

Mifepristone alleviates cerebral ischemia-reperfusion injury in rats by stimulating PPAR γ

X.-J. WU, X.-H. SUN, S.-W. WANG, J.-L. CHEN, Y.-H. BI, D.-X. JIANG

Department of Neurology, Weihai Central Hospital of Shandong, Weihai, China

Abstract. – **OBJECTIVE:** To investigate the changes of peroxisome proliferator-activated receptor gamma (PPAR γ) in focal cerebral ischemia-reperfusion injury, and to explore the effect and mechanism of mifepristone on the cerebral ischemia-reperfusion injury.

MATERIALS AND METHODS: Male Sprague-Dawley (SD) rats were selected, and the middle cerebral artery occlusion and reperfusion (MCAO/R) rat model was constructed using the long's suture-occluded method. The sham operation group was not inserted with occlusion sutures. All experimental rats were divided into four groups: the sham operation group (SHA group), the MCAO/R model group (MCR group), the mifepristone intervention group (MIF group) (3 mg/kg, intragastric administration), and the mifepristone + bisphenol A diglycidyl ether (BADGE) intervention group (MIF+BAD group) [3 mg/kg mifepristone (intragastric administration) + 30 mg/kg BADGE (intraperitoneal injection)]. The long's scoring method (5 grades) was applied for scoring after reperfusion, at the time when the animals woke up, and at 48 h after awaking before execution, respectively. 48 h after the model was successfully established, triphenyl tetrazolium chloride (TTC) staining was performed to calculate the volume of cerebral infarction, and Nissl staining was conducted to observe the cranial nerve tissue morphology. Meanwhile, immune-histochemical staining was used to detect PPAR γ . Moreover, the protein expression levels of PPAR γ , tumor necrosis factor-alpha (TNF- α), interleukin-1 beta (IL-1 β), interleukin-6 (IL-6), matrix metalloproteinase-2 (MMP-2), MMP-9 and tissue inhibitor of metalloproteinase 1 (TIMP-1) were examined by Western blotting (WB).

RESULTS: Mifepristone could significantly enhance the neurological function after cerebral ischemia-reperfusion injury, reduce the volume of cerebral infarction, and improve the morphology of nerve tissues in rats. The expression of PPAR γ in the brain tissues of rats after cerebral ischemia-reperfusion injury markedly declined, whereas mifepristone could remarkably increase the protein expression of PPAR γ . After mifepristone intervention, the protein levels of TNF- α , IL-1 β , IL-6, MMP-2, and MMP-9 in

the infarcted brain tissues of rats were markedly decreased, while the expression of the TIMP-1 protein was increased. When combined with BADGE, the effect of mifepristone was partially offset.

CONCLUSIONS: Mifepristone acts as a PPAR γ agonist, and relieves cerebral ischemia-reperfusion injury by restoring the balance between MMPs and TIMPs and inhibiting inflammatory cytokines.

Key Words:

Mifepristone, Cerebral ischemia-reperfusion injury, PPAR γ , MMPs, Inflammation.

Introduction

Ischemic stroke (IS) is one of the three major causes of deaths in the global population, following malignant tumors and heart diseases¹. With the characteristics of high morbidity, high disability, high mortality, and high recurrence rate, IS brings a heavy burden on the patient's family and the whole society^{2,3}. However, revascularization occurs in IS patients due to thrombolytic therapy or spontaneous movement of emboli. Meanwhile, reperfusion further aggravates brain injury after ischemia^{4,5}. Therefore, the research direction of clinical treatment for acute IS mainly focuses on reducing cerebral ischemia-reperfusion injury.

With the development of an in-depth study on pathological changes after cerebral infarction, it is believed that the activation of inflammatory cytokines induced by cerebral ischemia-reperfusion plays an important role in the pathological changes of post-ischemic brain injury⁶⁻⁸. Peroxisome proliferator-activated receptor gamma (PPAR γ) is a ligand-activated transcriptional regulator that belongs to the ligand-regulated nuclear receptor superfamily. At present, the neuroprotective effect of PPAR γ in cerebral ischemia-reperfusion

injury has aroused increasing attention. Studies⁹⁻¹¹ have revealed that in the animal models of focal cerebral ischemia, the activation degree of PPAR γ is reduced at the corresponding time point after cerebral ischemia-reperfusion injury. In addition, PPAR γ agonists can effectively protect the brain by reducing cell apoptosis and the IS area.

