

Magnesium isoglycyrrhizinate protects against renal-ischemia-reperfusion injury in a rat model via anti-inflammation, anti-oxidation and anti-apoptosis

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Abstract. The present study aimed to investigate whether magnesium isoglycyrrhizinate protects against renal ischemia-reperfusion injury (RIRI), and to verify the underlying mechanisms. An RIRI rat model was induced by removing the right kidney, and exposing and clamping the left kidney. RIRI model rats were administered 30 mg/kg magnesium isoglycyrrhizinate for 3 days. Blood urea nitrogen (BUN) and serum creatinine levels in the blood of RIRI model rat were examined, compared with sham-operated controls. Magnesium isoglycyrrhizinate suppressed the activities of tumor necrosis factor- α , interleukin (IL)-1 β , IL-6, superoxide dismutase, glutathione peroxidase, inducible nitric oxide synthase (iNOS) and caspase-3 in RIRI model rats. Renal iNOS, matrix metalloproteinase (MMP)-2, phosphorylated-signal transducers and activators of transcription 3 (STAT3) and intercellular adhesion molecule-1 (ICAM-1) protein expression levels were suppressed by magnesium isoglycyrrhizinate treatment in RIRI model rats. These findings suggested that magnesium isoglycyrrhizinate protects RIRI via anti-inflammatory, -oxidative and -apoptotic mechanisms in an RIRI rat model. These results implicate magnesium isoglycyrrhizinate pretreatment as a potential approach to protect against RIRI via suppression of the iNOS, ICAM-1, MMP-2 and STAT3 signaling pathways.

Introduction

Ischemia-reperfusion injury (IRI) refers to the damage effect generated by tissues or organs following ischemia, and recovery blood perfusion or oxygen supply (1). Clinically, renal IRI (RIRI) is relatively common (2). RIRI is a common cause

of acute renal failure. It is one of the main factors impacting functional recovery, and early-phase and long-term survival following renal transplantation (3). Therefore, it is important to understand the underlying mechanisms of RIRI to reduce the degree of injury.

Previous studies have indicated that adhesion of vascular endothelial cells in circulation, aggregation and infiltration serve important roles in IR (4). During this process, β 2 integrins interact with intercellular adhesion molecules (ICAMs) on the surface of vascular endothelial cells, and mediate IR injury (5). Under normal circumstances, intercellular adhesion molecule-1 (ICAM-1) is not expressed on glomerular epithelial, intercapillary and glomerular cells; however, its expression markedly increases following exposure to toxins, including tumor necrosis factor (TNF)- α and interleukin (IL)-1 (6).

Extracellular matrix metabolism imbalance is an important cause of IR injury (7). The matrix metalloproteinases (MMPs) are a major enzyme family that degrade the extracellular matrix. MMP-2 is a principle member of the MMP family (8). Previous studies have demonstrated that abnormal MMP-2 expression in kidneys serves a role in the development and progression of IR injury (7,9).

In addition to hypoxia-induced damage, renal IR may induce an inflammatory response of kidney parenchyma cells directly and cause ischemic acute renal failure (10). The induction of inflammation results in a systemic response reaction to RIRI (11).

Signal transducers and activators of transcription 3 (STAT3) may regulate biological behaviors of tumor and immune cells by mediating extracellular signal molecules of inflammatory mediators, and is an indispensably critically module for chronic inflammation to promote tumorigenesis and the inflammatory process (12).

Magnesium isoglycyrrhizinate is a magnesium salt with a stereoisomer for national glycyrrhizic acids (13). Previous studies have demonstrated that magnesium isoglycyrrhizinate impacts ion channels (inhibits calcium ions), activates or inhibits enzyme activity, regulates substance metabolism, and regulates excitability of cholinergic neurons by acting on hormone receptors. It has similar effects to adrenocortical hormones, and presents obvious anti-inflammatory and immunoregulatory effects (14-16). The present study aimed to investigate whether magnesium isoglycyrrhizinate protects RIRI, and to assess the potential underlying mechanisms.

