

# Melodinhennine B attenuates NLRP3 expression in a cerebral ischemia/reperfusion-induced neuronal injury rat model

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## Abstract

*This investigation evaluated the neuroprotective effect of melodinhennine B in a cerebral ischemia/reperfusion (I/R)-induced neuronal injury rat model. The effect of melodinhennine B was determined by evaluating the neurological deficit score, cerebral infarcted area, and blood-brain barrier (BBB) permeability. Moreover, the level of inflammatory cytokines and expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), interleukin-1 $\beta$  (IL-1 $\beta$ ), NLRP3, zonula occludens-1 (ZO-1), and occluding proteins were estimated by Western blotting. Histopathological changes and immunohistochemical analysis were performed to estimate the effect of melodinhennine B on neuronal injury. The neurological deficit score, percentage of infarcted area, and BBB permeability were improved in the melodinhennine B-treated group of rats. Treatment with melodinhennine B attenuated the altered expression of NF- $\kappa$ B, IL-1 $\beta$ , NLRP3, ZO-1, and occluding proteins in the brain tissue of I/R-induced neuronal injury rats. The inflammatory cytokine levels were reduced in the melodinhennine B-treated group. Histopathologically, melodinhennine B reversed the pathological changes in the brain tissues of I/R-induced neuronal injury rats. In conclusion, melodinhennine B protects against neuronal injury in cerebral ischemia-reperfusion injury rats by regulating the inflammasomes.*

**Key words:** melodinhennine B, neuronal injury, ischemia-reperfusion, inflammasomes.

## Introduction

Cerebral ischemia is a major cause of physical disability and mortality worldwide [6]. Reperfusion after the management of ischemia leads to secondary cerebral injury, commonly known as ischemia/reperfusion (I/R) injury. Several pathways are involved in the development of secondary injury, including autophagy, apoptosis, calcium overload, excitotoxicity, oxidative stress, impaired energy production, and inflammation [12]. The management of secondary injury due to cerebral I/R-induced neuronal injury has several limitations.

Autophagy in neuronal cells is regulated by the inflammatory cascade via inflammasomes [4]. Inflammation and immunity are regulated by inflammasomes, and NLRP3 is a major intracellular multiprotein complex that recognises pathogen-associated molecular patterns [11]. NLRP3 enhances the inflammatory response by activating cytokines and caspase-1 at the site of injury [1]. NLRP3 is involved in the development of disorders such as Alzheimer's disease, atherosclerosis, cancer, and diabetes [10]. Moreover NLRP3 is involved in the development of cerebral I/R-induced neuronal injury [16].

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Several molecules from natural/herbal sources have proven roles in the treatment of chronic disorders. Traditionally, *Melodinus henryi* (Apocynaceae) has been used in China for treating bone fractures and meningitis [8]. Chemically, melodinenine B is an eburnean-vindoline-type bisindole alkaloid isolated from *M. henryi* [15] which has strong anti-inflammatory and nitric oxide inhibitory activity [2,15]. Therefore, this study evaluated the neuroprotective effects of melodinenine B in a cerebral I/R-induced injury rat model.

## Material and methods

### Animals

Male Sprague-Dawley rats weighing 150-200 g were kept under a 12-h light/dark cycle at 60 ±5% humidity and 24 ±3°C. The study protocols were approved by the animal ethics committee of the General Hospital of Northern Theater Command, China (IAEC/GHNTC/2017/09).

### Chemicals

Melodinenine B was supplied by the Laboratory of Natural Medicines, Nanjing, China. ELISA kits were procured from Thermo Fisher Scientific, China. Primary antibodies used in the Western blot assay were procured from Cell Signaling Technology (Beverly, MA, USA), and secondary antibodies from Tianjin Sungene Biotech (Tianjin, China).