Mifepristone, a steroid compound, is a regular prescription of emergency contraceptives and aborticide. Some studies^{12,13} have manifested that mifepristone exerts its effects by acting on the nuclear receptor PPAR γ and the activity of mediated target genes. However, no researches have been found on mifepristone and its application in cerebral ischemia-reperfusion injury. If mifepristone can affect the activation of PPAR γ during cerebral ischemia-reperfusion injury, it is expected to provide new suggestions for the drug treatment of IS. In this study, the rat model of middle cerebral artery occlusion and reperfusion (MCAO/R) was established by using the suture-occluded method, and we observed the changes of PPAR γ in focal IS. Moreover, mifepristone was combined used with Bisphenol Adiglycidyl Ether (BADGE) (a PPAR γ antagonist) to observe the effects of mifepristone on focal IS injury and the role of PPAR γ in infarcted brain tissues.

Materials and Methods

Construction of the Rat Model

A total of 72 healthy adult male Sprague-Dawley (SD) rats weighing 250-280 g were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). This study was approved by the Animal Ethics Committee of Weihai Central Hospital of Shandong Animal Center. The long's middle cerebral artery suture-occluded method¹⁴ was applied to prepare the MCAO/R rat model. Briefly, 10% chloral hydrate (0.3 mL/100 g) was intraperitoneally injected in rats for anesthesia, and a 2-cm incision was made in the middle of the neck. The left common carotid artery, the left internal carotid artery, the left external carotid artery and the left vagus nerve were isolated. The common carotid artery was ligated with 3.0 surgical sutures, and the external carotid artery was ligated before bifurcation. The common carotid artery close to the bifurcation remained with sutures but was not ligated. First, the internal carotid artery was clipped with a bulldog clamp. An oblique cut

was made using a piece of ophthalmic scissors at the common carotid artery 2 mm away from the bifurcation of the internal carotid artery. The occlusion sutures held by ophthalmologic forceps were inserted into the head and were then gently pushed to the bulldog clamp. Subsequently, the bulldog clamp was removed, and the occlusion sutures were further slowly inserted into the internal carotid artery with ophthalmologic forceps. The occlusion sutures were inserted deeply into approximately 20-22 mm away from the bifurcation of the common carotid artery. A sense of resistance indicated that the occlusion sutures reached the initial segment of the middle cerebral artery. Then, the insertion of the occlusion sutures was stopped, and the surgical sutures at the bifurcation of the internal carotid artery were tightly tied. After the operation, the neck incision was closed with intermittent sutures. The occlusion sutures were set aside about 10 mm outside the incision for reperfusion after 2 h. The sham operation group (SHA group) was not subjected to ligation and suture insertion, but the rest procedures were the same. 2 h after ischemia, the occlusion sutures were pulled to the site where the common carotid artery was ligated to re-canalize the middle cerebral artery.

Determination of Cerebral Infarction Volume

48 h after reperfusion, the rats were decapitated after deep anesthesia. Brain tissues were completely removed, stored at -20°C for 20 min, and evenly cut into 5-6 coronal sections. The brain sections were then immersed in the ready-to-use 1% triphenyl tetrazolium chloride (TTC) solution, followed by incubation in the thermostatic water bath at 37°C for 40 min without light. Subsequently, the sections were removed and fixed in 4% paraformaldehyde for 20 min. Finally, cerebral infarction volume was detected by capturing the sections with a digital camera.

Protein Extraction and Preparation for Paraffin Sections

48 h after reperfusion, the rats were quickly decapitated to remove the brains after deep anesthesia. A total of 50-60 mg brain tissue was weighed and cut into slices on ice, as well as homogenized with a tissue homogenizer. 100-200 μ L radioimmunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China) was then added for cell lysis. After that, the samples were centrifuged at 4°C, 12,000 rpm for 30 min,

and the supernatant was collected. The protein concentration was measured by using the bicinchoninic acid (BCA) kit (Thermo Fisher Scientific, Waltham, MA, USA). Additionally, brain tissues were gradient dehydrated with ethanol, transparent with xylene and immersed into liquid paraffin. After 12 h, paraffin blocks were fixed on a paraffin slicer, and the serial coronal sections were conducted from the frontal pole to the occipital pole to obtain 5- μ m-thick sections. Finally, paraffin sections were prepared.

