical structure of pentoxifylline.

**Cerebral IRI model.** Intraluminal filamentous occlusion of the middle cerebral artery (MCA) was performed to induce the cerebral IRI model for 1 h. Reperfusion of the MCA was initiated by removing the MCA-occlusive filament after the 1 h of occlusion and then reperfusion was performed for 1 h. The mean arterial blood pressure, arterial blood gases and pH were monitored following cannulation of the right femoral artery. A laser Doppler flowmeter (Periflux System 5000; Perimed AB, Järfälla, Sweden) was used to monitor regional cerebral blood flow. Sham-operated rats were anesthetized (35 mg/kg pentobarbital sodium) and surgically opened up without cerebral IRI induction. Brain samples were fixed using 4% paraformaldehyde for 24 h at room temperature and processed by routine histological methods, embedded in paraffin blocks and sectioned coronally into sequential 5- $\mu$ m sections. Then, section samples were stained using haematoxylin and eosin for 20 min at room temperature. Subsequently, neurological deficit scores were analyzed (0, normal; 1, moderate; 2, considerable; 3, severe) following treatment with pentoxifylline for 3 days using a fluorescence microscope (OLYMPUS BX51; Olympus Corporation, Tokyo, Japan).

**Evaluation of cerebral infarct volume.** Rats were sacrificed by decapitation and cerebral infarct tissues were rapidly acquired. Cerebral infarct tissues were frozen at -80°C for storage. Subsequently, cerebral infarct tissues were fixed with 4% paraformaldehyde in PBS for 24 h at room temperature. Then, brains tissues were sliced into 5- $\mu$ m uniform coronal sections, which were stained with 2% 2,3,5-triphenyltetrazolium chloride solution for 1 h at 37°C. Cerebral infarct volume was using a fluorescence microscope (OLYMPUS BX51) and measured using OlyVIA software version 2.6 (both Olympus Corporation).

**Histology.** Rats were sacrificed by decapitation and cerebral infarct tissues were rapidly acquired. Cerebral infarct tissues were fixed in 3% paraformaldehyde in PBS for 4 h on ice and then transferred to 50% ethanol for 2 h. Tissues samples were cut into histological sections (4- $\mu$ m) and were stained with hematoxylin and eosin (H&E) for 15 min at room temperature using light microscope (magnification, x20; Metallurgical Microscope; Shanghai Optical Instrument Import & Export Co., Ltd., Shanghai, China) to analyze the number of neurocytes.

**Evaluation of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6, malondialdehyde (MDA) and superoxide dismutase (SOD) activities.** Rats were sacrificed by decapitation and

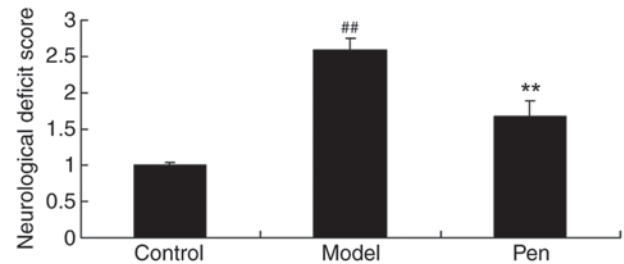


Figure 2. Protective effects of pentoxifylline on neurological deficit score. <sup>##</sup>P<0.01 vs. control group; <sup>\*\*</sup>P<0.01 vs. model group. Control, sham-operated group; model, cerebral ischemia reperfusion injury model group; pen, pentoxifylline-treated group.

blood samples (0.5 ml) were collected from the femoral vein. Serum was isolated from the blood following centrifugation at 1,000 x g for 20 min at 4°C. ELISA analyses were performed using ELISA kits for TNF- $\alpha$  (cat. no. KRC3012; Biosource; Thermo Fisher Scientific, Inc., Waltham, MA, USA), IL-6 (cat. no. BMS625TEN; Biosource; Thermo Fisher Scientific, Inc.), MDA (cat. no. A003-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and SOD (cat. no. A001-1-1; Nanjing Jiancheng Bioengineering Institute) to measure their activities in serum samples, according to the manufacturer's protocol.