### Experimental study

Ischemia/reperfusion-induced neuronal injury was obtained using a middle cerebral artery occlusion model, as previously reported [3]. The left external and common carotid arteries were ligated after exposing them in anesthetised animals. A monofilament nylon suture was used to ligate the middle cerebral artery (MCA). The monofilament was inserted until it reached the origin of the MCA and local cerebral blood flow decreased to < 16% of the baseline level. Reperfusion involved restoring the cerebral blood flow to 75% after 1 hour of ischemia by releasing the ligature.

Animals were separated into three groups: the sham group underwent surgery without I/R induction; the I/R group underwent I/R; and the melodinenine B group received melodinenine B 100 mg/kg intraperitoneally at the time of reperfusion.

### Neurological dysfunction

Neurological dysfunction was determined by neurological scores in I/R-induced neuronal injury rats using Longa's method [20]. The scoring was as follows: 0 – no deficit; 1 – forelimb failed to extend entirely and weak; 2 – circling to the contralateral side; 3 – weight-bearing capacity reduced on the injured side; and 4 – no spontaneous locomotor activity.

### Estimation of cerebral infarction

The brain was isolated one day after reperfusion, and brain coronal slices were sectioned into 2-mm-thick sections and stained for 30 min at 37°C with 2% 2,3,5-triphenyltetrazolium chloride. Image J software was used to determine the percentage of the infarcted area in the slices from photographs of the slices.

### Estimation of blood-brain barrier permeability

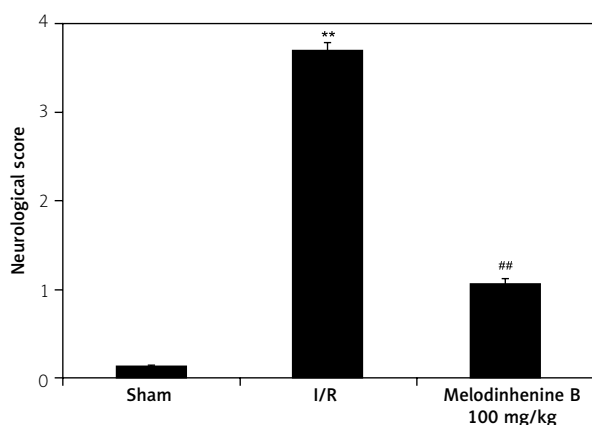
The blood-brain barrier (BBB) permeability was estimated by examining the extravasation of Evans Blue (EB) into the brain. Animals were injected with 2% EB at a dose of 2 ml/kg through the tail vein immediately after reperfusion. The brain was dissected from each animal after sacrifice, and the brain tissue was homogenised in phosphate-buffered saline. The supernatant of the homogenate was separated by centrifugation for 30 min at 15,000 rpm. Trichloroacetic acid solution (50%) was used to dilute the supernatant, which was then incubated for 2 h at room temperature. A 160A spectrophotometer was used to determine the absorbance of the supernatant at 610 nm.

### Estimation of cytokines

The levels of the inflammatory mediators interleukin (IL)-1 $\beta$ , IL-6, and tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) were determined in the brain tissue homogenate using enzyme-linked immunosorbent assays (ELISA) as per the kit protocols.

### Western blotting

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), IL-1 $\beta$ , NLRP3, zonula occludens-1 (ZO-1), and occluding proteins expressions were assessed using Western blot assays of



**Fig. 1.** Effect of melodinhennine B on the neurological deficit score in an I/R-induced neuronal injury rat model. Mean ± SEM ( $n = 10$ ). \*\* $p < 0.01$  compared with the sham group; ## $p < 0.01$  compared with the I/R group.

brain tissue homogenates. A BCA assay kit was used to quantify the protein from each tissue homogenate, and 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis was used to separate the proteins, which were transferred to a nitrocellulose membrane by electroblotting. Subsequently, each membrane was blocked with 5% blocking solution (non-fat milk) and incubated in blocking buffer with primary antibodies overnight at 4°C. Goat secondary antibodies conjugated with horseradish peroxidase were added to the blocking buffer, and a chemiluminescence kit was used to detect the proteins.