Nissl Staining

Three sections of each experimental subject were stained at the same location. All the sections were dewaxed, hydrated and immersed in prepared Nissl staining solution twice, with 20s each time. Subsequently, the sections were soaked in 70% ethanol for 2 min and immersed in 95% ethanol. Nissl staining was observed under an optical microscope. If blue-purple Nissl body appeared, the color separation was stopped, and the neutral gum was added dropwise for mounting.

Immunohistochemistry

Paraffin sections were routinely dewaxed, hydrated and repaired with sodium citrate antigen for three times. After the sections were dropwise added with peroxidase to block solution, the PPAR γ antibody (diluted at 1:200) was added dropwise for incubation at 4°C overnight. Subsequently, the biotin-labeled secondary antibody was added for incubation in a wet box at room temperature for 10 min. The ready-to-use 3,3'-diaminobenzidine (DAB) solution was added, and the color development process was observed under a microscope. Finally, hematoxylin was used to counterstain the nucleus, and the sections were let naturally dry and sealed with neutral gum.

Western Blotting (WB)

The proteins were heated in boiling water bath for 3-5 min to completely denature. After cooling to room temperature, the protein samples were loaded into sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel wells for electrophoresis, and were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) by using a wet transfer device. The membrane transfer current was 300-400 mA. Subsequently, the milk blot was added, and the cells were blocked at room temperature for 60 min. The membranes were then incubated with target protein antibodies at

4°C overnight, followed by incubation with relevant secondary antibodies at room temperature for 60 min. Subsequently, the color solution was added for incubation in the dark for 15 s. Finally, LAS-3000 was applied to detect light-emitting bands.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 Software (IBM, Armonk, NY, USA) was used for all statistical analysis. Data were expressed as mean \pm standard deviation. One-way analysis of variance was employed to compare the differences among multiple groups. The least significant difference (LSD) *t*-test was used for the comparison between two groups, and independent samples *t*-test was applied for the comparison of mean values between two groups. The significance level α was set as 0.05. The Kruskal-Wallis test was applied to compare the score of neurological function among multiple groups, followed by Bonferroni correction by using the Mann-Whitney U test. $p < 0.005$ was considered statistically significant.

Results

Effect of Mifepristone on the Neurological Function Injury in MCAO/R Rats

After 48 h of reperfusion, the neurological deficit score in the MCAO/R model group (MCR group) was significantly higher than that of the SHA group, suggesting that the focal IS model was successfully established. Meanwhile, the score was remarkably improved after mifepristone intervention, indicating that mifepristone could significantly improve neurological deficits after cerebral ischemia-reperfusion injury in rats. After the combination of mifepristone and BADGE, the neurological deficit score of rats was significantly different from that of the mifepristone intervention group (MIF group), which was still markedly lower than that of the MCR group (Figure 1A). This suggested that mifepristone could improve the neurological function after cerebral ischemia-reperfusion injury in rats partly through activating the production of PPAR γ .

Effect of Mifepristone on Cerebral Infarction Volume in MCAO/R rats

TTC staining of rat brain tissues revealed that there was no white infarcted area in the

