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Retigabine attenuates focal cerebral ischemic injury through inhibiting mitochondria-dependent apoptotic pathway

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Abstract. – OBJECTIVE: We explored the protective effect of retigabine (RTG) on focal cerebral ischemic injury and the potential molecular mechanism.

MATERIALS AND METHODS: A mouse model of middle cerebral artery occlusion (MCAO) was established to induce cerebral ischemic injury. Blood samples were collected for the measurement of malondialdehyde (MDA), superoxide dismutase (SOD) and reduced glutathione (GSH). The brain infarct volume was stained by triphenyltetrazolium chloride. The cell apoptosis was observed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining. The expression of B-cell lymphoma-2 (Bcl-2), BCL2-Associated X (Bax), cleaved caspase 3, p-p38 and p-JNK, were determined by Western blot.

RESULTS: RTG treatment reduced the MCAO-induced increase in brain infarct volume and neurological deficit scores. RTG treatment reduced the level of MDA and increased the activity of SOD and GSH. RTG treatment also decreased the Bax/Bcl-2 ratio and cleaved caspase 3 expression in the ischemic tissues. Further, RTG treatment decreased the phosphorylation levels of p38 and JNK in the ischemic tissues.

CONCLUSIONS: RTG attenuated cerebral ischemic injury through reducing oxidative stress and mitochondria-mediated apoptosis via inhibiting p38 and JNK phosphorylation.

Key Words:
Retigabine (RTG), Cerebral ischemic injury, Apoptosis, Oxidative stress, Mitogen-activated protein kinase.

Introduction

Ischemic stroke due to the occlusion of blood vessels or thrombosis still remains a leading cause of mortality and disability worldwide[1]. Ischemic stroke results in cell necrosis in the central region of ischemia and apoptosis in the potentially salvaged penumbra region.. The pathological events involved in the penumbra are characterized by glutamate excitotoxicity, oxidative stress, and apoptosis[2,3]. However, the effective treatment for ischemic stroke is extremely limited. Currently, recombinant tissue plasminogen activator (rt-PA) is the only clinically effective drug for ischemic stroke. However, because of the narrow treatment window and an increased risk of hemorrhage[4], the therapeutic effect is not satisfactory. Therefore, the development of novel neuroprotective agents for the treatment of ischemic stroke is critical needed.

Retigabine (RTG) is a novel antiepileptic drug and currently used for partial seizures in adults. It has the characteristics of stable neuronal membrane potential and anti-excitabilit[5]. In addition, RTG also has potential therapeutic effect for diseases such as neuropathic pain[6], neurodegenerative diseases[7] and dystonia[8]. Moreover, RTG has been reported to exert antioxidant effect and inhibit apoptosis on cultured hippocampal cells after serum and oxygen deprivation *in vitro*[9,10]. However, the role of RTG on ischemic stroke has not been reported. In the present study, we established a mouse model of ischemic stroke induced by middle cerebral artery occlusion (MCAO), and investigated the role and mechanism of RTG in focal cerebral ischemic injury.

Materials and Methods

Ethics Statements

Animal experiments were implemented in accordance with the Guide for the Care and Use of Laboratory Animals published by the NIH. 10 weeks old male C57BL/6 mice weigh-

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ing 20-30 g were housed in a room at 22°C with 12:12 hour light/dark cycles and fed standard mouse food and water. This study was approved by the Animal Ethics Committee of The First Hospital of Jilin University Animal Center.

Mouse Model of MCAO

The mouse model of middle cerebral artery occlusion (MCAO) was performed as previously described[11]. Briefly, the mice were anaesthetized by pentobarbital (50 mg/kg, intraperitoneal injection) and fixed. A longitudinal incision was made at the middle of neck. After the ligation and abscission of the external carotid artery (ECA), a 6-0 surgical monofilament with a rounded tip was inserted into the internal carotid artery through the ECA. The filament was used to block the origin of the left middle cerebral artery (MCA) at approximately 10-11 mm from the carotid bifurcation. The sham-operated mice were underwent the same surgical procedure without the occlusion of the MCA. There were three groups in this study: i) sham group; ii) MCAO group, saline (10 mL/kg) was injected intraperitoneally 1 h after ischemia; iii) MCAO + retigabine (RTG) group, RTG (10 mg/kg) was injected intraperitoneally 1 h after ischemia. n=15 in each group. After MCAO for 24 h, mice were sacrificed and the brain samples were harvested for analysis.