### Immunohistochemical analysis

The 3,3'-diaminobenzidine method was used to stain the brain tissue immunohistochemically, as previously reported. Isolated brain tissue was sectioned and then incubated with NLRP3, ZO-1, or occluding protein antibodies at 4°C overnight. A BX51 light microscope was used to determine the immunoreactive proteins, and protein densities were estimated using Image-Pro Plus.

### Histopathological analysis

Formaldehyde (4%) was used to fix the brain tissues, which were embedded in paraffin, sectioned to 5-µm thicknesses using a microtome, and stained with haematoxylin and eosin (H&E). A trinocular microscope was used to evaluate pathophysiological changes in the brain tissue.

### Statistical analysis

All data are expressed as mean ± the standard error of the mean (SEM;  $n = 10$ ). The statistical analysis consisted of one-way analysis of variance (ANOVA). The means were compared with Dunnett's *post hoc* test using GraphPad Prism (ver. 6.1; San Diego, CA, USA).  $P$ -values  $< 0.05$  were considered to indicate statistical significance.

### Results

#### Melodinhennine B improves the neurological function

Figure 1 shows the effect of melodinhennine B on the neurological deficit score in I/R-induced neuronal injury rats. The score was 3.7 in the I/R group and 1.07 in the melodinhennine B-treated I/R-induced neuronal injury rats.

#### Melodinhennine B reduces the cerebral infarcted area

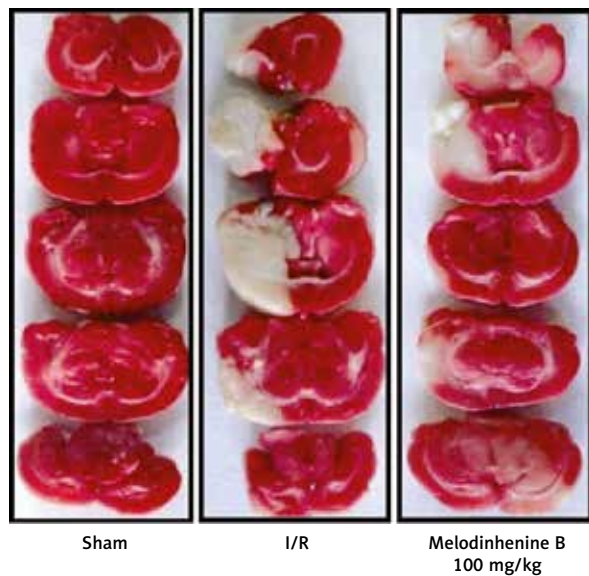
The area of cerebral infarction was determined in the brains of I/R-induced neuronal injury rats (Fig. 2). The percentage area of cerebral infarction was 29% higher in the I/R group than in the sham-operated group, and it was reduced by 14% in the melodinhennine B-treated I/R-induced neuronal injury rat.

#### Melodinhennine B reduces blood-brain barrier permeability

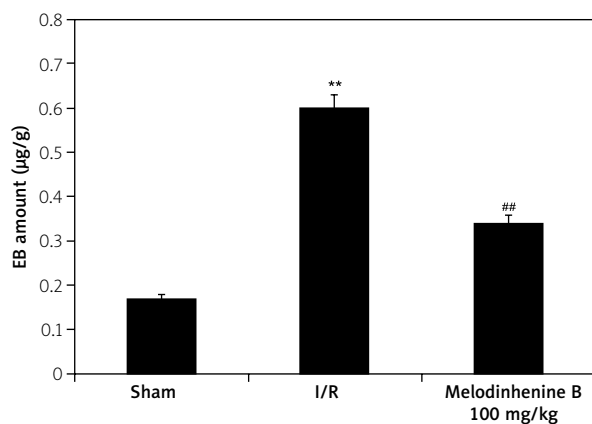
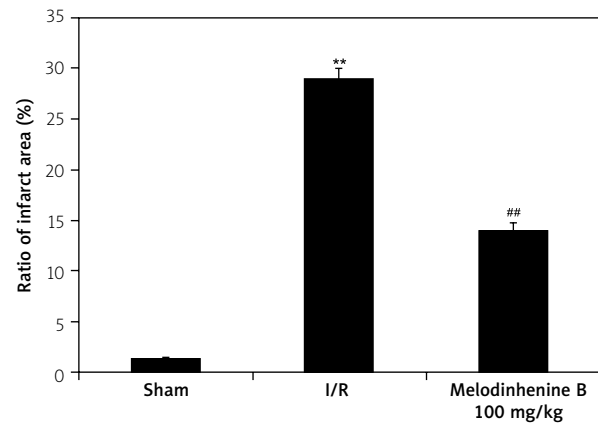
Using EB staining, the permeability of the BBB was observed in the cerebral tissues of I/R-induced neuronal injury rats treated with melodinhennine B. There was a significant increase in the EB staining in the cerebral tissue of the I/R group compared with the sham-operated group. Treatment with melodinhennine B ameliorated the altered permeability of the BBB in the cerebral tissue of I/R-induced neuronal injury rats (Fig. 3).

