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ORIGINAL RESEARCH

Protective Effects of Remimazolam on Cerebral Ischemia/Reperfusion Injury in Rats by Inhibiting of NLRP3 Inflammasome-Dependent Pyroptosis

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Introduction: Remimazolam is a novel benzodiazepine γ-aminobutyric acid A (GABAa) receptor agonist used for sedation and the induction as well as maintenance of general anesthesia. Previous research proved that anesthetic agents acting on GABAa receptor, such as thiopentone, propofol and midazolam, have protective actions for cerebral ischemia/reperfusion (I/R) injury. We here probed into remimazolam for its protective effect and potential mechanism of action against cerebral I/R injury.

Material and Methods: A rat model of middle cerebral artery occlusion (MCAO) with focal transient cerebral I/R injury was established and was given tail vein injection of gradient remimazolam (5, 10, 20 mg/kg) after 2 h of ischemia. Following 24 h of reperfusion, neurological function, brain infarct volume, morphology of cerebral cortical neurons, and expressions of corticocerebral NLRP3, ASC, caspase-1, GSDMD, IL-1β and IL-18 were evaluated.

Results: The results showed that remimazolam could effectively improve the neurological dysfunction, reduce the infarct volume and alleviate the damage of cortical neurons after I/R injury. Notably, the expression of NLRP3 inflammasome pathway was down-regulated, suggesting that remimazolam exerted protective actions on I/R injury by suppressing pyroptosis with decreased expression and release of inflammatory factors, and the involvement of the NLRP3 inflammasome pathway might be the core during that process. Overall, our results indicate that NLRP3 inflammation is a promising target.

Conclusion: Based on this mechanism, remimazolam may be one of the ideal anesthetic drugs for patients with ischemic stroke.

Keywords: remimazolam, NLRP3 inflammasome, ischemia/reperfusion injury, I/R, pyroptosis, inflammation

Introduction

Stroke is one of the primary causes of death in human with an annually increasing mortality.[1] In most cases, there is a need of surgery, such as stent implantation and cerebrovascular recanalization.[2] General anesthesia can contribute to lesser stress response and hemodynamics stability during surgery and has become the best choice for critical stroke. However, such management can be potentially risky for perioperative brain injury,[3] and the use of improper anesthetic agent may cause severe conditions. Given the fact, perioperative brain protection is always been a challenge in anesthesiologists and neurologists, and the selection of optimal anesthetic agent appears to be much more prominent.

Archer et al[4] reported that general anesthesia by propofol, isoflurane and sevoflurane could alleviate the ischemic nerve injury in rodents of focal cerebral ischemia, yet the underlying mechanism was not clarified. In studies by Yang et al,[5] Peng et al,[6] Shi et al,[7] Liu et al[8] and Jiang et al[9] it was also found that the use of propofol, isoflurane, sevoflurane, midazolam and dexmedetomidine contributed to improved outcome of stroke with improved cerebral infarction and I/R injury. Inhaled anesthetics are rarely used for stroke due to the possible risk for increased intracranial pressure. Instead, intravenous anesthetics, such as propofol, dexmedetomidine and midazolam, are popular while with certain side effects

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which limit their use in clinic, for example, propofol has significant suppressive effect on circulation, dexmedetomidine is relatively poor in sedation and midazolam leads to a high incidence of delayed awakening from anesthesia.

Remimazolam is a novel benzodiazepine agonist targeting γ-aminobutyric acid A (GABAa) receptor. It can stabilize hemodynamics while providing rapid anesthesia and awakening, and produce lesser suppressive effect toward breathing. In the meantime, it allows for prolonged infusions without accumulation and the metabolites have no any pharmacological action. Owing to the advantages, remimazolam is promising in clinical applications.[10,11] GABAergic signaling participates in multiple functions of macrophages, but the underlying mechanism has not been clarified yet.[12] It was reported that GABA receptor-associated protein, GABARAP, affected nucleotide-binding oligomerization domain-like receptors pyrin domain containing 3 (NLRP3) inflammasome-dependent inflammation via mediating the mitochondrial mass of relevant macrophages.[13] In addition, higher GABA receptor expression and functions could be protective for the acute injury resulting from transient focal cerebral ischemia.[14]

NLRP3 inflammasome pathway was proven to be vital significant in cerebral I/R and following pyroptosis.[15,16] Specifically, NLRP3 is activated in microglial cells in response to I/R injury, subsequently expresses in neuron and microvascular endothelial cells.[17] In that way, blocking NLRP3 activation and expression of related proteins in neuron and microglial cells was reported to be an effective approach against focal cerebral I/R injury, contributing to improved neurological function score, decreased infarct volume and lighter cerebral edema.[18–20]

We here propose a hypothesis that remimazolam can play protective actions on brain with the involvement of NLRP3 inflammasome pathway, which is important in pyroptosis and further inflammatory cascade to reduce the neuronal damage resulted from I/R. To this end, rat models of MCAO were constructed to study possible effects and corresponding molecular mechanism.