**Measurement of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) mRNA expression.** Total RNA was prepared from cerebral infarct tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA samples (1 mg) were used for cDNA synthesis, which was performed using the RevertAid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The temperature protocol was as follows: 37°C for 1 h and 42°C for 10 min. Quantitative (q)PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) by the ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) to measure the mRNA expression of COX-2 and iNOS (n=3). The following primers were used: COX-2 sense, 5'-GTGGGA TGACGAGCGACTGT-3' and antisense 5'-TTTCAGGGA GAAGCGTTTGC-3'; iNOS sense, 5'-GCATCCCAAGTA CGAGTGGT-3' and antisense 5'-GAAGGCGTAGCTGAA CAAGG-3'; and GAPDH sense, 5'-CCATCACTGCCACTC AGAAGA-3' and antisense 5'-CATGAGGTCCACCACCCT GT-3'. The PCR conditions were 94°C for 5 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, and then

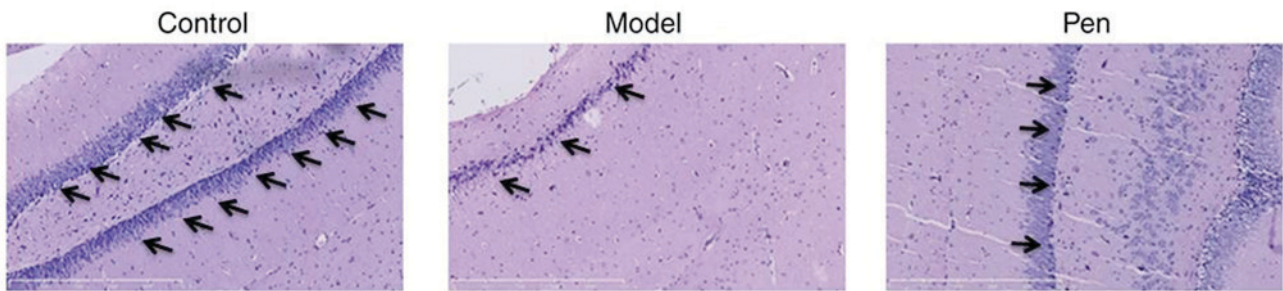


Figure 3. Protective effects of pentoxifylline on neurocytes. Tissues were stained with hematoxylin and eosin (magnification, x20). Control, sham-operated group; model, cerebral ischemia reperfusion injury model group; pen, pentoxifylline-treated group.

4°C for 10 min. miRNA expression were measured using the  $2^{-\Delta\Delta Ct}$  method (15). Experiments were repeated three times.

**Western blotting.** Rats were sacrificed by decapitation and cerebral infarct tissues were rapidly acquired. Cerebral infarct tissues were frozen at -80°C. Subsequently, brain tissues were ground into a powder with liquid nitrogen and lysed in radio-immunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) for 30 min at 4°C, which was followed by centrifugation at 12,000 x g for 5 min at 4°C. The supernatant was collected and the protein concentration was determined using a BCA Protein assay kit (Sangon Biotech Co., Ltd., Shanghai, China). Proteins (50 µg) were separated on 12% SDS polyacrylamide gels (Sangon Biotech Co., Ltd.) and electrophoretically transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA), which were then blocked using 5% non-fat milk in TBST (TBS containing 0.1% Tween-20) for 1 h at 37°C. Membranes were incubated overnight at 4°C with anti-COX-2 (cat. no. sc-7951; 1:2,000; Santa Cruz Biotechnology), anti-iNOS (cat. no. sc-649; 1:2,000; Santa Cruz Biotechnology), anti-cleaved caspase-3 (cat. no. 9662; 1:2,000; Santa Cruz Biotechnology), anti-matrix metalloproteinase (MMP)-9 (cat. no. sc-10737; 1:2,000; Santa Cruz Biotechnology), anti-phosphorylated (p)-p38 mitogen-activated protein kinase (cat. no. 4511; 1:3,000; Cell Signaling Technology, Inc.) and anti-GAPDH (cat. no. sc-25778; 1:500; Santa Cruz Biotechnology). Membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated IgG (cat. no. sc-2004; 1:5,000; Santa Cruz Biotechnology) for 1 h at 37°C. Protein expression was observed with the BeyoECL Plus chemiluminescence reagent (Beyotime Institute of Biotechnology) and analyzed using Image\_Lab\_3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Statistical analysis.** Data are presented as the mean + standard error of the mean. Statistical analysis was performed by one-way analysis of variance and Dunnett's post-hoc test, which was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Protective effects of pentoxifylline on neurological deficit score.** To determine the protective effects of pentoxifylline