#### Melodinhennine B reduces the levels of inflammatory mediators

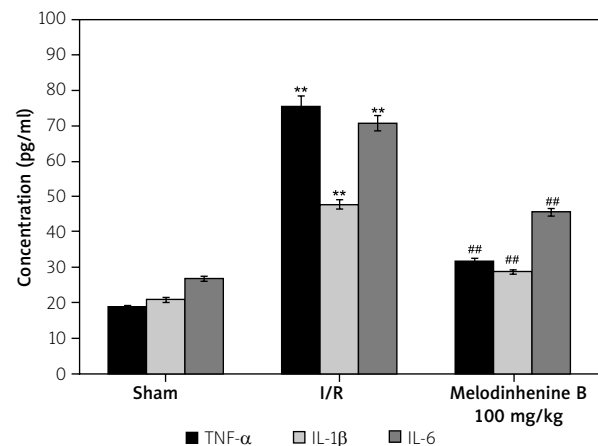
Figure 4 shows the levels of inflammatory cytokines in the brain tissue of I/R-induced neuronal injury rats. The TNF-α, IL-1β, and IL-6 levels were increased in the brain tissue homogenate of the I/R group compared with the sham-operated group. How-



**Fig. 2.** Effect of melodinhene B on the percentage of the cerebral infarct area in an I/R-induced neuronal injury rat model. Mean  $\pm$ SEM ( $n = 10$ ).  $**p < 0.01$  compared with the sham group;  $##p < 0.01$  compared with the I/R group.



**Fig. 3.** Effect of melodinhene B on BBB permeability in an I/R-induced neuronal injury rat model. Mean  $\pm$ SEM ( $n = 10$ ).  $**p < 0.01$  compared with the sham group;  $##p < 0.01$  compared with the I/R group.



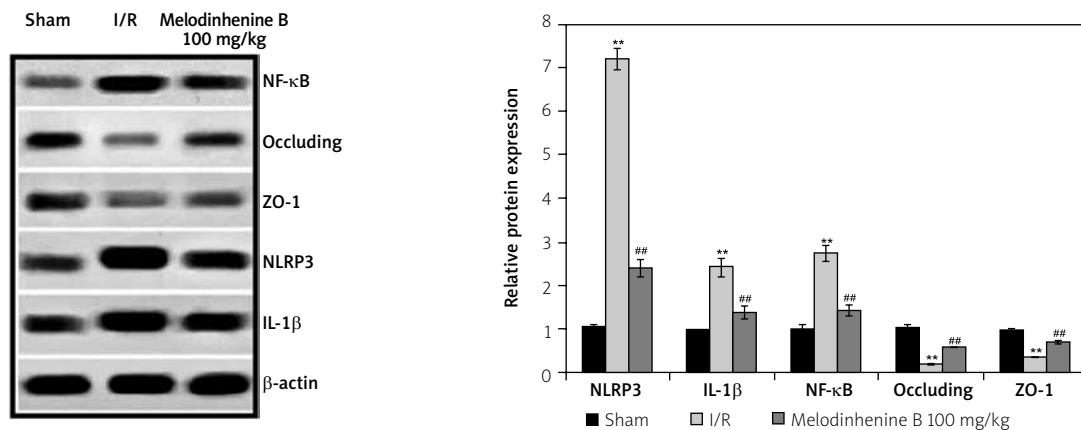
**Fig. 4.** Effect of melodinhene B on inflammatory mediators in an I/R-induced neuronal injury rat model. Mean  $\pm$ SEM ( $n = 10$ ).  $**p < 0.01$  compared with the sham group;  $##p < 0.01$  compared with the I/R group.