Neurological Deficit Scores

After MCAO for 24 h, neurological deficit scores were evaluated with a single-blind method according to Longa et al[11]: 0 points for no neurological deficit; 1 point when the contralateral forelimb of the lesion cannot be completely extended when the tail is lifted up; 2 points for circling to the contralateral side when walking; 3 points for falling to the contralateral side when walking; 4 points for unable to walk spontaneously and losing consciousness. The higher of the neurological deficit score, the more severe of the cerebral ischemic injury.

Brain Infarct Volume

Brain samples were excised and sliced. The brain sections were stained with 1% triphenyltetrazolium chloride at 37°C for 20 min and then fixed with 4% paraformaldehyde overnight at room temperature. Infracted tissue was carefully separated from the non-infarcted tissue and the brain infarct volume was calculated according to the following formula: brain infarct volume (%) = [(normal hemisphere volume - non-infarct volume of infarct side) / normal hemispheric volume] × 100%.

Oxidative Stress Markers

After MCAO for 24 h, the ischemic tissues were separated and homogenized. The level of malondialdehyde (MDA), as well as the activities of superoxide dismutase (SOD) and reduced glutathione (GSH), were measured according to the instructions provided by the assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The results were normalized to the protein concentration and expressed as nmol/mg protein or U/mg protein.

Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP Nick-End Labeling (TUNEL) Assay

Cell apoptosis was detected using *in situ* cell death detection kit (Roche, Mannheim, Germany). Brain tissue sections were stained with the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) reaction mixture and Converter-POD, and then observed under a microscope (Eclipse, Nikon, Tokyo, Japan). Apoptotic nuclei was shown in brown, while normal nuclei was shown in blue. The amount of TUNEL-positive nuclei was expressed as a percentage of total nuclei.

Western Blotting

Western blot analysis was used to determine the expression of proteins extracted from the ischemic tissues. Briefly, gel electrophoresis was performed to separate the proteins with different molecular weight, which were then transferred onto polyvinylidene difluoride (PVDF)membranes. These membranes were incubated with anti-Bcl-2 (B-cell lymphoma-2), anti-Bax (BCL2-Associated X), anti-cleaved caspase3, anti-p-p38, anti-p38, anti-p-JNK and anti-JNK (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. After incubated with these primary antibodies, the membranes were washed in Tris-buffered saline and Tween 20 (TBST) (Beyotime, Shanghai, China) and then incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for another 2 h. Western Blot Detection kit and Image J software (NIH) were used to measure the blot signal and density.

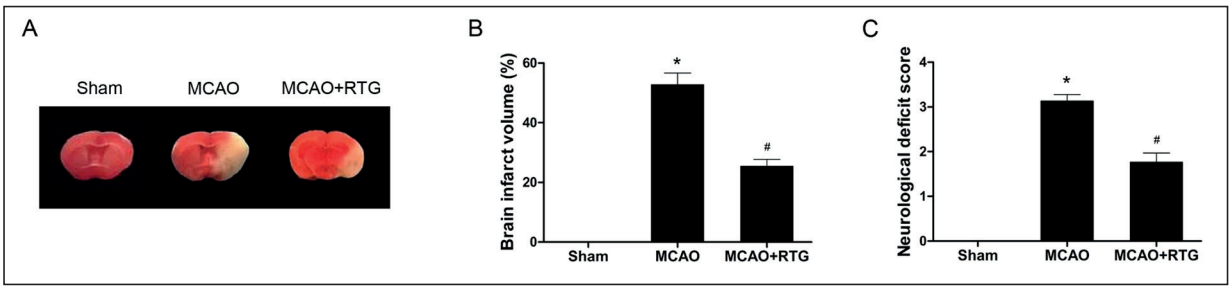

Figure 1. Figure 1. RTG reduced brain infarct volume and neurological deficit scores. ***A,*** Representative brain sections; ***B,*** Brain infarct volume; ***C,*** Neurological deficit scores. *$p < 0.05$ *vs.* sham group; #$p < 0.05$ *vs.* MCAO group.

Statistical Analysis

All results were presented as means ± standard deviations (SD). Differences among different groups were analyzed by One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). A value of $p < 0.05$ was confirmed to be statistically significant.

Results

RTG Reduced Infarct Volume and Neurological Deficit Scores

As shown in Figure 1, the infarct tissue was white, while the normal tissue was red. The infarct volume and neurological deficit scores were significantly increased in the MCAO group compared with the sham group. RTG treatment significantly reduced the infarct volume and neurological deficit scores in contrast to the MCAO group.