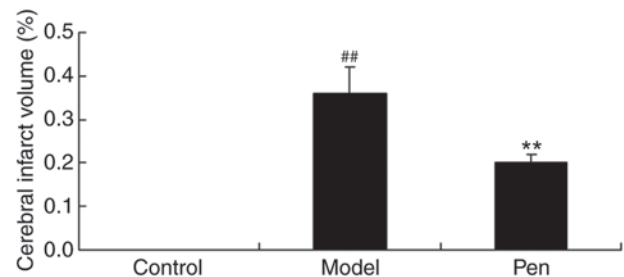


Figure 4. Protective effects of pentoxifylline on cerebral infarct volume. <sup>##</sup> $P < 0.01$  vs. control group; <sup>\*\*</sup> $P < 0.01$  vs. model group. Control, sham-operated group; model, cerebral ischemia reperfusion injury model group; pen, pentoxifylline-treated group.

on neurological deficit score, neurological deficit scores were evaluated. As presented in Fig. 2, there was a significant increase in the neurological deficit score of the cerebral IRI model group, compared with the sham-operated rats (control) group. However, pretreatment with pentoxifylline significantly reduced the neurological deficit score of cerebral IRI rats (Fig. 2).

**Protective effects of pentoxifylline on neurocytes.** To further determine the protective effects of pentoxifylline on neurocytes, neurocytes in brain sections were stained using H&E. The number of neurocytes in the sham group was higher compared with the cerebral IRI model group (Fig. 3). However, pentoxifylline treatment increased neurocytes compared with the cerebral IRI model group (Fig. 3).

**Protective effects of pentoxifylline on cerebral infarct volume.** The present study also determined the protective effects of pentoxifylline on cerebral infarct volume. In the sham group, cerebral infarct volume was lower compared with the cerebral IRI model group (Fig. 4). However, treatment with pentoxifylline significantly reduced the cerebral infarct volume in rats with cerebral IRI (Fig. 4).

**Effects of pentoxifylline on IL-6 and TNF- $\alpha$  levels in cerebral IRI rats.** The present study also determined the effects of pentoxifylline on IL-6 and TNF- $\alpha$  levels in the serum of cerebral IRI rats using ELISA kits. As demonstrated in Fig. 5, a significant increase in the levels of TNF- $\alpha$  and IL-6 was observed in cerebral IRI rats compared with the sham-operated group. Pretreatment with pentoxifylline

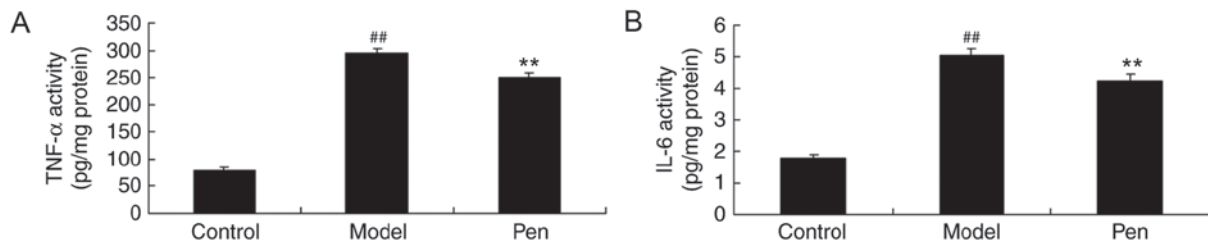


Figure 5. Pentoxifylline treatment affects IL-6 and TNF- $\alpha$  serum levels in cerebral IRI rats. Effects of pentoxifylline on (A) TNF- $\alpha$  and (B) IL-6 levels in cerebral IRI rats. <sup>##</sup>P<0.01 vs. control group; <sup>\*\*</sup>P<0.01 vs. model group. TNF, tumor necrosis factor; IL, interleukin; IRI, ischemia reperfusion injury; control, sham-operated group; model, cerebral ischemia reperfusion injury model group; pen, pentoxifylline-treated group.