ever, there was a significant reduction in the cytokine levels in the brain tissue homogenate of the melodinhene-B-treated group compared with the I/R group.

### Melodinhene B attenuates the expression of NF- $\kappa$ B, IL-1 $\beta$ , NLRP3, ZO-1, and occluding proteins

Figure 5 shows the expression of NF- $\kappa$ B, IL-1 $\beta$ , NLRP3, ZO-1, and occluding proteins in melodinhene-

hene-B-treated I/R-induced neuronal injury rats by Western blotting. The expression of NLRP3, IL-1 $\beta$ , and NF- $\kappa$ B proteins was enhanced and that of ZO-1 and occluding proteins was reduced in the brain tissues of the I/R group compared with the sham-operated group. However treatment with melodinhene B attenuated the expression of NF- $\kappa$ B, IL-1 $\beta$ , NLRP3, ZO-1, and occluding proteins in the I/R-induced neuronal injury rats.



**Fig. 5.** Effect of melodinhene B on the expression of NF-κB, IL-1β, NLRP3, ZO-1, and occluding proteins in an I/R-induced neuronal injury rat model by Western blot assay. Mean ± SEM (n = 10). \*\*p < 0.01 compared with the sham group; ##p < 0.01 compared with the I/R group.

### Melodinhene B reduces the expression of NLRP3, ZO-1 and occluding proteins

The expression of NLRP3, ZO-1, and occluding proteins in the brain tissue of melodinhene-B-treated I/R-induced neuronal injury rats was determined immunohistochemically (Fig. 6). The expression of NLRP3 was enhanced, and that of ZO-1 and occluding proteins was reduced, in the brain tissue of I/R-induced neuronal injury rats. However, the expression of NLRP3 was reduced, and that of ZO-1 and occluding proteins was enhanced, in the brain tissue of the melodinhene-B-treated group compared with the I/R group.

### Melodinhene B ameliorates histopathological changes in the brain tissue

Figure 7 shows the histopathological changes in the brain tissues of I/R-induced neuronal injury rats. Brain tissue sections of the sham-operated group appeared normal. The brain tissues of the I/R group showed nuclear and cytoplasmic vacuolation in the white matter and cortex. Moreover, the neurocytes were unconsolidated and disordered. However, treatment with melodinhene B ameliorated these pathological changes in the brain tissues of I/R-induced neuronal injury rats.