Materials and Methods

Animals

Seventy-five male, SPF, Sprague-Dawley rats (7–8 weeks, 250–300 g) were obtained from laboratory animal center of Guangxi Medical University. All rats were allowed to acclimate for 1 week before modeling (22–25°C, relative humidity 55–60%, 12/12 light/dark), with free water and diet. They were fasted and deprived of water, respectively, 12 h and 4 h before the experiment. The animal experiment was conducted with the approval of the ethics committee of Guangxi Medical University, strictly following The Guide for Care and Use of Laboratory Animals released by National Institutes of Health.

Materials

Remimazolam tosilate for injection was purchased from HengRui Medicine Co., Ltd. (Jiangsu, China). Antibodies included anti-NLRP3, anti-ASC, anti-caspase-1, anti-GSDMD, anti-β-actin, anti-IL-1β and anti-IL-18 antibodies (Abcam and Cell Signaling Technology, USA). Detection kits included SP kit (Zhongshan Golden Bridge Biotechnology, Beijing, China), BCA protein assay kit and hematoxylin-eosin (HE) staining kit (Solaibao Biotechnology Co., Ltd., Beijing, China).

Model Establishment and Grouping

Rat models of MCAO were constructed following the Longa method.[21] The right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were isolated by dissection. A intraluminal filament was placed from ECA to ICA, passing through the bifurcation of CCA. The intraluminal filament reached the intracranial part of ICA to block the right MCA blood flow, with the strand-forward length as (18±2) mm from the bifurcation of CCA.

A random method was applied to equally divide the 75 rats into 5 groups: Sham, MCAO, MCAO+R_L, MCAO+R_M, and MCAO+R_H. Blood flow blocking was managed 2 h in all MCAO rats and reperfusion was performed after that by removing the strand. Rats from MCAO+R_L, MCAO+R_M, and MCAO+R_H groups were, respectively, assigned to receive additional gradient remimazolam by tail vein injection instantly after reperfusion (5 mg/kg, 10 mg/kg, 20 mg/kg). All the rats were sacrificed under anesthesia after 24 h of reperfusion, and the brain was immediately isolated for further analysis.

Neurological Function Score

Five-grade (0–4 points) Longa method was applied to confer a neurological deficit score (NDS) at 2 h of ischemia and 24 h of reperfusion, respectively. Grade I (0-point): normal behavior without any neurological deficit; Grade II (1-point): the left front paw fails to fully extend; Grade III (2-point): turn to the left (paralyzed side) while walking; Grade IV (3-point): tip to the left (paralyzed side) while walking; Grade V (4-point): unable spontaneous walking with impairment of consciousness. Models with 2 h of ischemia scored 1–3 points were included, otherwise excluded from the study.

Triphenyltetrazolium-Chloride (TTC) Staining

Following model establishment, the rats were decapitated under deep anesthesia and the brain tissue excluding cerebellum, olfactory bulb and lower brain stem was extracted, transferred to PBS solution (0–4°C) for cleaning and then frozen at −20°C for 20 min. The tissue block was cut to obtain coronal brain slices (2 mm). Being exposed to 2% TTC solution, the slices were water-bathed at 37°C in the dark for 30 min. Shaking at 5-min intervals was performed to ensure uniform staining. Subsequently, the stained tissue samples were fixed overnight with 4% paraformaldehyde, and dried on the following day for photography. Image-ProPlus 6.0 software was run to obtain the infarct area (white) on the tail side, and the infarct volume was calculated according to the slice thickness.

Hematoxylin-Eosin (HE) Staining

The rats were transcardially perfused with 4% paraformaldehyde after anesthesia. Intact brain tissue was isolated, fixed, washed, dehydrated, and then processed for transparency and preparation of paraffin-embedded blocks. Coronal sections (5 μm) at the posterior optic chiasma were obtained. The sections were then deparaffinized through xylene and ethanol to water, followed by staining with hematoxylin and eosin. Tissue slides were prepared and observed microscopically for morphology of cortical neurons.

Immunohistochemistry (IHC) Staining

Paraffin tissue sections were prepared and then processed according to the instructions provided by SP kit. In short, the sections were deparaffinized through gradient xylene and ethanol to water, followed by high-temperature antigen repair with sodium citrate. 0.3% hydrogen peroxide and goat serum were sequentially added for blocking with each 15 min. Afterwards, the tissue samples were consecutively exposed to primary antibodies (NLRP3, GSDMD, ASC, caspase-1, IL-1β and IL-18) for 1 h, biotin-labeled secondary antibody for 15 min, and HRP-conjugated SPTM for 15 min. DAB was applied to develop color and hematoxylin was supplemented for counterstaining. The results were observed microscopically. Negative control was free of primary antibody. Image-Pro Plus 6.0 was operated to perform quantitative analysis. The positive rate was represented by average optical density (AOD=IOD/Area).