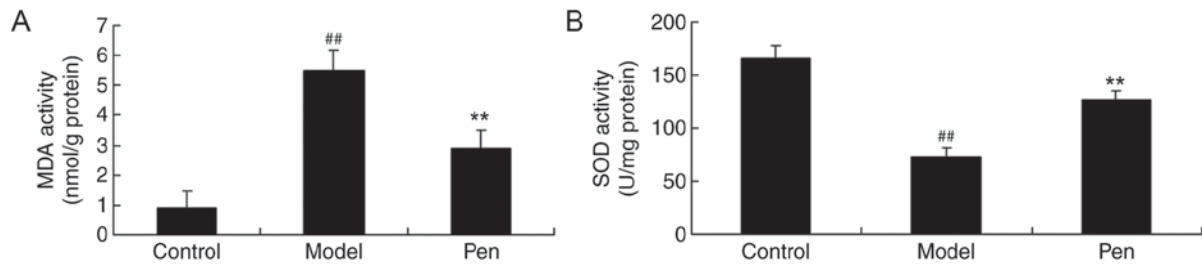


Figure 6. Pentoxifylline treatment affects MDA and SOD levels in cerebral IRI rats. Effects of pentoxifylline on (A) MDA and (B) SOD levels in cerebral IRI rats. <sup>##</sup>P<0.01 vs. control group; <sup>\*\*</sup>P<0.01 vs. model group. MDA, malondialdehyde; SOD, superoxide dismutase; IRI, ischemia reperfusion injury; control, sham-operated group; model, cerebral ischemia reperfusion injury model group; pen, pentoxifylline-treated group.

significantly suppressed the levels of IL-6 and TNF- $\alpha$  in the serum of cerebral IRI rats (Fig. 5).

*Effects of pentoxifylline on MDA and SOD levels in cerebral IRI rats.* Furthermore, the effects of pentoxifylline on MDA and SOD levels in the serum of cerebral IRI rats were investigated by ELISA. Compared with the sham-operated rats, there was a significant increase in MDA and reduction in SOD levels in the serum of cerebral IRI model rats (Fig. 6). However, the MDA and SOD levels were significantly reversed by treatment with pentoxifylline in cerebral IRI rats (Fig. 6).

*Effects of pentoxifylline on COX-2 and iNOS mRNA and protein expression in cerebral IRI rats.* Reverse transcription-qPCR (RT-qPCR) analysis and western blotting was performed to determine the changes in COX-2 and iNOS mRNA and protein expression, respectively. The sham-operated group exhibited a significant decrease in COX-2 and iNOS mRNA and protein expression, compared with cerebral IRI model rats (Fig. 7). However, treatment with pentoxifylline significantly suppressed the COX-2 and iNOS mRNA and protein expression in cerebral IRI rats (Fig. 7).

*Effects of pentoxifylline on cleaved caspase-3, MMP-9 and p38 protein expression in cerebral IRI rats.* In order to determine the effects of pentoxifylline on cleaved caspase-3, MMP-9 and p-p38 MAPK protein expression in cerebral IRI rats, western blotting was performed. The protein expression of cleaved caspase-3 and p-p38 MAPK was significantly increased in cerebral IRI rats, while the expression of MMP-9 was significantly reduced, compared with sham control rats (Fig. 8). However, the protein expression of cleaved caspase-3,

MMP-9 and p38 was significantly reversed with pentoxifylline administration in cerebral IRI rats (Fig. 8).

## Discussion

Ischemic cerebral vascular disease is a common neurological disease, and its high disability and death rates negatively affect families and society (16). At present, the principle of treatment is to restore the blood supply to the ischemic area, however, the reperfusion injury that follows this may exacerbate the brain dysfunction and cause further damage to the tissue, which is termed IRI injury (17). IRI often occurs in the recanalization of cerebral vascular embolization, heart failure correction, shock correction, IRI microcirculation recanalization and cardiopulmonary-cerebral resuscitation (18). With the increased understanding of the pathophysiological mechanism of cerebral IRI, the inhibition of reperfusion injury has become an important part of the treatment of ischemic cerebrovascular disease (19), and the effectiveness and safety of these treatments is the focus in the clinic. In the present study, pretreatment with pentoxifylline significantly reduced the neurological deficit score and cerebral infarct volume in rats with cerebral IRI. Taken together, these results indicate that pentoxifylline may be a potential candidate drug for cerebral IRI.

COX is a type of rate-limiting enzyme that catalyzes the synthesis of prostaglandin and thromboxane from arachidonic acid (20). There are two isomers of COX; COX-1 exists in the majority of tissues as a structural type and catalyzes the production of prostaglandins required to maintain the normal physiological function (21,22), and COX-2, which is abnormally expressed following cerebral ischemia, is a key enzyme in the production of free radicals and inflammatory mediators, and is involved in the development of ischemic brain