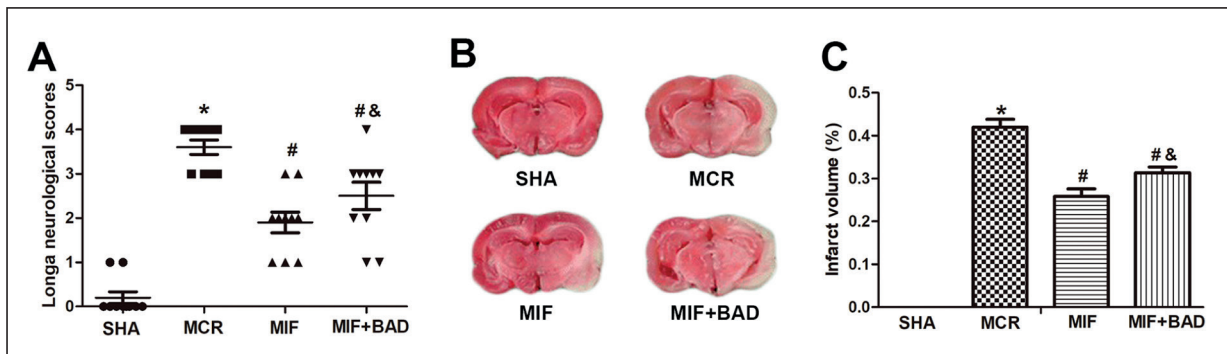


Figure 1. The beneficial effect of mifepristone on neurological function and cerebral infarction volume in MCAO/R rats. *A*, Analysis of neurological deficit scores by the Longa's modified scoring method. *B*, Representative images of cerebral infarct volume by TTC. *C*, Analysis of cerebral infarct volume by TTC. * $p < 0.05$ vs. the SHA group, # $p < 0.05$ vs. the MCR group, & $p < 0.05$ vs. the MIF group.

SHA group. However, results found that white infarcted area appeared in the left middle cerebral artery blood supply area in the MCR group (Figure 1B). Compared with the MCR group, the application of mifepristone could significantly reduce the volume of cerebral infarction in rats. Besides, after combined use of mifepristone and BADGE, the volume of cerebral infarction in rats was significantly different from that of the MIF group. However, it was still markedly decreased when compared with the MCR group (Figure 1C), indicating that mifepristone remarkably improves cerebral ischemia-reperfusion injury in rats and this effect is partly induced by the activation of PPAR γ .

Effect of Mifepristone on the Nerve Tissue Morphology in MCAO/R Rats

Nissl staining indicated that the morphology of neurons in the rat brain tissues of the SHA group was normal. The Nissl body was manifested as a large number of dark blue lumps or particles markedly stained and distributed in tabby shape. In the infarcted brain tissues of the MCR group, neurons were found to be swollen in disorder, and some were detached in the ischemic region of the middle cerebral artery. Besides, Nissl bodies were partially dissolved and disappeared with only the contour left, and the number of intact neurons was markedly declined when compared with the SHA group (Figure 2A). Mifepristone could significantly increase the number of intact

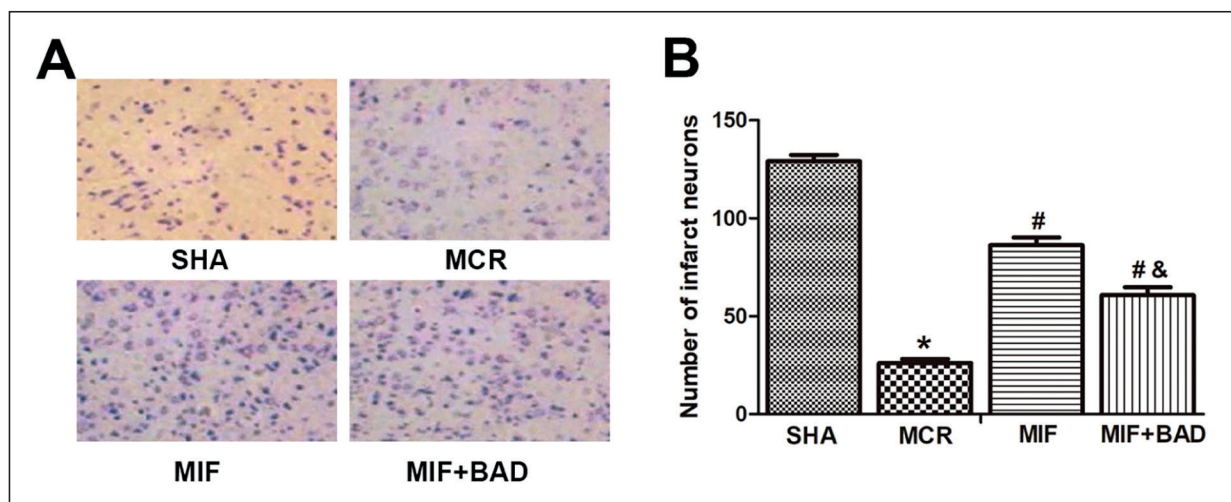


Figure 2. Effect of mifepristone on nerve tissue morphology of MCAO/R rats. *A*, Representative images of the morphology of neurons by Nissl staining (X 200). *B*, Analysis of infarct neurons in each group. * $p < 0.05$ vs. the SHA group, # $p < 0.05$ vs. the MCR group, & $p < 0.05$ vs. the MIF group.