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Materials and methods

Ethics and animals. Male Sprague-Dawley (SD) rats (age, 8 weeks; weight, 240–280 g) were obtained from the Animal Research Center of Wuhan University (Wuhan, China), housed at 22–24°C in a 12-h light/dark cycle and were provided with standard rat food and water *ad libitum*. This study was approved by the ethics committee of Wuhan General Hospital of Guangzhou Military Command (Wuhan, China).

IR induction. SD rats were administered with 35 mg/kg of pentobarbital at 36–37°C. A midline abdominal incision was made following sterilization. Subsequently, the right kidney was removed, and the left kidney was exposed and the renal artery blood vessel was clamped for inspection of ischemia for 1 h. Then, reperfusion was performed by releasing the clamps and blood flow was restored. Finally, the incisions were closed. Meanwhile, rats in the control (sham-operated) group were administered with pentobarbital and midline abdominal incision was performed without ischemia.

Groups. SD rats were randomly divided into three groups: Control (sham-operated group, n=6), administered with saline for 3 days; RIRI model (model group, n=8), administered with saline for 3 days; and magnesium isoglycyrrhizinate-treated (MAG group, n=8), administered with 30 mg/kg magnesium isoglycyrrhizinate for 3 days after RIRI induction. Magnesium isoglycyrrhizinate was purchased from Chia Tai Tianqing Pharmaceutical Group Co., Ltd (Jinan, China) and its chemical structure is presented in Fig. 1.

Evaluation of renal function. The left kidney from all rats were dissected and immediately fixed in 10% neutral formalin solution for 24 h. Subsequently, tissue samples were dehydrated with different concentrations of alcohol, embedded in paraffin and sectioned into 5- μ m thick pieces. Following this, sections were stained with hematoxylin and eosin.

After 3 days of magnesium isoglycyrrhizinate treatment, blood samples were collected from SD rats. Blood urea nitrogen (BUN) and serum creatinine (Cr) levels were measured with using ELISA kits (cat. nos. C013-2 and C011-2, respectively; Nanjing Jiancheng Biology Engineering Institute, Nanjing, China) and a spectrophotometer (SpectroLab UV 7500) at 450 nm.

ELISA. Blood (100 μ l) was drawn from the vena cava and centrifuged at 4,000 \times g for 10 min at 4°C. Serum was collected and analyzed by ELISA for the levels of TNF- α (cat. no. CSB-E11987r), IL-1 β (cat. no. CSB-E08055r), IL-6 (cat. no. CSB-E04640r), superoxide dismutase (SOD; cat. no. CSB-E14981r), glutathione peroxidase (GSH-Px; cat. no. CSB-E12146r), inducible nitric oxide synthase (iNOS; cat. no. CSB-E08325r) and caspase-3 (cat. no. CSB-E08857r) activity using a spectrophotometer (SpectroLab 7500 UV) at 450 or 405 nm, according to manufacturer's protocol. All ELISA kits were purchased from Cusabio (Wuhan, China).

Western blotting. After 3 days of magnesium isoglycyrrhizinate treatment, the left kidney from all rats was dissected and immediately homogenized with Tissue Protein Extraction

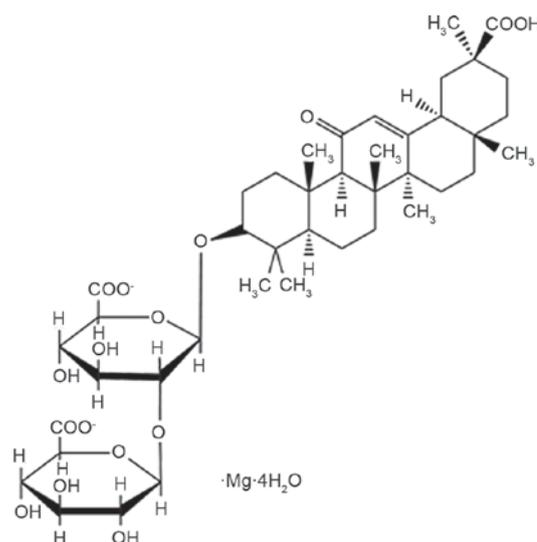


Figure 1. The chemical structure of magnesium isoglycyrrhizinate.