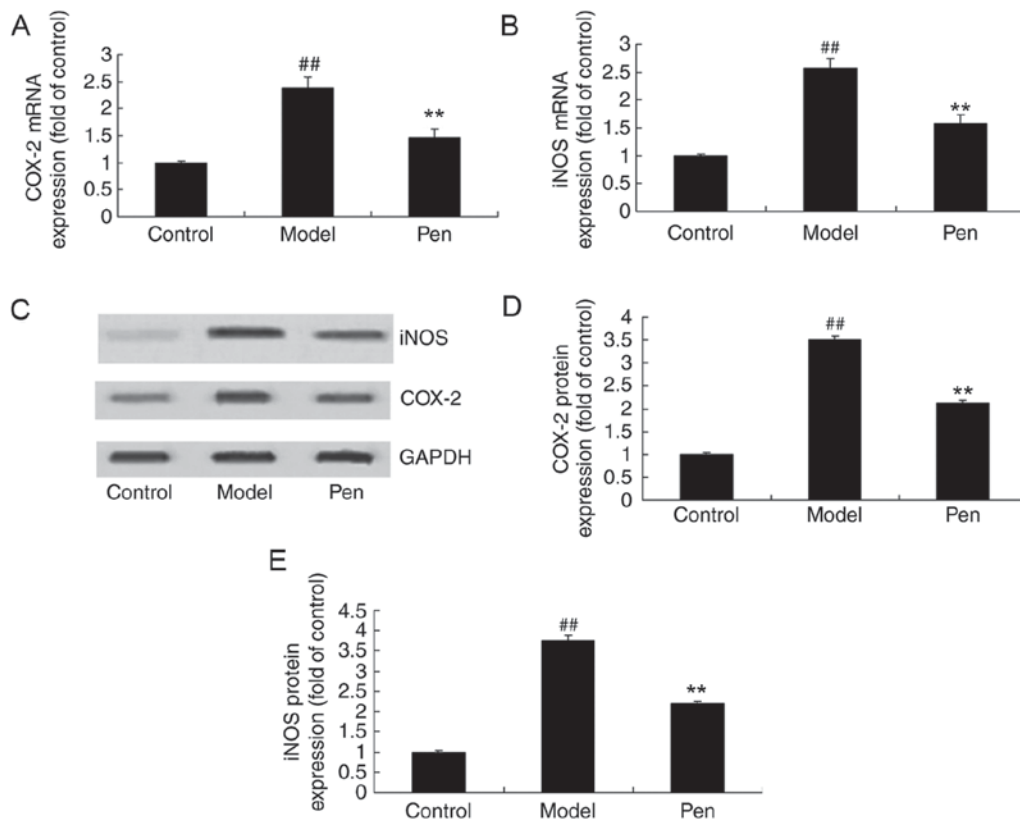


Figure 7. Pentoxifylline treatment affects COX-2 and iNOS mRNA and protein expression in cerebral IRI rats. Effects of pentoxifylline on the mRNA expression of (A) COX-2 and (B) iNOS. (C) Representative western blot for COX-2 and iNOS protein expression. Densitometric and statistical analysis of (D) COX-2 and (E) iNOS protein expression in cerebral IRI rats. <sup>##</sup>P<0.01 vs. control group; <sup>\*\*</sup>P<0.01 vs. model group. COX, cyclooxygenase; iNOS, inducible nitric oxide synthase; IRI, ischemia reperfusion injury; control, sham-operated group; model, cerebral ischemia reperfusion injury model group; pen, pentoxifylline-treated group.