neurons within the infarcted area of rats. Meanwhile, after the combined use of mifepristone and BADGE, the number of intact neurons was significantly different from that of the MIF group, but was still significantly higher than that of the MCR group (Figure 2B). These results suggested that mifepristone could improve cerebral ischemia-reperfusion injury in rats, and this effect is partially triggered by activating PPAR γ .

Effect of Mifepristone on PPAR γ in the Infarcted Brain Tissues of MCAO/R Rats

Immunohistochemistry demonstrated that compared with the SHA group, the percentage of PPAR γ -positive cells in the brain tissues of the MCR group was significantly decreased, indicating that the function of PPAR γ was significantly weakened 48 h after ischemia-reperfusion (Figure 3A). The percentage of PPAR γ -positive cells in the MIF group was remarkably increased, indi-

cating that mifepristone activated PPAR γ . After the combined use of mifepristone and BADGE, the percentage of PPAR γ -positive cells was not significantly different from that of the MCR group, suggesting that BADGE totally blocked the activation of PPAR γ through mifepristone (Figure 3B). WB results illustrated that compared with the SHA group, the protein expression of PPAR γ in the brain tissues was remarkably declined in the MCR group, but was significantly increased in the MIF group. After the combined application of mifepristone and BADGE, there were no significant differences in PPAR γ function and protein expression when compared with those of the MCR group. The above results all indicated that mifepristone not only enhances the nuclear translocation of PPAR γ , but also increases its protein expression level. However, BADGE completely retarded the effect of mifepristone on PPAR γ (Figure 3C and 3D).