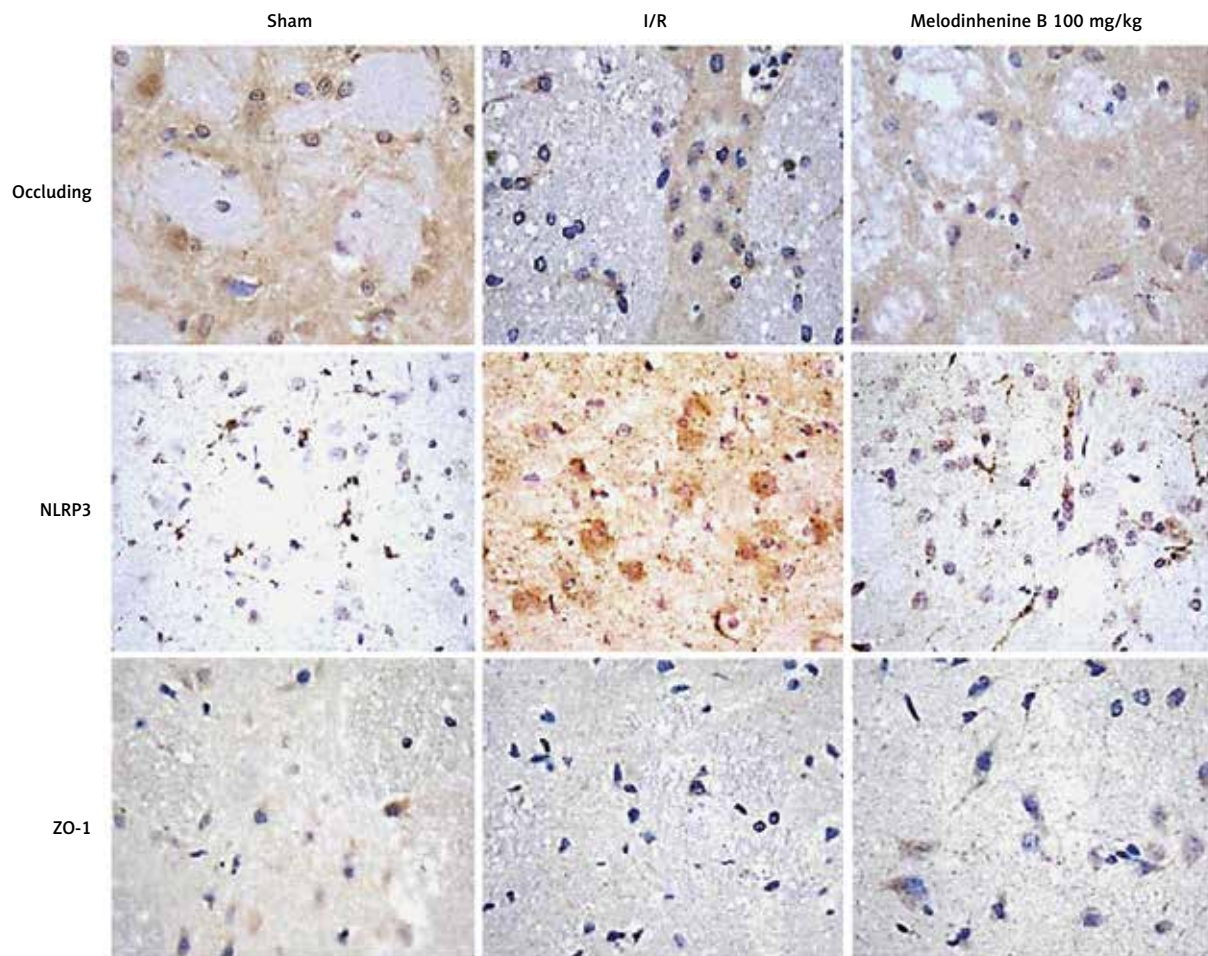
## Discussion

Cerebral ischemia is a major cause of brain injury, and its management remains challenging. Therefore,

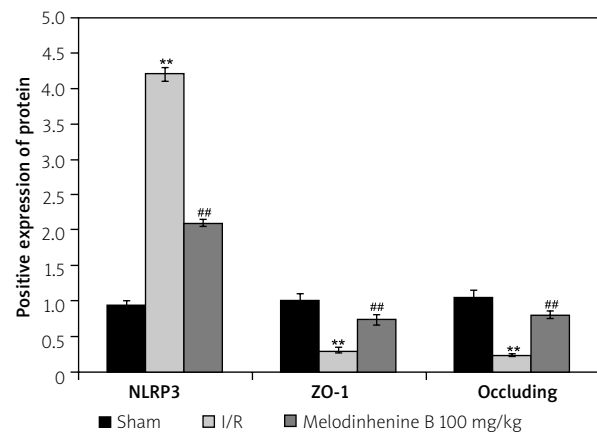
we evaluated the protective effect of melodinhene B against I/R-induced neuronal injury by evaluating the neurological deficit score, cerebral infarcted area, and BBB permeability. In addition, the levels of inflammatory cytokines and expression of NF-κB, IL-1β, NLRP3, ZO-1, and occluding proteins were estimated by ELISA and Western blotting, respectively. Histopathological and immunohistochemical analyses were performed to examine the effect of melodinhene B on neuronal injury.