RTG Reduced the Levels of Oxidative Stress Markers

As shown in Figure 2, the level of MDA was significantly increased, while the levels of SOD and GSH were significantly decreased in the MCAO group compared with the sham group. RTG treatment significantly reduced the level of MDA and increased the levels of SOD and GSH in contrast to the MCAO group.

RTG Inhibited MCAO-Induced Apoptosis

As shown in Figure 3, the TUNEL staining was used to detect apoptosis. The number of apoptotic nuclei (shown in brown) was significantly increased in the MCAO group compared with the sham group. In contrast, RTG treatment significantly reduced the number of apoptotic nuclei induced by MCAO.

As shown in Figure 4, the expression of Bax was significantly increased, whereas the expression of Bcl-2 was significantly decreased, which resulted in a higher ratio of Bax/Bcl-2 in the MCAO group than that in the sham group. The expression of cleaved caspase-3 was also increased in the MCAO group compared with the sham group. RTG treatment significantly reduced the ratio of Bax/Bcl-2 and the expression of cleaved caspase-3 in contrast to the MCAO group.

RTG Inhibited MCAO-Induced p38 and JNK Phosphorylation

As shown in Figure 5, the phosphorylation levels of p38 and JNK were both significantly

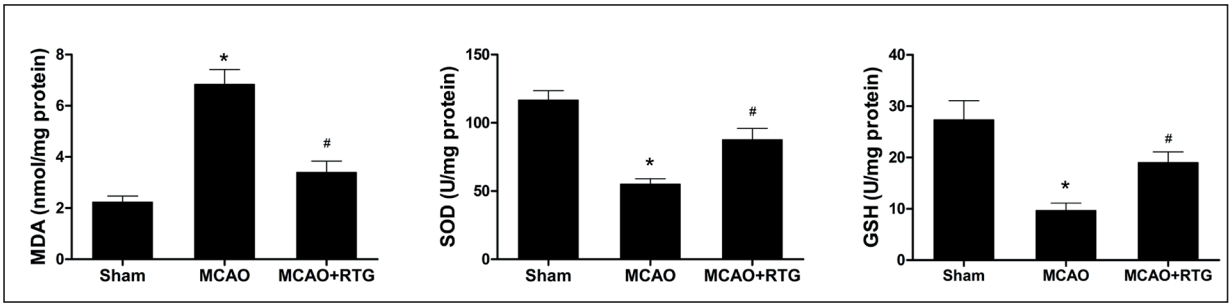

Figure 2. RTG reduced the levels of oxidative stress markers. *$p < 0.05$ *vs.* sham group; #$p < 0.05$ *vs.* MCAO group.