Western Blot

Peripheral cortex (0.1 g) of infarct area was collected by cryogenic grinding and lysed on ice with 1 mL lysis (RIPA + PMSF + phosphatase inhibitor) for half an hour. The lysates were cryogenically centrifuged for 15 min. The products were cryogenically centrifuged, and the supernatant was then harvested to determine protein concentration with the BCA method. The protein samples were mixed with 5× sample buffer at 4:1, degenerated by boiling at 100°C for 10 min, and then processed by SDS-PAGE. The obtained proteins were loaded to a PVDF membrane by wet-transfer, and then exposed to 5% skim milk for 1 h at room temperature to avoid unspecific binding. Primary antibodies, including anti-NLRP3, anti-GSDMD, anti-ASC, anti-caspase-1, anti-IL-1β and anti-IL-18 were added overnight at 4°C, followed by fluorescent dye-coupled secondary antibody for 1 h at room temperature. Images were captured by Odyssey system (LI-COR Biosciences), and analyzed on Image J to obtain protein expression. β-actin acted as the internal control.

Statistical Analysis

Data were presented as mean±SD and processed on SPSS 25.0 and GraphPad Prism 8.0.2. Data normality was assessed with QQ plots (see the Supplementary Materials). Normally distributed data were analysed with one-way ANOVA followed by SNK-q. Skewed data were analysed with Kruskal–Wallis test followed by Dunn's multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

Neurological Function Score

Sham rats were scored 0, regarded normal without any neurological deficit. MCAO rats were presented with evident neurological deficits, such as failure to fully extend the left front paw, turning or tipping to the left while walking. In the meantime, they had higher scores after 2 h of ischemia relative to the Sham group ($p<0.001$). Moreover, the injection of remimazolam at 5 and 10 mg/kg after 24 h of reperfusion contributed to significantly lower scores, demonstrating alleviated neurological disorders ($p < 0.05$; $p < 0.01$; Figure 1).

Brain Infarct Volume

Brain tissue of Sham rats was in uniform red without white infarct area. To the contrary, significant white infarct areas were present in the MCAO group ($p<0.001$). Following injection of remimazolam, the areas were evidently reduced ($p<0.001$), which was much more significant at a medium concentration in the MCAO+R_M group ($p<0.01$ versus the MCAO+R_L, and $p<0.001$ versus the MCAO+R_H; Figure 2A and B).

Morphology of Ischemic Cortical Neuron

In the HE staining results, Sham rats showed well-organized neurons, round or cone-shaped cells with intact structure, clear nucleoli and boundaries; in the MCAO group, there was evidence of cell death, including significant neuronal degeneration, loose tissue structure, interstitial edema, swollen neural cells in triangular or irregular shapes, which were

