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Methylene blue attenuates renal ischemia-reperfusion injury by negative regulation of NLRP3 signaling pathway

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Abstract. – OBJECTIVE: To investigate the effect of methylene blue (MB) on renal ischemia-reperfusion (IR) injury in mice and its possible relevant mechanisms.

MATERIALS AND METHODS: A total of 30 male C57/BL6 mice aged 4 months old were randomly divided into the following three groups: Sham group (n=10), IR group (n=10), and MB group (n=10). Mice in MB group were treated with gavage continuously using methylene blue solution (dosage: 25 mg·kg^{-1}·d^{-1}) until they were 7 months old. Mice in the other two groups were administrated with the same amount of normal saline for gavage. After that, the abdomen of mice in Sham group was opened and closed, and bilateral renal pedicles of mice in IR group and MB group were occluded using a micro-artery clamp for 45 min for modeling. After modeling, the renal tissues and blood samples of mice were taken for detection. The levels of serum creatinine (Scr), blood urea nitrogen (BUN), and malondialdehyde (MDA), and activity of superoxide dismutase (SOD) in mice in each experimental group were detected and statistically analyzed, respectively. The degree of renal tubular necrosis in renal tissues of mice was observed under an optical microscope. Enzyme-linked immunosorbent assay (ELISA) was used to detect the levels of inflammatory factors [interleukin-1β (IL-1β), IL-18, IL-10 and transforming growth factor-β1 (TGF-β1)] in renal tissues of mice in each experimental group, followed by relevant statistical analyses. The expressions of relevant proteins [NACHT, LRR, and PYD domains-containing protein 3 (NLRP3), nuclear factor-κB (NF-κB), caspase-1, pro-IL-1β, and IL-1β] in renal tissues of mice in each experimental group were detected via Western blotting, and the gray value of each band was detected for statistical analysis.

RESULTS: It was found in this experimental study that Scr and BUN levels in MB group were significantly lower than those in IR group, and the differences were statistically significant ($p<0.05$). Compared with that in IR group, the degree of renal tubular necrosis in MB group was significantly alleviated, and the difference was statistically significant ($p<0.05$). Compared with those in IR group, the levels of inflammatory factors (IL-1β and IL-18) in MB group were significantly decreased, but the levels of IL-10 and TGF-β1 in MB group were significantly increased, and the differences were statistically significant ($p<0.05$). Compared with those in IR group, the activity of SOD in MB group was increased significantly, but the level of MDA was decreased, and the differences were statistically significant ($p<0.05$). The protein expressions of NLRP3, NF-κB, caspase-1, and pro-IL-1β in MB group were decreased compared with those in IR group, but the expression of IL-1β in MB group was increased, and the differences were statistically significant ($p<0.05$).

CONCLUSIONS: We showed that methylene blue can alleviate the apoptosis and inflammatory response induced by renal IR injury in mice, and its relevant mechanism may be related to the fact that methylene blue can negatively regulate NLRP3 signaling pathway.

Key Words
Methylene blue, Ischemia-reperfusion, NLRP3, NF-kb.

Introduction

Methylene blue (MB), as a kind of promethazine drug, is widely used in the treatment of methemoglobinemia, malaria, cyanide poisoning, septicopyemia, and other diseases because of its anti-microbial, anti-inflammatory, and antioxidant effects[1,2]. Besides, due to its non-toxic biological staining characteristics, MB is widely used as a tracer agent for lymph node dissection in gastrointestinal, thyroid, and breast surgery[3]. In recent years, the neuroprotective effect of MB has attracted more and more attention. A study[4] has confirmed that MB has a definite therapeutic effect in the treatment of Alzheimer's disease (AD), and the AD is a chronic inflammatory response of the nervous system, whose occurrence and development are closely related to the excessive activation of astrocytes and a series of resulting

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inflammatory responses and apoptosis[5]. It is reported that the effect of MB in the treatment of AD may be related to its ability to inhibit the abnormal activation of astrocytes and reduce the oxidative stress response. Some studies[6] have also reported that MB can down-regulate the expression of NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) inflammasome produced by bone marrow-derived macrophages induced by adenosine triphosphate (ATP), thus alleviating its inflammatory response, whose mechanism may be also related to its influence on the classic pro-inflammatory signaling pathway, nuclear factor-κB (NF-κB)[7]. Related studies[8] have also confirmed that MB can inhibit caspase-1 activation to reduce apoptosis, and such an effect may be closely related to the fact that MB can reduce NLRP3 expression and inhibit oxidative stress response[3]. It was found in clinical work that after the treatment with MB for a period of time, the renal function of AD patients complicated with renal insufficiency was also improved. However, there are no relevant research reports and its mechanism of action is not yet clear. Therefore, the following animal experiment, based on the renal ischemia-reperfusion (IR) injury model of mice, was performed to investigate the effect of MB on renal IR injury and its relevant mechanism involved.