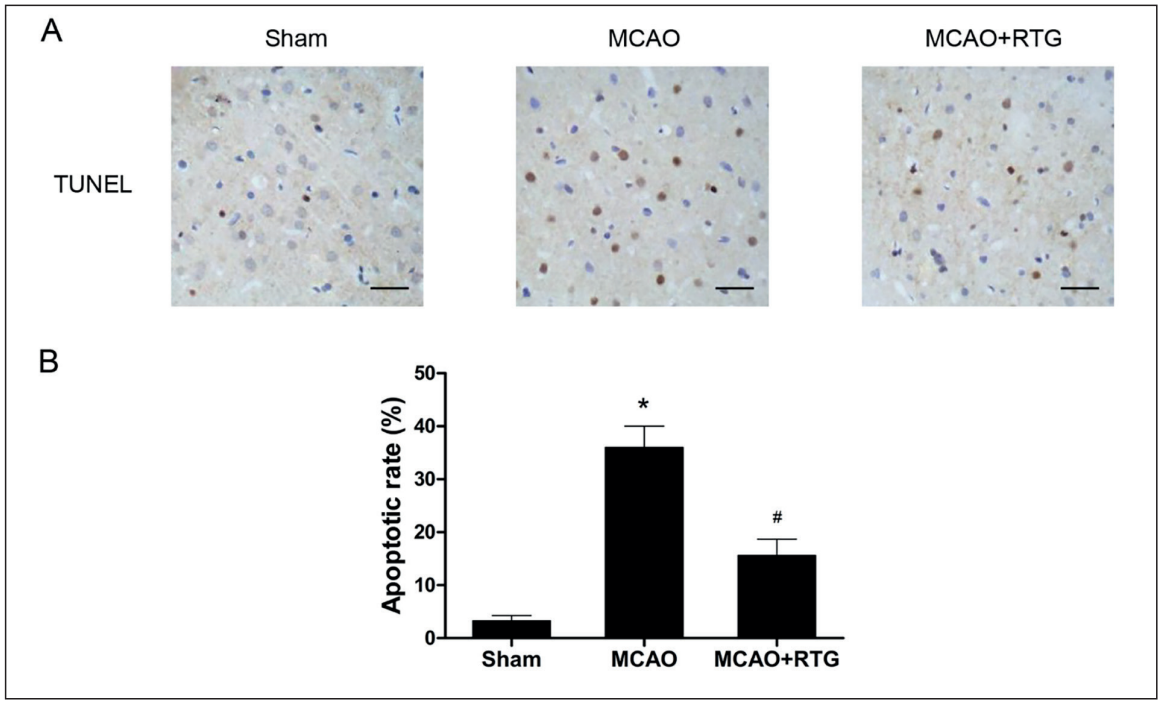

Figure 3. RTG inhibited MCAO-induced apoptosis. ***A,*** Representative TUNEL staining; ***B,*** Apoptotic rate in each group. *$p < 0.05$ *vs.* sham group; #$p < 0.05$ *vs.* MCAO group.

increased in the MCAO group compared with the sham group. RTG treatment significantly inhibited the phosphorylation levels of p38 and JNK induced by MCAO.

Discussion

The present study focused on the protective effect of retigabine (RTG) on focal cerebral ischemic injury in a mouse model of MCAO. Our results showed that RTG treatment markedly attenuated cerebral ischemic injury, as confirmed by the decrease in brain infarct volume and neurological deficit scores. This protective effect was associated with the inhibition of oxidative stress and mitochondria-mediated apoptosis, as well as the blockage of p38 and JNK signals.

Oxidative stress plays a critical role in cerebral ischemic injury[12,13] and results in mitochondria damage, cell apoptosis and brain dysfunction[14,15]. MDA is a toxic product of lipid peroxidation and is a sensitive marker of oxidative stress[16]. As endogenous antioxidant enzymes, SOD and GSH play important roles in the maintenance of redox homeostasis through inhibiting excessive ROS generation[17,18]. In this work, we demonstrated that RTG treatment significantly decreased MDA level and increased the activity of SOD and GSH in the ischemic tissues. These

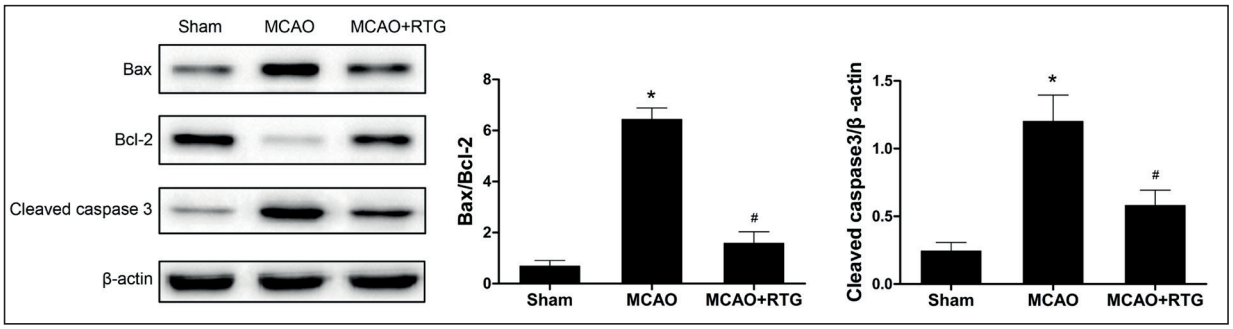

Figure 4. RTG inhibited mitochondria-mediated apoptosis. *$p < 0.05$ *vs.* sham group; #$p < 0.05$.*vs.* MCAO group.