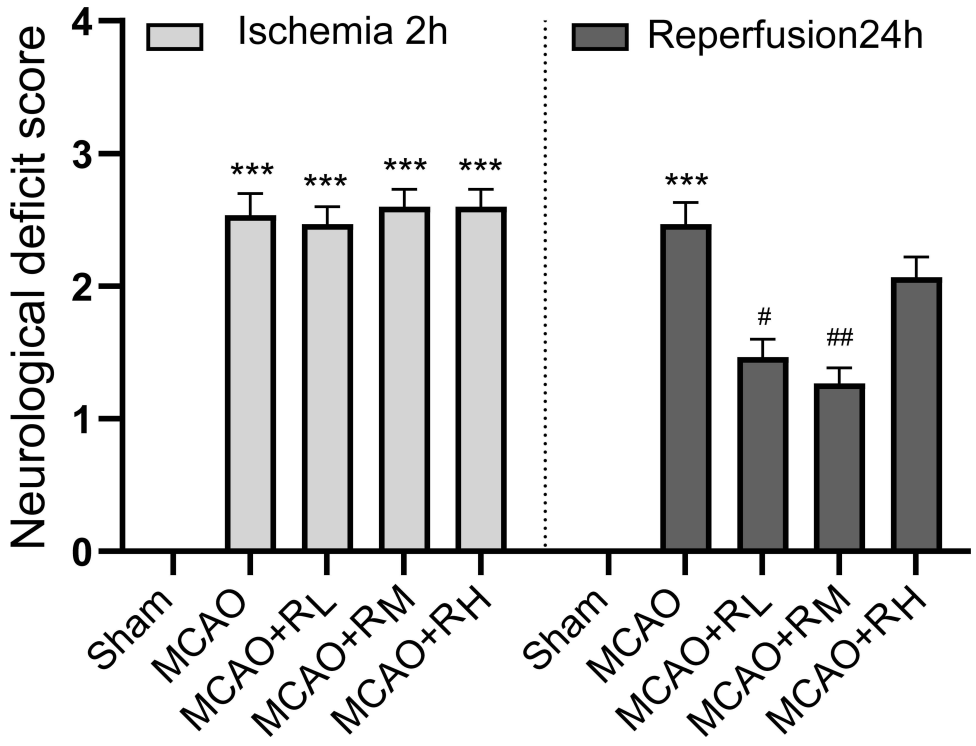

Figure 1 NDS at 2 h of ischemia and 24 h of reperfusion.
Notes: ***$p < 0.001$, compared to Sham group; $^{\#}p < 0.05$, $^{\#\#}p<0.01$, compared to MCAO group; n = 15.

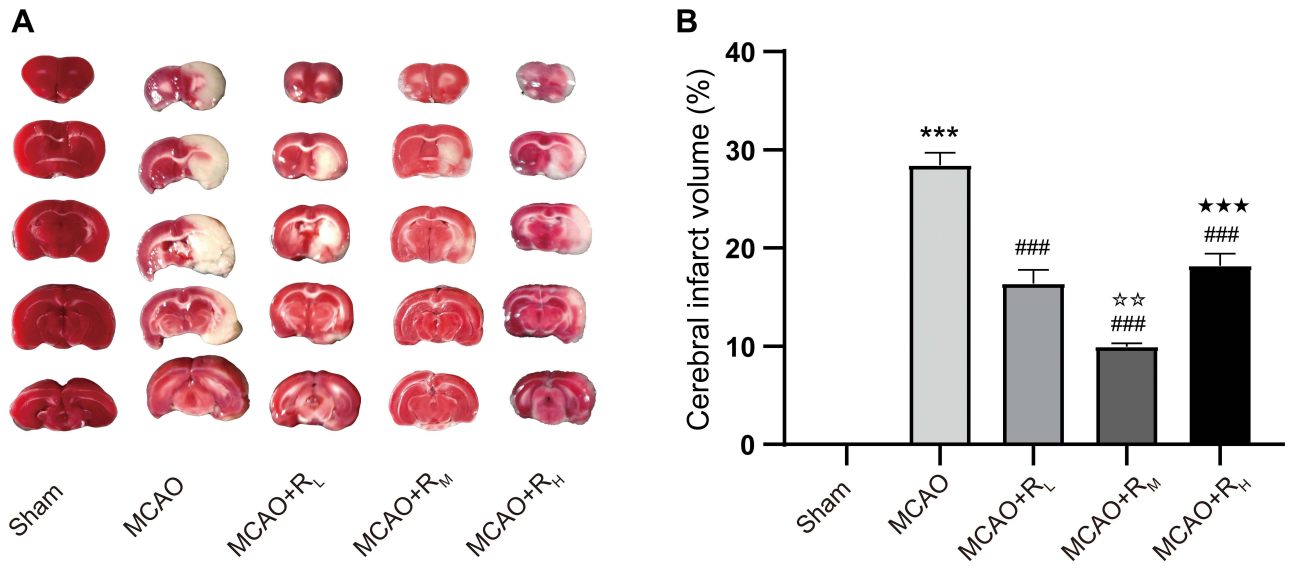

Figure 2 TTC staining for brain infarct volume.
Notes: (**A**) Representative picture; (**B**) Infarct volume after 24 h of reperfusion. $^{***}p < 0.001$, compared to Sham group; $^{\#\#\#}p < 0.001$, compared to MCAO group; $^{\star\star}p < 0.01$, compared to MCAO+R_L group; $^{\star\star\star}p < 0.001$, compared to MCAO+$R_M$ group; n = 5.

disordered, with nuclear pyknosis and fragmentation. Treatment of remimazolam contributed to significantly alleviated injury (Figure 3).

Brain NLRP3, ASC, Caspase-1, GSDMD, IL-1β and IL-18 Expression

IHC Staining

Expressions of NLRP3, ASC, caspase-1, GSDMD, IL-1β and IL-18 at the border zone of infarcts were measured by IHC (Figure 4). Relative to the Sham group, higher expressions were present in the MCAO group ($p<0.001$, Figure 4B–G). Relative to the MCAO group, significant reductions were found in NLRP3, ASC, caspase-1, GSDMD, IL-18 in MCAO +R_L ($p<0.05$; $p<0.01$; $p<0.001$; Figure 4B–E and G), NLRP3, ASC, caspase-1, GSDMD, IL-1β and IL-18 in MCAO+R_M ($p<0.001$; Figure 4B–G), and NLRP3, ASC, GSDMD, IL-1β and IL-18 in MACO+R_H ($p<0.05$; $p<0.01$; $p<0.001$; Figure 4B, C, E–G) groups. Relative to the MCAO+R_L group, ASC, GSDMD, IL-1β and IL-18 expressions were significantly decreased in the MCAO+R_M group ($p<0.05$; $p<0.01$; Figure 4C, E–G). Relative to the MCAO+R_M group, ASC, caspase-1, GSDMD and IL-18 were profoundly elevated in the MCAO+R_H group ($p<0.05$, $p<0.01$ Figure 4C–E and G).