Materials and Methods

Animals and Treatments

A total of 30 male clean-grade C57/BL6 mice aged 4 months old were randomly divided into Sham group (n=10), IR group (n=10), and MB group (n=10). This study was approved by the Animal Ethics Committee of Hubei University of Arts and Science Animal Center. Mice in MB group were treated with gavage continuously using MB solution (dosage: 25 $mg \cdot kg^{-1} \cdot d^{-1}$)[9] until they were 7 months old. Mice in Sham group and MB group were administrated with the same amount of normal saline for gavage. After fasting for 8 h, mice in Sham group were anesthetized *via* intraperitoneal injection of tribromoethanol solution (200 mg/kg), a 2 cm-long incision was made directly, and the abdomen was closed immediately. Mice in IR group and MB group were anesthetized using the same method, the abdomen was opened, and bilateral renal pedicles were carefully separated and occluded using a micro-artery clamp. The occlusion was successful if the renal color turned from bright red into purple-black. After 45 min, the artery clamp was removed, the kidney turned red rapidly, and the abdomen was closed layer by layer. After modeling, the blood was drawn from the retrobulbar venous plexus of mice in each experimental group and centrifuged at 3,000 rpm for 10 min to separate the serum. Then, the abdomen was opened to take renal tissues. Part of the renal tissues was taken, prepared into 10% liver homogenate using normal saline at 4°C, and centrifuged at 3000 rpm for 10 min. The supernatant was taken to detect superoxide dismutase (SOD) and malondialdehyde (MDA) in renal tissues in each group. Part of the renal tissues was taken and immersed in 4% formaldehyde solution, and the remaining renal tissues were cut into small pieces and stored at -80°C for subsequent detection.

Detection of Serum Creatinine (Scr) and Blood Urea Nitrogen (BUN)

The levels of Scr and BUN in serum collected in each experimental group were determined using a full-automatic biochemical analyzer for laboratory animals (KeyGen, Nanjing, China), followed by relevant statistical analyses.

The degree of renal tubular necrosis in renal tissues was observed under an optical microscope (Table I).

Renal tissues fixed in 10% formaldehyde solution in each experimental group were prepared into paraffin sections according to the method in literature[10]. The pathological changes in renal tissues were observed under the optical microscope (400×), and the degree of renal tubular necrosis in the same field of view was observed, followed by relevant statistical analyses based on the degree of renal tubular necrosis[11].

Detection of Levels of Relevant Inflammatory Factors in Renal Tissues via Enzyme-Linked Immunosorbent Assay (ELISA)

The same mass of renal tissue samples was weighed, added with protein extraction reagent, homogenized and centrifuged at 2,000-3,000 rpm for about 20 min. Then, the supernatant was carefully collected and detected step by step according to the instructions of ELISA kit. Each index was detected for 3 times and the average was taken.

Table I. Scoring criteria of degree of renal tubular necrosis.

No injury	≤10%	11-25%	26-45%	46-75%	≥76%
0	1	2	3	4	5

Detection of SOD and MDA in Renal Tissues

Using the renal tissue homogenate collected above, oxidative stress indexes, including the MDA level and SOD activity, were detected. The MDA level was determined using thiobarbituric acid (TBA) colorimetric method, and nitrite method was used to detect the SOD activity[12].

Detection of Protein Levels in Renal Tissues via Western Blotting

Frozen renal tissue samples of mice obtained in each experimental group were thawed and lysed, followed by protein detection. The expressions of NLRP3, NF-κB, caspase-1, pro-interleukin-1β (IL-1β), and IL-1β in renal tissues of mice in each experimental group were detected *via* Western blotting. In this study, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference, and antibodies used in mice in each experimental group were purchased from Abcam (Abcam, Cambridge, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of data obtained in this experiment. All data were presented as mean ± standard deviation, and *t*-test was used to compare the differences between two experimental groups. $p<0.05$ suggested that the difference was statistically significant.