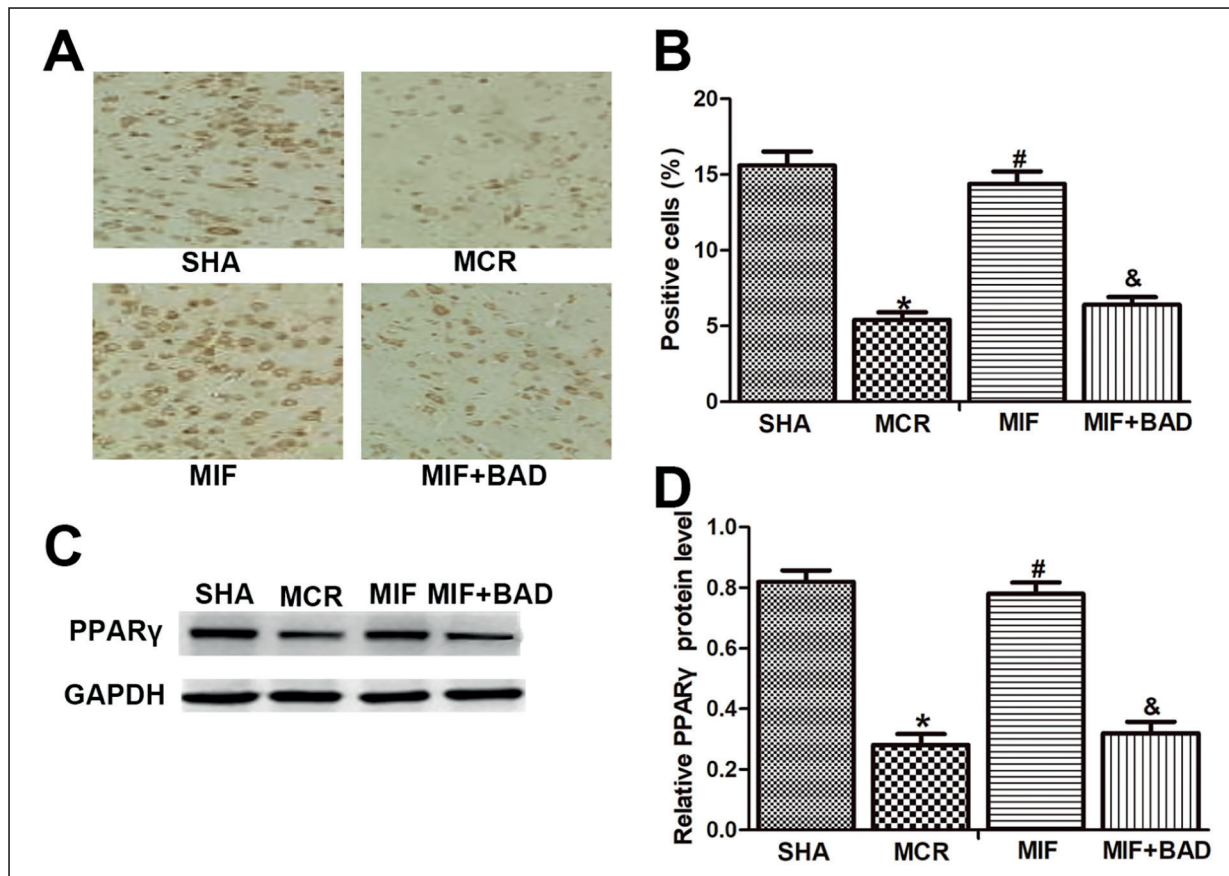


Figure 3. Effect of mifepristone on PPAR γ in infarcted brain tissues of MCAO/R rats. **A**, Representative images of PPAR γ -positive cells in brain tissues by immunohistochemistry (X100). **B**, Analysis of PPAR γ -positive cells. **C**, Western blotting showed that mifepristone increased the expression levels of PPAR γ . **D**, Analysis of the protein level of PPAR γ . * p < 0.05 vs. the SHA group, # p < 0.05 vs. the MCR group, & p < 0.05 vs. the MIF group.

Effects of Mifepristone on the Contents of Tumor Necrosis Factor-Alpha (TNF- α), Interleukin-1 Beta (IL-1 β) and IL-6 in the Infarcted Brain Tissues of MCAO/R Rats

WB results found that, compared with those of the SHA group, the levels of TNF- α , IL-1 β , and IL-6 in the infarcted brain tissues of the MCR group were significantly increased. Compared with those in the MCR group, the levels of TNF- α , IL-1 β , and IL-6 in the infarcted brain tissues of the MIF group were remarkably decreased. After the combined application of mifepristone and BADGE, the levels of TNF- α , IL-1 β , and IL-6 in the infarcted brain tissues were markedly different from those of the MIF group. However, there were no significant differences when compared with the MCR group (Figure 4).

Effects of Mifepristone on Matrix Metalloproteinase-2 (MMP-2), MMP-9 and Tissue Inhibitor of Metalloproteinase 1 (TIMP-1) in the Infarcted Brain Tissues of MCAO/R Rats

WB results demonstrated that compared with the SHA group, the protein expression levels of MMP-2 and MMP-9 in the infarcted brain tissues of the MAR group were significantly increased. Compared with those of the MCR group, the protein expressions of MMP-2 and MMP-9 in the MIF group were remarkably decreased. After the combined use of mifepristone and BADGE, the protein expressions of MMP-2 and MMP-9 were significantly different from those of the MIF group, and the expression level and function of

MMP-9 were still markedly lower than the MCR group (Figure 5A and 5B). Moreover, the expression of the TIMP-1 protein in the infarcted brain tissues of the MCR group was decreased. Compared with that of the MCR group, the protein expression of TIMP-1 in the MIF group was significantly increased. After the combined application of mifepristone and BADGE, the protein expression of TIMP-1 was markedly different from that of the MIF group, which was still significantly higher than the MCR group (Figure 5C).

Discussion

Clinically, some patients undergo distal vascular recanalization due to thrombolytic therapy or spontaneous movement of emboli, which may lead to more severe cerebral ischemia-reperfusion injury^{4,5}. Therefore, animal models of cerebral ischemia-reperfusion are often established for the research of IS. In this study, it was found that after mifepristone was administered in the cerebral ischemia-reperfusion rat model, both the neurological deficit score and the cerebral infarction volume were significantly reduced. Nissl staining results revealed that mifepristone could improve the nerve tissue morphology after cerebral ischemia-reperfusion injury in rats, indicating that mifepristone was an effective treatment drug for cerebral ischemia-reperfusion injury. The combination of mifepristone and BADGE could still significantly reduce the volume of cerebral infarction, suggesting that mifepristone