Western Blot

Figure 5A shows the protein bands of NLRP3, ASC, caspase-1, GSDMD, IL-1β and IL-18 in each group. Higher protein expressions were present in the MCAO group versus the Sham group ($p<0.05$; $p<0.001$; Figure 5B–G). Relative to the MCAO group, significant reductions were found in ASC, caspase-1, IL-1β and IL-18 in MCAO+R_L ($p<0.05$; Figure 5C, D, F and G); NLRP3, ASC, caspase-1, GSDMD, IL-1β and IL-18 in MCAO+R_M ($p<0.05$; $p<0.01$; $p<0.001$; Figure 5B–G); and NLRP3, caspase-1 in MACO+R_H ($p<0.05$, $p<0.01$, Figure 5B and D) groups. Relative to the MCAO+R_L group, NLRP3 and caspase-1 expressions were highly decreased in the MCAO+R_M group ($p<0.05$; Figure 5B and D). Relative to the MCAO +R_M group, NLRP3 and ASC were profoundly increased in the MCAO+R_H group ($p<0.05$; Figure 5B and C).

Discussion

MCAO models are commonly established to mimic the pathological conditions following the onset of middle cerebral artery occlusion. It allows the induction of focal I/R injury and provides a subject view toward relevant functions.[22] Here, rat models of right MCAO were established to investigate the effect of remimazolam on cerebral I/R injury. It is known that focal cerebral I/R injury generally can cause morphological changes and dysfunction of ischemic cortical neurons,

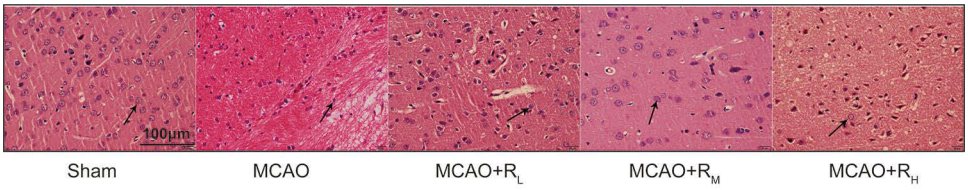

Figure 3 HE staining for morphology of peripheral cortical neuron of cerebral ischemic area (Scale: 100 μm).
Notes: Sham group: well-organized neural cells, with intact structure, large and round nuclei, and clear nucleoli (arrow); MCAO group: irregular, disordered neuralcells, with nuclear pyknosis and fragmentation (arrow); MCAO+R_L/MCAO+R_M/MACO+R_H: better cell morphology (arrow) than the MCAO group, much more significant in the MCAO+R_M group.

and result in cerebral infarction to the most critical degree after 24 h.[23] The rats here suffered from acute neurological disorders of varying degrees after 24 of reperfusion. TTC and HE staining revealed significant infarct lesions in the MCA blood supply area, in the meantime, pathologically injured or dead neurons were present. Following reperfusion and instant remimazolam injection, great improvements were found in neurological function scores, neuronal injury and brain infarct volume, suggesting the effectiveness of remimazolam in improving the neurological disorders and decreasing the damage to cortical neurons after I/R injury.

Cerebral I/R injury is a result of a series of complex cascade reactions. Major pathological changes after ischemia include microvascular injury, blood-brain barrier dysfunction, inflammation, oxidative stress/reactive oxygen species increase, and reduced neuronal survival.[24] During reperfusion process, oxygen radical, cytokine and inflammatory factor will be largely released. In the meantime, there will be an increase in inflammatory cell infiltration because of the increased permeability of blood-brain barrier, thus propagating inflammatory response to cause microvascular blocking and further secondary hypoperfusion.[25] Inflammation, one of the pathological factors for cerebral I/R injury, has been recognized as a vital part in cerebral ischemic stroke. Both the formation and development of local inflammation are involved in the injury and necrosis of neurons. Consistently, we here found an evident increase in cortical IL-1β and IL-18 in the ischemic area after MCAO establishment, which also supported the vital role of inflammation in acute cerebral injury.