Results

MB Could Reduce the Scr and BUN Levels in Patients with Renal Insufficiency

A total of 20 patients with renal insufficiency were randomly selected, and the fasting blood was drawn in the morning to detect the Scr and BUN levels. After that, patients orally took MB (1 mg/kg, q.d.) for 4 consecutive weeks[5]. The fasting blood was drawn in the morning to detect the Scr and BUN levels again. Results showed that Scr (Figure 1A) and BUN (Figure 1B) levels in patients after application of MB were decreased compared with those before oral administration of MB, and the differences were statistically significant ($p<0.05$). To study its relevant mechanism of action, this animal experimental research was performed.

MB Could Alleviate the Renal Function Damage and Pathological Changes in Renal Tissues of IR Mice

First of all, Scr and BUN were used as common indexes of evaluating renal function in this experiment. Results showed that Scr (Figure 2A) and BUN (Figure 2B) levels in mice in IR group were significantly higher than those in MB group, and the differences were statistically significant ($p<0.05$), indicating that MB can alleviate the renal function damage in IR mice. Secondly, renal tubular necrosis was used as a classic pathological change in renal tissues[11]. The degree of renal tubular necrosis in renal tissues of mice in each experimental group was observed using the optical microscope (400×), scored and statistically analyzed. Results showed that the renal tubule necrosis in renal tissues of mice in MB group was significantly alleviated compared with that in IR group, and the difference in the score of renal tubular necrosis was statistically significant ($p<0.05$), suggesting that MB can alleviate the pathological changes in renal tissues of IR mice. In summary, MB can reduce the IR injury in mice.

MB Could Negatively Regulate the Expressions of IL-1ß and IL-18, and Positively Regulate the Expressions of IL-10 and TGF-ß1 in Renal Tissues of IR Mice

IL-1β and IL-18 are important pro-inflammatory factors in the occurrence and development processes of renal IR injury in mice[13]. Results of

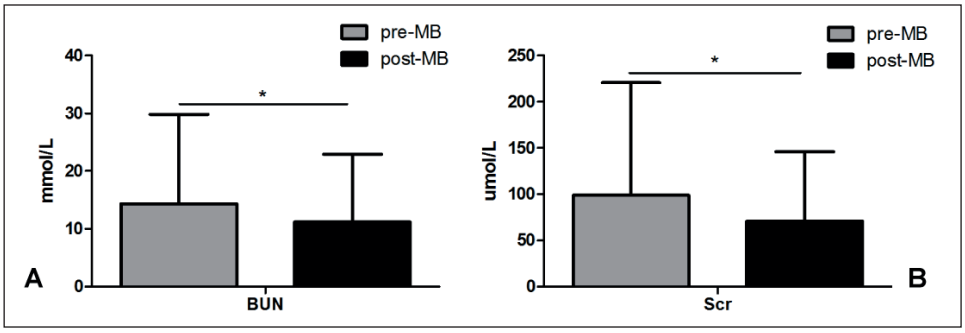

Figure 1. Comparisons of Scr/BUN levels in patients with renal insufficiency before and after application of MB in clinical work (mean ± standard deviation, *$p<0.05$).