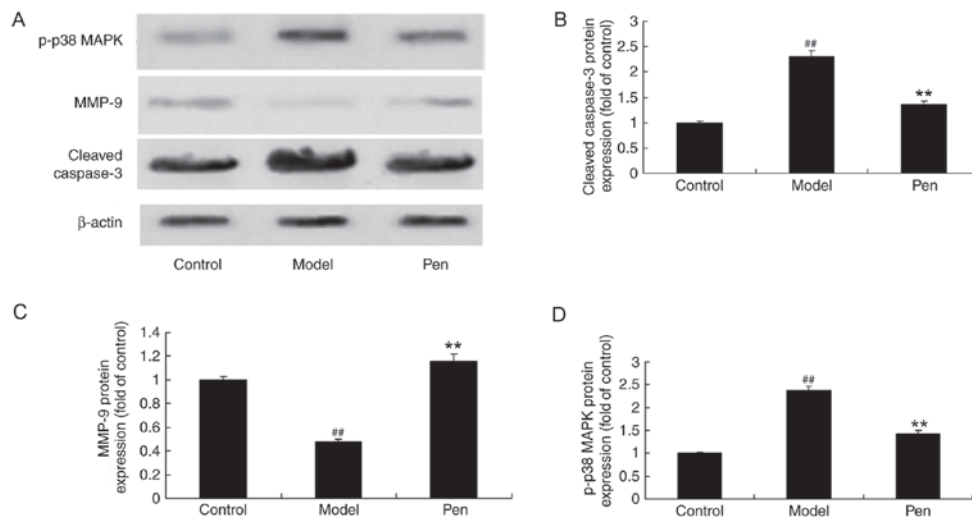


Figure 8. Pentoxifylline treatment affects cleaved caspase-3, MMP-9 and p-p38 MAPK protein expression in cerebral IRI rats. (A) Representative western blot demonstrating the effects of pentoxifylline on cleaved caspase-3, MMP-9 and p-p38 MAPK protein expression. Densitometric and statistical analysis of (B) cleaved caspase-3, (C) MMP-9 and (D) p-p38 MAPK protein expression in cerebral IRI rats. <sup>##</sup>P<0.01 vs. control group; <sup>\*\*</sup>P<0.01 vs. model group. MMP, matrix metalloproteinase; p-, phosphorylated-; MAPK, mitogen-activated protein kinase; IRI, ischemia reperfusion injury; control, sham-operated group; model, cerebral ischemia reperfusion injury model group; pen, pentoxifylline-treated group.

injury and closely associated with the prognosis of cerebral ischemia (21,23). Oxidative damage and the mechanism of death in ischemic neurons has received increased attention in research. The results of the current study demonstrated that

pretreatment with pentoxifylline significantly suppressed IL-6 and TNF- $\alpha$  levels, inhibited MDA and increased SOD levels, and reduced COX-2 mRNA and protein expression in cerebral IRI rats. Marques *et al* (24) reported that pentoxifylline may

attenuate the inflammatory process and apoptosis via cleaved caspase-3 and COX-2 in rats with intestinal IRI. In addition, Mayyas *et al* (25) observed that pentoxifylline suppressed myocardial oxidative status following intake of a western diet.

MMP-9 is a type of zinc-dependent proteolytic enzyme (26). In the neuroinflammation reaction, it is released by the stimulation of cytokines and immediate-early factors, reacts to various proinflammatory stimuli and is involved in the inflammatory reaction and pathophysiological process of various nervous system diseases (27). MMP-9 has an important role in the physiological and pathological processes of the central nervous system (28). A previous study indicated that MMP-9 also has an important role in the development of ischemic cerebrovascular disease (29). It has been demonstrated that MMP-9 is expressed in the hippocampus, cortex and striatum of normal brain tissue, although the expression level is very low (30). Following cerebral IRI injury, the expression of MMP-9 in the ischemic brain tissue is reported to be markedly enhanced (30). Notably, the present study demonstrated that treatment with pentoxifylline significantly reduced the iNOS mRNA and protein expression, and induced MMP-9 protein expression in cerebral IRI rats. Garcia *et al* (31) reported that pentoxifylline decreased glycemia levels through suppression of NOS and COX-2 expression in the pancreas of diabetic rats. In addition, de Campos *et al* (32) demonstrated that pentoxifylline attenuated pulmonary inflammation via MMP-9 in experimental acute pancreatitis.

A previous study confirmed that the p38 MAPK signaling pathway is closely associated with nerve cell damage (33). During cerebral IRI, p38 MAPK is activated, and the inhibition of p38 MAPK may protect nerve cells from IRI (34). Activation of the p38 MAPK pathway is reported to regulate nitric oxide levels by influencing the expression of iNOS (35). Nitric oxide is an important factor that affects the expression of MMP-9, which directly degrades collagen IV and subsequently leads to increases in blood brain barrier permeability and the exosmosis of IgG from the blood vessel (35). The results of the current study indicate that treatment with pentoxifylline significantly attenuated caspase-3 and p-p38 protein expression in cerebral IRI rats. Costantini *et al* (36) reported that pentoxifylline suppressed leukoreduced stored blood-induced neutrophil activation via p38 MAPK and extracellular signal-regulated kinase phosphorylation.

In conclusion, the results of the present study demonstrated that pentoxifylline inhibits the neurological deficit score and cerebral infarct volume in cerebral IRI rats, which may occur via anti-inflammation, antioxidation and antiapoptotic mechanisms as pentoxifylline suppressed COX-2 expression, increased MMP-9 expression and downregulated p38 pathways in cerebral IRI rats *in vivo*. Further research is required to evaluate the potential beneficial effect of pentoxifylline on cerebral IRI in the clinic.

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