Reagent and 1% protease inhibitor (KangChen Bio-Tech, Shanghai, China). Following centrifugation at 11,000 \times g for 10 min at 4°C, the supernatant was obtained to measure protein concentrations with a Bicinchoninic Acid Protein Assay kit (Tiangen, Beijing, China). Total proteins (50 μ g) were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (0.45 μ m). The membrane was blocked with 5% bovine serum albumin in TBS with Tween-20 (TBST) for 1 h and incubated with following primary antibodies overnight at 4°C: Anti-iNOS (1:2,000; cat. no. 13120), anti-MMP-2 (1:4,000; cat. no. 87809), anti-phosphorylated (p)-STAT3 (1:2,000; cat. no. 9145), anti-ICAM-1 (1:4,000; cat. no. 4915), anti-B-cell lymphoma 2 X-associated protein (Bax; 1:3,000; cat. no. 14796), anti- β -actin (1:2,000; cat. no. 4970), all purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA), and anti-B-cell lymphoma 2 (Bcl-2; 1:2,000; cat. no. ab59348), which was purchased from Abcam (Cambridge, UK). The membrane was washed with TBST and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5,000; cat. no. 7074; Cell Signaling Technology, Inc.) at 37°C for 1 h. Immunoreactivity was detected using an Enhanced Chemiluminescence kit (EMD Millipore, Billerica, MA, USA). Protein expression was detected using the Molecular Imager[®] Gel Doc[™] XR+ System with Image Lab[™] Software and quantified using the Bio-Rad Laboratories Quantity One software (version 3.0; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Statistical analyses were performed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as mean \pm standard deviation. Differences between groups were analyzed by one-way analysis of variance followed by Tukey's test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Magnesium isoglycyrrhizinate protects BUN and Cr levels in RIRI model rats. In the RIRI rat model group, RIRI

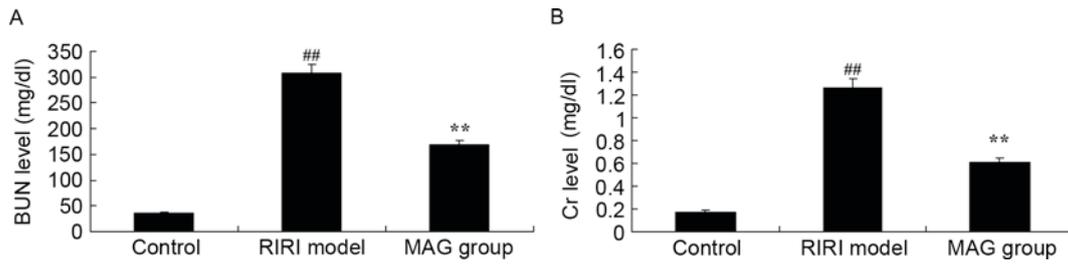


Figure 2. Magnesium isoglycyrrhizinate protects BUN and Cr levels in the blood of RIRI model rats. Quantification of (A) BUN and (B) Cr levels in the three groups. Data are expressed as the mean \pm standard deviation. ##P<0.01 vs. control; **P<0.01 vs. RIRI model. RIRI, renal ischemia-reperfusion injury; MAG, magnesium isoglycyrrhizinate; Cr, creatinine; BUN, blood urea nitrogen.