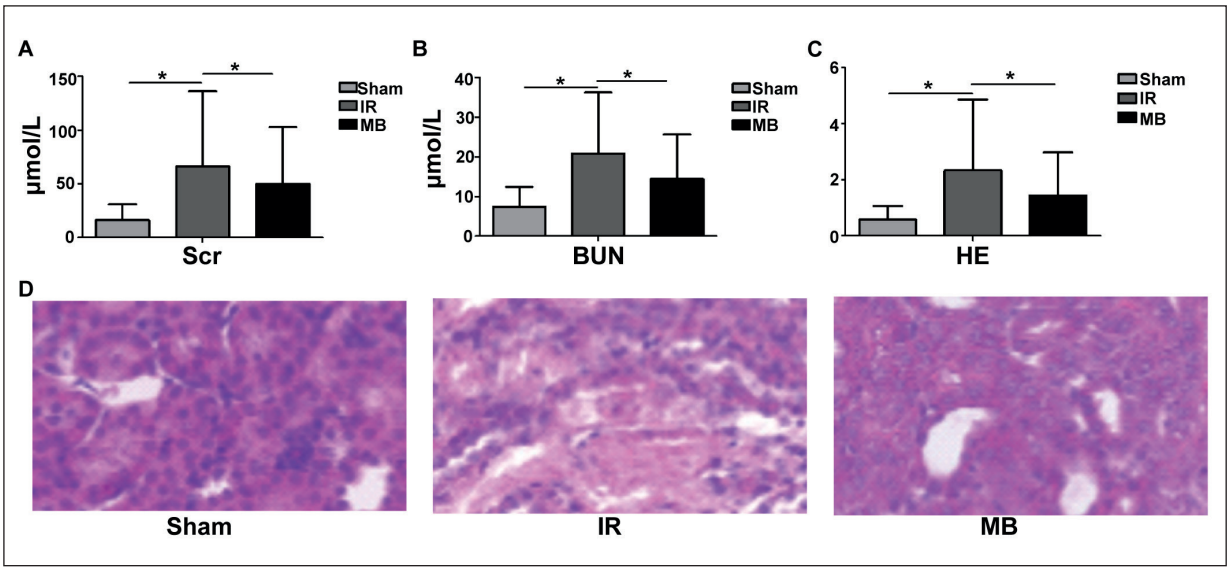

Figure 2. A-B, Comparisons of Scr/BUN levels in mice in each experimental group (n=10) (mean ± standard deviation, *p<0.05). **C**, Comparisons of morphological changes in renal tissues of mice in each experimental group (n=10). **D**, Comparison of the degree of renal tubular necrosis of mice in each experimental group under the optical microscope (400×) (mean ± standard deviation, *p<0.05).

this experiment showed that IL-1β and IL-18 levels in mice in MB group were significantly decreased compared with those in IR group. However, the levels of IL-10 and TGF-β1, important anti-inflammatory factors in the process of inflammatory response, were opposite, and the differences were statistically significant (p<0.05) (Figure 3). In summary, MB attenuates IR injury in mice, which is manifested as alleviating the inflammatory response through increasing the expressions of anti-inflammatory factors and decreasing the expressions of pro-inflammatory factors.

MB Could Negatively Regulate Reactive Oxygen Species (ROS)/NF-κB-NLRP3 Signaling Pathway

To further investigate the mechanism of MB in reducing inflammatory response in IR renal tissues in mice, the SOD activity and MDA level, key indexes to evaluate the oxidative stress response in IR injury, in renal tissues of mice in each experimental group were measured[14]. Results revealed that the SOD activity in mice in MB group was increased compared with that in IR group, but the MDA level was the opposite, and the differences were statistically significant (p<0.05) (Figure 4), suggesting that MB can exert its effect through its antioxidant response. Further protein detection showed that NF-κB and NLRP3 protein expressions in mice in MB group were significantly decreased compared with those in IR group, and the differences in comparisons of gray values were statistically significant (p<0.05) (Figure 5). The above results indicate that MB may alleviate renal tissue damage in IR mice *via* negatively regulating ROS/NF-κB-NLRP3 signaling pathway.

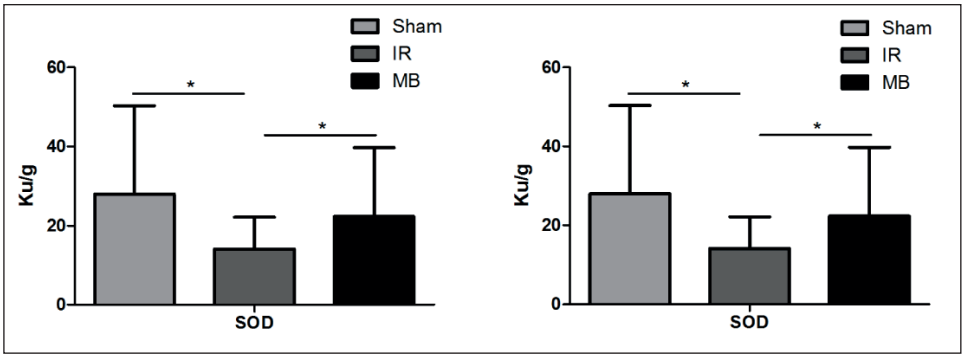

Figure 3. Levels of IL-1β and TGF-β1 in renal tissues of mice in each experimental group (n=10) (mean ± standard deviation, *p<0.05).