Pyroptosis is a type of programmed cell death dependent on cysteinyl aspartate specific proteinase 1 (caspase-1).[26,27] It is different from apoptosis and shows a close relationship with inflammation. Typical characteristics of pyroptosis are cell swelling, cell membrane blebbing, pore-forming resulting in cell death due to rupture, and outflow of intracellular substances.[28] Pyroptosis is majorly present in phagocytic cells such as macrophages and neutrophils, and also can be seen in endothelial cells and neurons.[29]

It is established that inflammasome activation is the typical and key part in the regulation of pyroptosis. It can be triggered by varying stimulatory signals involving two patterns: pathogen-associated molecular patterns (PAMP) and danger-associated molecular patterns (DAMP). Research revealed that following the onset of stroke there is typical NLRP3 inflammasome activation triggered by the DAMP pattern.[30,31] NLRP3 inflammasome is composed of nucleotide-binding oligomerization domain protein-like receptors (NLRs), adaptin including apoptosis-associated speck-like protein containing (ASC) and effector protein such as pro-caspase-1. It participates in regulating inflammatory cascades in multiple diseases, in the meantime, shows a role in pyroptosis by affecting pore-forming protein gasdermin D (GSDMD).[32,33]; The activated NLRP3 will promote pro-caspase-1 to transform into caspase-1 of hydrolysis activity, in turn activating several proinflammatory cytokines including IL-1β and IL-18.[34] GSDMD is a specific effector molecule involved in pore channel formation and the N-terminal fragment function is the key for the occurrence of pyroptosis.[35] In the context of caspase-1 activation, GSDMD can be lysed as GSDMD-NT, which affects the permeability of cell membrane, and in turn triggers pyroptosis and release of inflammatory cytokines such as IL-1β and IL-18.[36]

Fu et al[37] reported that neuronal injury was accompanied by activation of NLRP3 inflammasome and strong inflammatory reactions with vast IL-1β and IL-18 released. In this study, IHC and Western blot assays were devised to test cortical expressions of NLRP3, ASC, caspase-1, GSDMD, IL-1β and IL-18. Consistent with the previous studies,