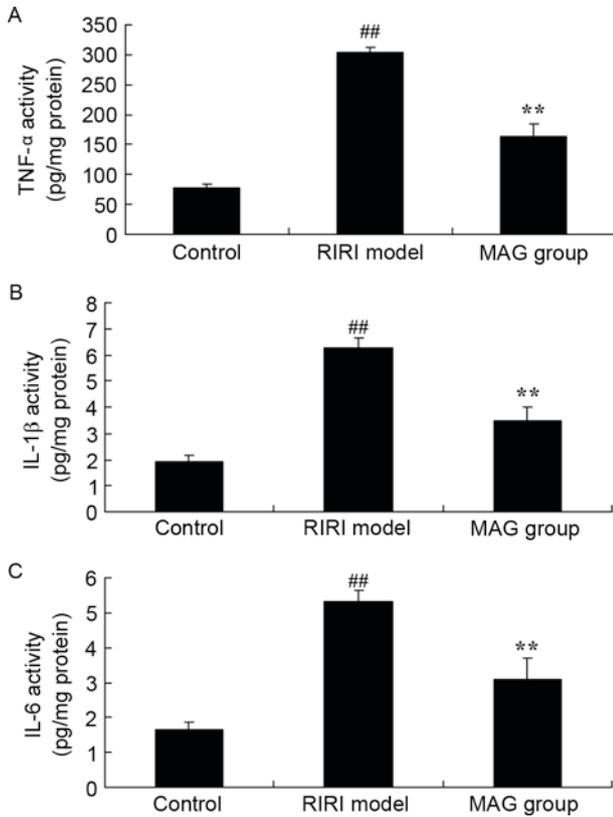


Figure 3. Magnesium isoglycyrrhizinate protects the activities of TNF- α , IL-1 β and IL-6 in the blood of RIRI model rats. Quantification of (A) TNF- α , (B) IL-1 β and (C) IL-6 levels in the three groups. Data are expressed as the mean \pm standard deviation. ##P<0.01 vs. control; **P<0.01 vs. RIRI model. RIRI, renal ischemia-reperfusion injury; MAG, magnesium isoglycyrrhizinate; TNF- α , tumor necrosis factor- α ; IL, interleukin.

significantly increased BUN (Fig. 2A) and Cr (Fig. 2B) levels in the blood of RIRI model rats, compared with the control group. However, treatment with magnesium isoglycyrrhizinate significantly ameliorated this effect (Fig. 2).

Magnesium isoglycyrrhizinate protects TNF- α , IL-1 β and IL-6 activity in RIRI model rats. Compared with the control group, TNF- α (Fig. 3A), IL-1 β (Fig. 3B) and IL-6 (Fig. 3C) activities in the blood of RIRI model rats were significantly increased. Magnesium isoglycyrrhizinate treatment significantly ameliorated this effect (Fig. 3).

Magnesium isoglycyrrhizinate promotes SOD and GSH-Px activities in an RIRI rat model. RIRI markedly decreased the

activities of SOD (Fig. 4A) and GSH-Px (Fig. 4B) in the RIRI rat model, compared with the control group. Following RIRI, magnesium isoglycyrrhizinate promoted RIRI-inhibited SOD and GSH-Px activities in the RIRI rat model (Fig. 4).

Magnesium isoglycyrrhizinate protects the activity and protein expression of iNOS in an RIRI rat model. There was a significant increase in iNOS activity (Fig. 5A) and protein expression (Fig. 5B and C) in RIRI model rats, compared with the control group. Magnesium isoglycyrrhizinate pretreatment significantly protected against RIRI-induced iNOS activity and protein expression in RIRI rats (Fig. 5).

Magnesium isoglycyrrhizinate protects the activity of caspase-3 in RIRI model rats. To evaluate the effect of magnesium isoglycyrrhizinate on apoptosis activity in the renal tissue of RIRI model rats, caspase-3 activity was assessed. RIRI significantly induced caspase-3 activity in RIRI rats, compared with controls (Fig. 6). RIRI-induced caspase-3 activity was inhibited significantly by magnesium isoglycyrrhizinate treatment (Fig. 6).