I/R-induced cerebral injury leads to disruption of the BBB, which causes further secondary neuronal injury [17]. This suggests that ischemic brain disease progresses due to the linear relationship between cerebral I/R injury and disruption of the BBB [19]. Maintaining the integrity of the BBB prevents neuronal injury and improves neurological function [18]. The permeability of the BBB was reduced in the melodinhene-B-treated group compared with the I/R group. Cerebral ischemia leads to an increase in the area of infarcted brain, which also reduces the neurological function [13]. Based on the neurological deficit score and percentage of the infarcted area, melodinhene-B treatment improved the neurological function compared with the untreated I/R rats.

Tight junction proteins such as ZO-1 and occluding proteins contribute to maintaining the integrity of the BBB [5]. These proteins form a barrier that separates the cellular compartments and facilitates the exchange of ions and other substances. Altered ZO-1 and occluding protein levels may lead to chang-



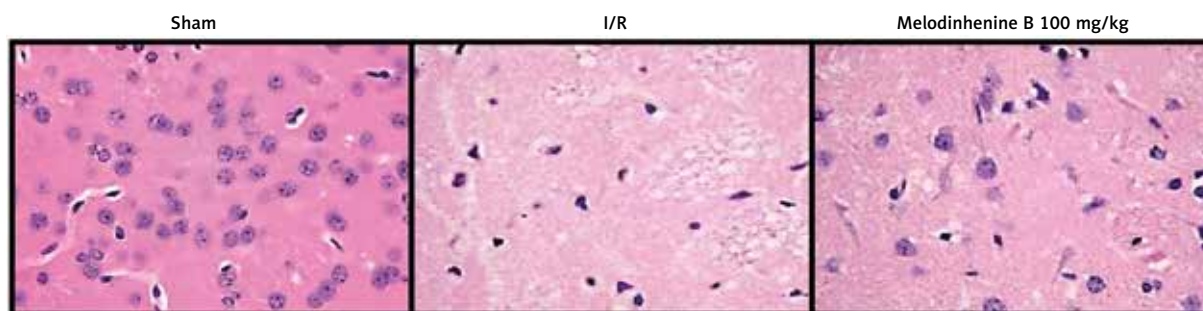
**Fig. 6.** Effect of melodininene B on the expression of NLRP3, ZO-1, and occluding proteins in the brain tissue of an I/R-induced neuronal injury rat model by immunohistochemical analysis. Mean  $\pm$ SEM ( $n = 10$ ).  $**p < 0.01$  compared with the sham group;  $##p < 0.01$  compared with the I/R group.



es in the integrity of the BBB [9]. Tight junction proteins have a role in neuronal injury, and the expression of ZO-1 and occluding proteins protects against neuronal injury [23]. We found that treatment with melodininene B normalized the altered expression of ZO-1 and occluding proteins in the cerebral tissues of I/R-induced neuronal injury rats.

Inflammatory cytokines contribute to the development of I/R-induced neuronal injury [14]. The levels of these cytokines are enhanced with disruption of the BBB. The inflammatory response is enhanced by the activation of inflammasomes (NLRP3) in cerebral I/R-induced neuronal injury [7,21]. In cerebral disease, NLRP3 contributes to the disruption of the





**Fig. 7.** Effect of melodinenine B on the histopathological changes in the brain tissue of an I/R-induced neuronal injury rat model by H&E staining.

BBB, which leads to neuronal injury, whereas inhibition of NLRP3 prevents neuronal injury [22]. The expression of NLRP3 was reduced in the cerebral tissues of melodinenine-B-treated I/R-induced neuronal injury rats.

## Conclusions

Melodinenine B protects against neuronal injury in cerebral ischemia-reperfusion injury rats by regulating the inflammasomes.

## Acknowledgments

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## Disclosure

The authors report no conflict of interest.

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