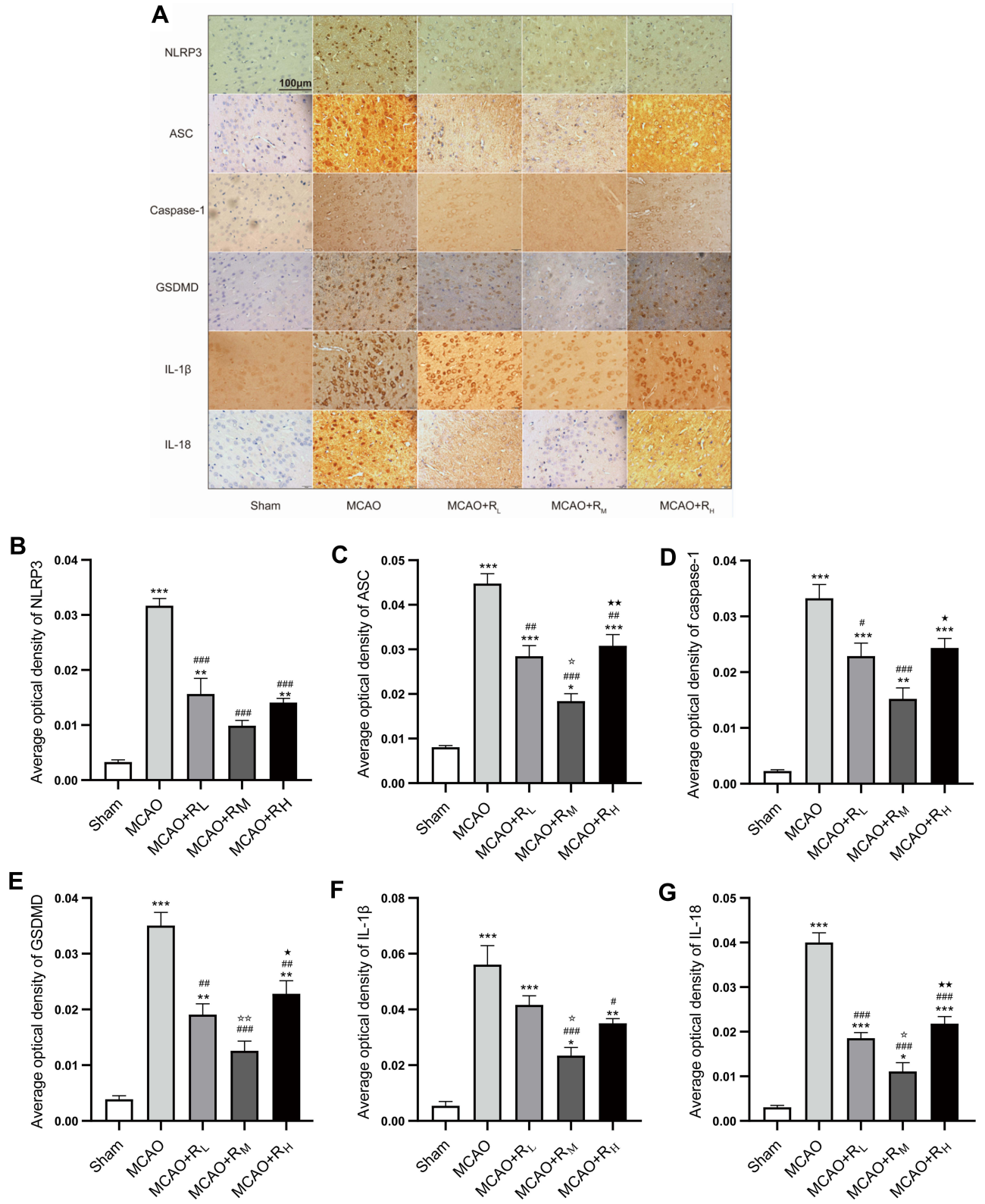

Figure 4 IHC staining for border zone infarcts. (**A**) Micrographs for NLRP3, ASC, caspase-1, GSDMD, IL-1β and IL-18 at 24 h of reperfusion (Scale: 100 μm).
Notes: Positive areas were in dark brown. The Sham group showed low positive expressions, while the MCAO group displayed abundant expressions, which were reduced by remimazolam treatment (**B**–**G**). Positive expression rate represented by AOD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared to Sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared to MCAO group; $^{\star}p < 0.05$, $^{\star\star}p < 0.01$, compared to MCAO+R_L group; $^{\star}p < 0.05$, $^{\star\star}p < 0.01$, compared to MCAO+R_M group; n = 5.

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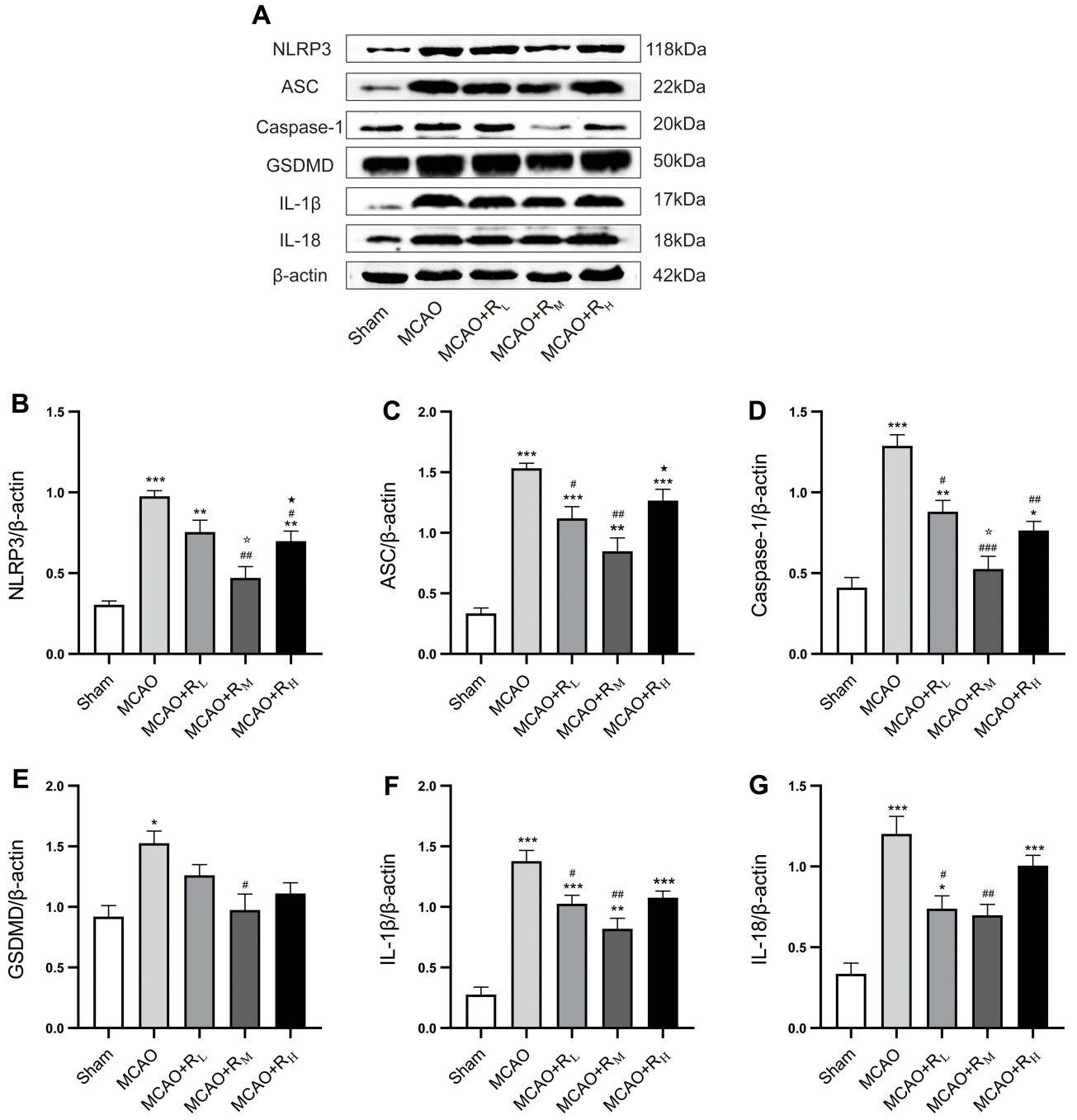