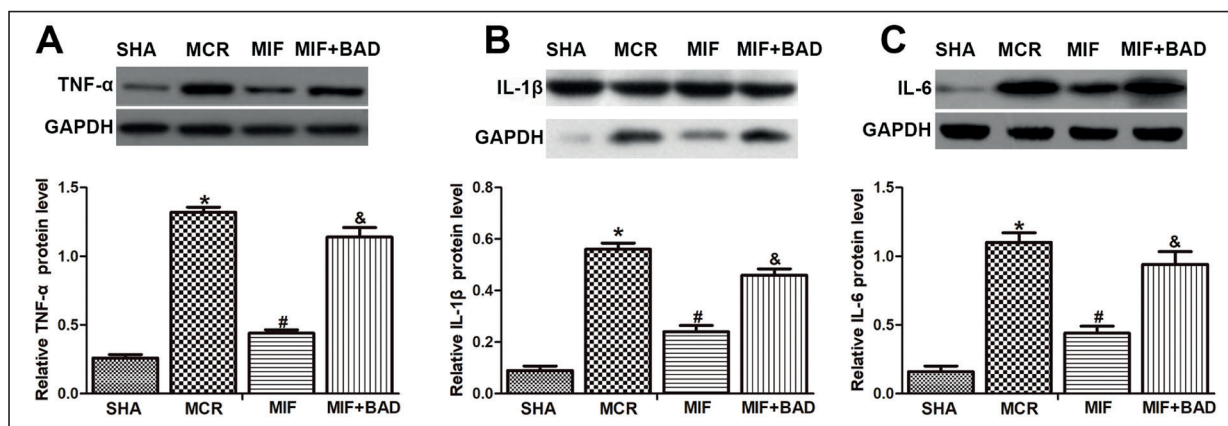


Figure 4. Effect of mifepristone on the contents of inflammatory cytokines in infarcted brain tissues of MCAO/R rats. **A**, Analysis of the protein level of *TNF- α* by Western blotting. **B**, Analysis of the protein level of *IL-1* by Western blotting. **C**, Analysis of the protein level of *IL-6* by Western blotting. * $p < 0.05$ vs. the SHA group, # $p < 0.05$ vs. the MCR group, & $p < 0.05$ vs. the MIF group.

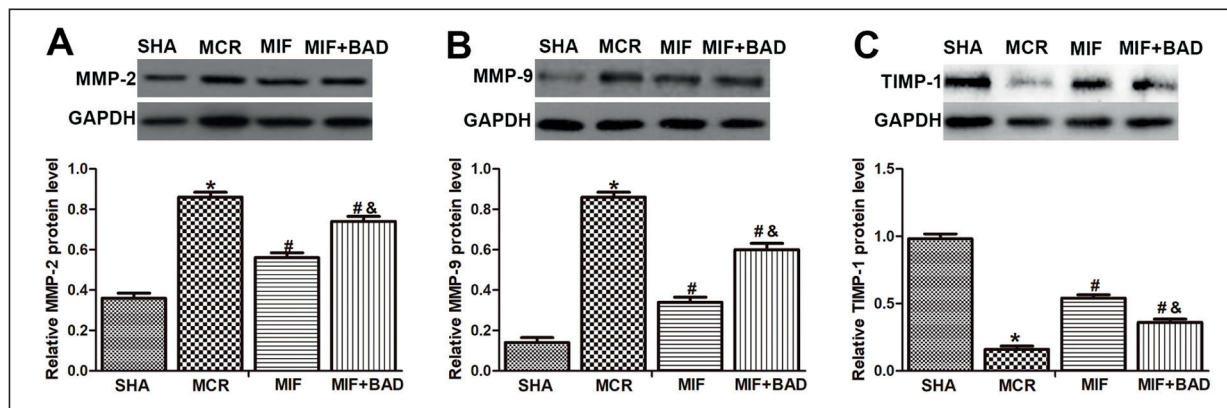


Figure 5. Effect of mifepristone on matrix metalloproteinase-2 (*MMP-2*), *MMP-9* and tissue inhibitor of metalloproteinase 1 (*TIMP-1*) in infarcted brain tissues of MCAO/R rats. **A**, Analysis of the protein level of *MMP-2* by Western blotting. **B**, Analysis of the protein level of *MMP-9* by Western blotting. **C**, Analysis of the protein level of *TIMP-1* by Western blotting. * $p < 0.05$ vs. the SHA group, # $p < 0.05$ vs. the MCR group, & $p < 0.05$ vs. the MIF group.

had a prominent neuroprotective effect on IS injury and was not only induced by the activation of PPAR γ .