Magnesium isoglycyrrhizinate protects the protein expression of MMP-2 in RIRI model rats. To investigate the effect of magnesium isoglycyrrhizinate on MMP-2 protein expression in the renal tissue of RIRI model rats, MMP-2 protein expression levels were measured by western blotting. RIRI model rats exhibited markedly increased protein expression levels of MMP-2 compared with control rats (Fig. 7). Pretreatment with magnesium isoglycyrrhizinate significantly protected against MMP-2 protein expression upregulation in the renal tissue of RIRI rats (Fig. 7).

Magnesium isoglycyrrhizinate protects the protein expression of p-STAT3 in RIRI model rats. The effect of magnesium isoglycyrrhizinate on p-STAT3 protein expression levels in the renal tissue of RIRI model rats was assessed by western blotting. As presented Fig. 8, p-STAT3 protein expression levels in renal tissue were significantly upregulated following RIRI, compared with control rats. The induction of p-STAT3 protein expression in renal tissue was significantly suppressed by magnesium isoglycyrrhizinate, compared with the RIRI model group (Fig. 8).

Magnesium isoglycyrrhizinate protects the protein expression of ICAM-1 in RIRI model rats. ICAM protein expression levels were detected in an RIRI rat model by western blotting. The

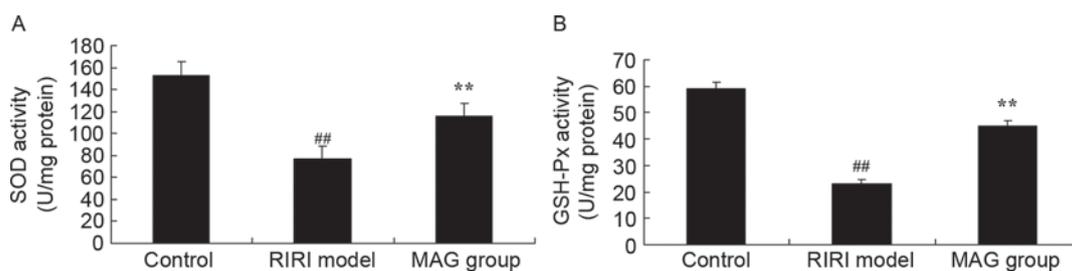


Figure 4. Magnesium isoglycrrhizinate protects the activities of SOD and GSH-Px in the blood of RIRI model rats. Quantification of (A) SOD and (B) GSH-Px activities in the three groups. Data are expressed as the mean \pm standard deviation. ## P <0.01 vs. control; ** P <0.01 vs. RIRI model. RIRI, renal ischemia-reperfusion injury; MAG, magnesium isoglycrrhizinate; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase.