Figure 5 Protein expressions of cortical NLRP3, ASC, caspase-1, GSDMD, IL-1β and IL-18 after remimazolam treatment.
Notes: (**A**) Representative protein bands; (**B**–**G**) Quantitative analysis for protein expression standardized by β-actin. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared to Sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared to MCAO group; $^{\star}p < 0.05$, compared to MCAO+R_L group; $^{\bigstar}p < 0.05$, compared to MCAO+R_M group; n = 5.

NLRP3 and ASC, vital components of NLRP3 inflammasome, were highly up-regulated following the onset of transient I/R injury, suggestive of NLRP3 inflammasome activation. The expressions of caspase-1 and GSDMD were also evidently increased resulting in the incidence of pyroptosis, and strong inflammatory reactions concurrently occurred with more activated inflammatory factors IL-1β, IL-18. Studies have revealed that inhibition of NLRP3-mediated pyroptosis might have neuroprotective effect.[38–40] Besides, neuronal injury could be alleviated after application of NLRP3 inhibitor MCC95 and caspase-1 inhibitor Ac-YUAD-CMK.[41] NLRP3-mediated pyroptosis and inflammatory cascade may be crucially potential targets for intervention in ischemic stroke. Notably, expressions of the mentioned

factors such as NLRP3, caspase-1, GSDMD IL-1β and IL-18 displayed a significant decreasing trend after remimazolam application. It demonstrated that remimazolam, to some extent, could suppress NLRP3-mediated pyroptosis and further inflammatory cascade. The above results were consistent with the morphological and functional changes of neurons in rats, altogether implied the possible mechanism of action of remimazolam, by which remimazolam suppresses NLRP3 inflammasome-induced pyroptosis to reduce activation and release of inflammatory factors and subsequent inflammatory cascade, finally alleviating cerebral I/R injury and improving neuronal morphology and function.

Perioperative organ protection is an important topic in Anesthesiology. Remimazolam has a broader application prospect because of its special advantages compared with existing GABA receptor agonists. The study of the effect of remimazolam on cerebral I/R injury is in line with the theme of organ protection, and has certain practical significance for guiding clinical medication in patients with ischemic stroke. Although we found and preliminarily verified the important role of NLRP3 inflammasome pathway in the protection of remimazolam on cerebral I/R injury in rats, considering the differences between animal models and clinical practice, as well as the ethical limitations of clinical studies, it is necessary to evaluate the safety and effectiveness of remimazolam in patients with ischemic stroke through high-quality, large-sample and multicenter prospective studies. In addition, the concentration gradients of 5 mg/kg, 10 mg/kg and 20 mg/kg were selected according to the ED_{50} of remimazolam in rats,[42] but the protective effect in this experiment was not dose-dependent. Moreover, this study did not use more methods to activate or block related signal pathways for further verification. Therefore, the appropriate dose and exact neuroprotective mechanism of remimazolam still need to be carried out in more in-depth researches in the future.

Conclusion

To sum up, this research identified the role of NLRP3 inflammasome-dependent pyroptosis in occurrence of cerebral I/R injury, which cooperates with secondary inflammatory cascade to cause morphological changes and dysfunction of cerebral cortical neurons. Remimazolam was proven to have protective actions for cerebral I/R injury in rats, since it could reduce neuronal injury and cerebral infarct as well as improve neurological function. The NLRP3 inflammasome-dependent pyrolysis pathway may play a vital significance regulatory role in the mechanism about neuroprotective of remimazolam.

Abbreviations

I/R, ischemia/reperfusion; MCAO, middle cerebral artery occlusion; GABAa, γ-aminobutyric acid A; NLRP3, nucleotide-binding oligomerization domain-like receptors pyrin domain containing 3; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; NDS, neurological deficit score; TTC, triphenyltetrazolium-chloride; HE, hematoxylin-eosin; IHC, immunohistochemistry; AOD, average optical density.

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Disclosure

The authors report no conflicts of interest in this work.

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