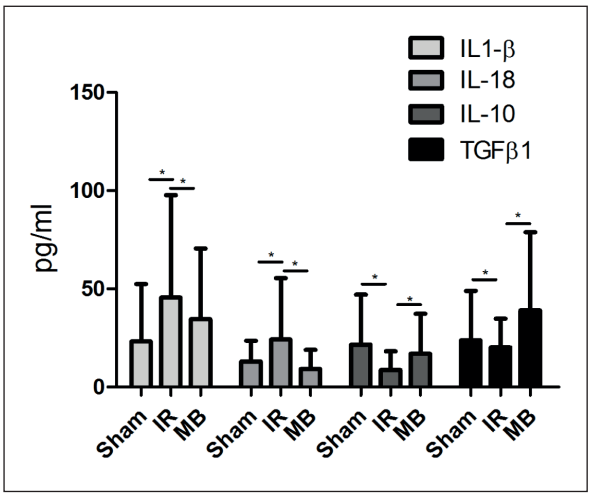

Figure 4. Comparisons of SOD activity and MDA level in renal tissues of mice in each experimental group (n=10) (mean ± standard deviation, *p<0.05).

MB Could Negatively Regulate Caspase-1-Related Signaling Pathway

A study[15] has confirmed that caspase-1 can mediate apoptosis, and plays an important role in the occurrence of a variety of kidney diseases. It has been demonstrated that[7] caspase-1 can promote the maturation of inflammatory factor IL-1β and exacerbate the renal tissue damage. Results of this experiment showed that caspase-1 and IL-1β protein expressions in MB group were significantly decreased compared with those in IR group, but the pro-IL-1β protein expression in MB group was increased, and the differences in comparisons of gray values were statistically significant (p<0.05) (Figure 5). The above experimental results suggest that MB may alleviate renal tissue damage in IR mice *via* negatively regulating the expression of caspase-1 and caspase-1-mediated signaling pathway.

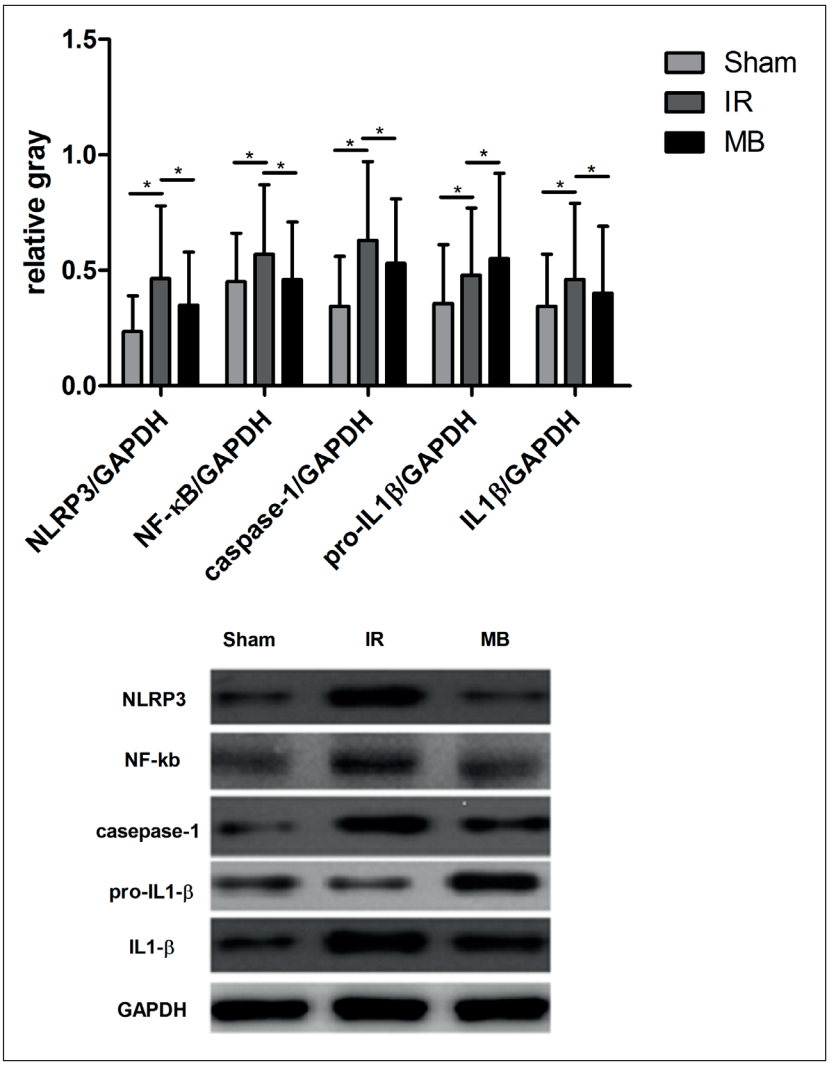

Figure 5. Comparisons of NLRP3, NF-κB, caspase-1, pro-IL-1β and IL-1β protein expressions in renal tissues of mice in each experimental group (n=10), and analyses of gray values (mean ± standard deviation, *p<0.05).