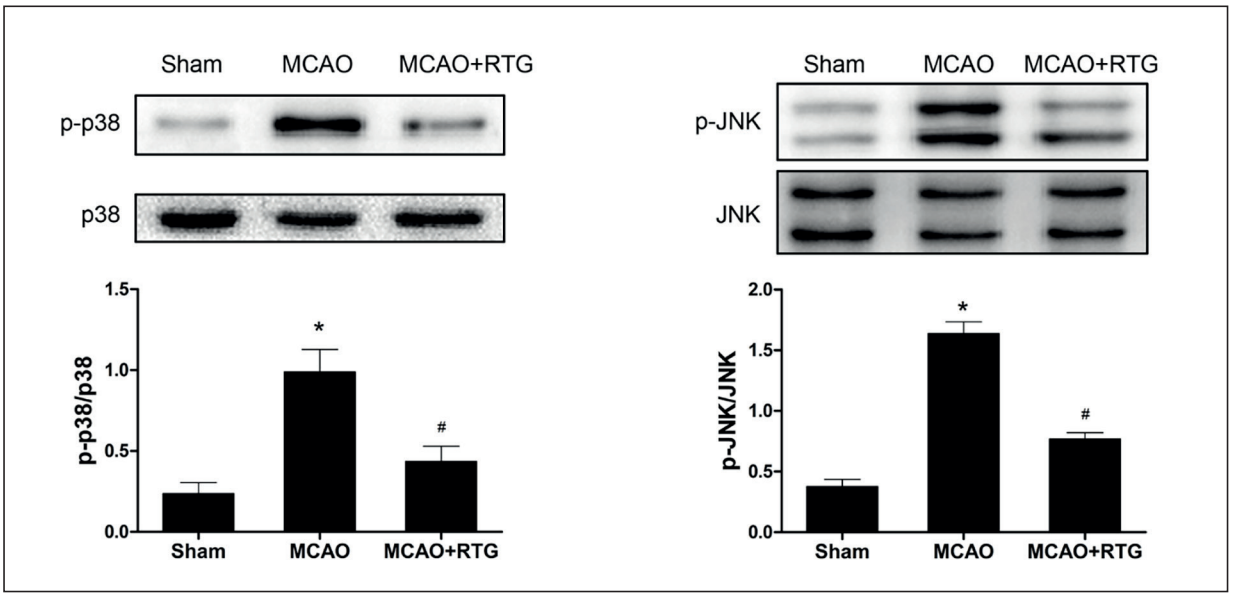

Figure 5. RTG inhibited the phosphorylation of p38 and JNK. *$p < 0.05$ *vs.* sham group; #$p < 0.05$ *vs.* MCAO group.

results suggested that RTG protected the brain against cerebral ischemic injury through exerting antioxidant effects.

Mitochondria have been considered as the central organelles of cell apoptosis[19]. Mitochondria-mediated apoptosis has also been reported as an important mechanism that involved in cerebral ischemic injury[20]. The dissipation of the mitochondrial membrane potential is the key step in the activation of mitochondrial-mediated apoptosis, which is predominantly modulated by the Bcl-2 family proteins, including Bcl-2 with anti-apoptotic effect and Bax with pro-apoptotic effect. The increase in the ratio of Bax/Bcl-2 led to the release of cytochrome c and activation of caspase-3, thereby resulting in apoptosis[21,22]. In the present study, TUNEL staining revealed that RTG treatment significantly inhibited nerve cell apoptosis induced by MCAO. RTG treatment could also decrease the ratio of Bax/Bcl-2 and the expression of cleaved caspase 3 in the ischemic tissues. Collectively, RTG exerted an anti-apoptotic effect by inhibiting the activation of mitochondrial-mediated apoptosis pathway.

p38 and c-Jun N-terminal kinase (JNK) are members of the mitogen-activated protein kinase (MAPK) signaling family[23] and have been viewed as two major effectors involved in mitochondria-dependent apoptosis[24]. The activated JNK and p38 can inhibit the anti-apoptotic role of Bcl-2 via phosphorylating Bcl-2[25,26]. Moreover, Kim et al[27] reported that the activation of p38 could facilitate apoptosis by increasing the expression of the pro-apoptotic factor Bax. Donovan et al[28] reported that phosphorylated JNK could facilitate the mitochondria-mediated apoptosis by activating the pro-apoptotic factors Bad and Bid. Conversely, cell apoptosis could be significantly inhibited by the specific inhibitors of JNK and p38 (SP600125 and SB203580) in response to numerous stimuli *in vitro*[24]. In this study, we found that the phosphorylation levels of p38 and JNK were significantly increased in the ischemic tissues. RTG inhibited the phosphorylation levels of p38 and JNK induced by MCAO. It is implicated that RTG attenuated-cerebral ischemic injury was associated with the inhibition of p38 and JNK phosphorylation.

Conclusions

We showed that RTG could inhibit cerebral ischemic injury induced by MCAO. The molecular mechanism involved in the protective effect of RTG was associated with the inhibition of oxidative stress and mitochondria-mediated apoptosis, as well as the blockage of p38 and JNK signals. Our results suggested that RTG could be applied as a potential treatment agent for ischemic stroke.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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