PPAR γ is a member of the ligand-activated nuclear transcription factor superfamily, and is involved in various physiological processes such as glucose metabolism¹⁵, fat metabolism¹⁶, cell growth¹⁷, and differentiation¹⁸. Recent animal studies^{19,20} have demonstrated that PPAR γ can regulate non-infectious chronic inflammation in the central nervous system and alleviate neurodegenerative changes. Moreover, it can provide new ideas for the drug treatment of neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease. After the ischemic injury, the expression levels of PPAR γ messenger ribonucleic acid (mRNA) and protein in neurons and microglial cells were rapidly increased. The highest mRNA and protein expression levels were reached 24 h after ischemia, the lowest 48 h were reached after ischemia, and finally maintained at a stable level. High levels of PPAR γ protein can still be detected 14 d after ischemic injury²¹. Studies^{22,23} have illustrated that increased PPAR γ nuclear translocation may exhibit protective effects on the cerebral ischemia-reperfusion injury. Our study found that the level of PPAR γ protein and the percentage of PPAR γ -positive cells were remarkably increased after mifepristone treatment. This indicated that mifepristone not only enhanced PPAR γ function, but also up-regulated the protein expression of PPAR γ , suggesting that mifepristone could activate PPAR γ . In addition, PPAR γ agonist BADGE fully impeded the ago-

nistic effect of mifepristone on PPAR γ . Meanwhile, the combined treatment of mifepristone and BADGE resulted in no difference in PPAR γ level from the control level. However, mifepristone could still markedly protect the nerve, suggesting that mifepristone might exhibit its neuroprotective effect not only via activating PPAR γ .

Inflammatory responses play an important role in the pathophysiology of ischemia-reperfusion injury after acute IS thrombolytic treatment^{6,7}. Therefore, more and more attention has been paid to the treatment of ischemia-reperfusion injury by suppressing inflammatory responses. The role of nuclear hormone receptor PPAR γ is also of great importance in many biological processes, including the prevention of acute inflammation^{24,25}. Inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 have been confirmed to be associated with IS severity and prognosis in both human and animal models^{26,27}. Hence, regulating any of these cytokines may be conducive to alleviate ischemic brain injury. Our study observed that mifepristone could reduce the levels of TNF- α , IL-1 β , and IL-6. The levels of these cytokines were significantly elevated after MCAO/R, but mifepristone markedly inhibited the expression of these cytokines in the brain. This study demonstrated that compared with the SHA group, cerebral ischemia-reperfusion injury activated *MMP-2* and *MMP-9*, inhibited *TIMP-1* and led to the imbalance of *MMP/anti-MMP*. The imbalance of *MMP/anti-MMP* was maintained by mifepristone through the activation of PPAR γ . Meanwhile, this imbalance degree was in line

with the infarcted volume. The combined use of PPAR γ and BADGE eliminated the activating effect of mifepristone on PPAR γ and remarkably inhibited the effects of mifepristone on the expression levels of TNF- α , IL-1 β , IL-6, MMP-2, MMP-9, and TIMP-1.

Conclusions

We demonstrated for the first time that mifepristone can act as a PPAR γ agonist and attenuate cerebral ischemia-reperfusion injury by restoring the balance between MMPs and TIMPs as well as inhibiting the inflammatory cytokines of TNF- α , IL-1B, and IL-6. However, although the combined effects of mifepristone and BADGE completely inhibit the activation of PPAR γ induced by mifepristone, it still has significant effects on the imbalance of MMP/anti-MMP and inflammatory cytokines, including IL-1B, TNF- α , and IL-6. This suggests that mifepristone may affect MMP/anti-MMP imbalance and inflammatory cytokines, and reduce ischemia-reperfusion injury through other mechanisms.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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