Discussion

In clinical work, it was found in the treatment of AD patients with MB that the renal function was improved. Therefore, patients with renal insufficiency were randomly selected for corresponding research, and it was found that MB could improve renal function in patients, and the measured values (Figure 1, Scr/BUN) had statistically significant differences. To explore its mechanism of action, the renal IR model of mice was used in this animal experiment.

Experimental results showed that compared with those in Sham group, Scr and BUN levels were increased and renal tubule necrosis was aggravated in IR group, and the differences were statistically significant, suggesting that the renal IR injury in mice is successfully simulated. Compared with those in IR group, Scr and BUN levels in MB group were reduced and renal tubule necrosis was alleviated, confirming that MB can attenuate the IR-induced renal function damage and pathological changes in renal tissues. Reports[16] have confirmed that injecting MB can effectively reduce the inflammatory response caused by traumatic brain injury in mice. To study the mechanism of MB in renal IR in mice, the levels of inflammatory factors (IL-1β, IL-18, IL-10, and TGF-β1) in the kidney of mice in each experimental group were detected first. Results showed that MB could negatively regulate the levels of IL-1β and IL-18, but positively regulate the levels of IL-10 and TGF-β1. Studies have proved that IL-1β and IL-18 are pro-inflammatory factors, while IL-10 and TGF-β1 are pro-inflammatory factors. Therefore, it is concluded that MB can increase the expressions of anti-inflammatory factors and decrease the expressions of pro-inflammatory factors to alleviate the renal IR injury. It has been reported in the literature[17] that mitochondrial dysfunction can lead to a variety of acute and chronic renal inflammatory injuries, and its dysfunction is often manifested as the accumulation of ROS. In addition, it has been reported[18] that ROS-induced oxidative stress response can induce the NLRP3 expression, thus promoting the pro-IL-1β secretion[17], and exacerbating the inflammatory response. Further experimental results (Figure 3) revealed that MB could reduce the level of MDA and increase the activity of SOD in renal tissues, indicating that MB can inhibit the oxidative stress response caused by the accumulation of ROS. It has been reported[19] that the increased protein expressions of inflammatory factors (IL-1β and IL-18) and NLRP3 are closely related to the NF-κB signaling pathway. Compared with those in IR group, the protein expressions of NF-κB and NLRP3 were decreased in MB group (Figure 5), indicating that MB can down-regulate the expressions of NF-κB and NLRP3. Therefore, it is hypothesized that MB may alleviate the inflammatory response through negatively regulating the ROS/NF-κB-NLRP3 signaling pathway. Some studies[20] have also confirmed that caspase-1-mediated apoptosis plays an important role in the occurrence of a variety of kidney diseases, while some[6] have demonstrated that caspase-1 can promote the maturation of pro-IL-1β and transformation into IL-1β, aggravating the injury. However, relevant reports[21] showed that MB can inhibit the activation of caspase-1 through the oxidation catalysis of cysteine. MB can attenuate the inflammatory cascade by down-regulating the protein expressions of pro-IL-1β and NLRP3[21], proving once again that MB alleviates IR injury in renal tissues *via* down-regulation of NLRP3. However, results in this experiment showed that caspase-1 and IL-1β protein expressions in MB group were significantly decreased compared with those in IR group, but the pro-IL-1β protein expression in MB group was increased, and the differences in gray values were statistically significant (Figure 5), indicating that the negative regulation of caspase-1-pro-IL-1β/IL-1β signaling pathway *via* MB may be one of its protective mechanisms. In conclusion, MB may attenuate renal IR injury through negatively regulating ROS/NF-κB-NLRP3 signaling pathway and caspase-1-related signaling pathway.

Conclusions

We observed that MB can alleviate the renal IR injury in mice, and its mechanism may be related to the fact that MB can negatively regulate ROS/NF-κB-NLRP3 signaling pathway and caspase-1-related signaling pathway *via* its anti-inflammatory and anti-oxidative effects.

Conflict of Interest

The authors declared no conflict of interest.

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