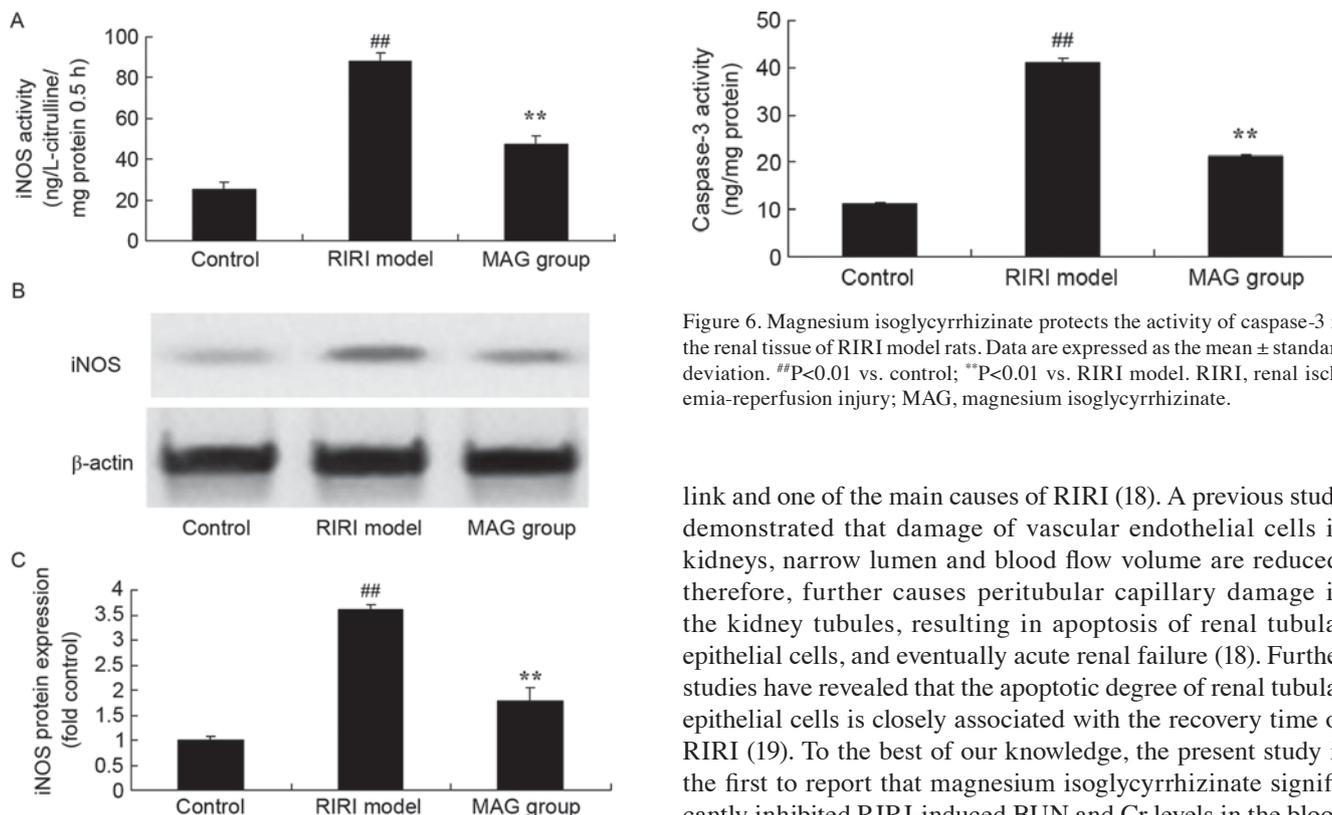


Figure 5. Magnesium isoglycrrhizinate protects the activity and protein expression levels of iNOS in the blood of RIRI model rats. (A) iNOS activity. (B) Representative western blot images and (C) quantification of iNOS protein expression levels in the three groups. β -actin served as an internal control. Data are expressed as the mean \pm standard deviation. ## P <0.01 vs. control; ** P <0.01 vs. RIRI model. RIRI, renal ischemia-reperfusion injury; MAG, magnesium isoglycrrhizinate; iNOS, inducible nitric oxide synthase.

results demonstrated that there was a significant increase in ICAM protein expression levels in RIRI model rats, compared with the control group (Fig. 9). Magnesium isoglycrrhizinate treatment significantly reduced these levels in RIRI rats (Fig. 9).

Discussion

RIRI is a relatively common and severe disease in clinics, and often occurs in patients with kidney transplantation, trauma hemorrhage and hemorrhagic shock (2). Treatment options, including hemodialysis, have improved; however, RIRI mortality rates remain >30% (17). IR injury is an important

Figure 6. Magnesium isoglycrrhizinate protects the activity of caspase-3 in the renal tissue of RIRI model rats. Data are expressed as the mean \pm standard deviation. ## P <0.01 vs. control; ** P <0.01 vs. RIRI model. RIRI, renal ischemia-reperfusion injury; MAG, magnesium isoglycrrhizinate.

link and one of the main causes of RIRI (18). A previous study demonstrated that damage of vascular endothelial cells in kidneys, narrow lumen and blood flow volume are reduced; therefore, further causes peritubular capillary damage in the kidney tubules, resulting in apoptosis of renal tubular epithelial cells, and eventually acute renal failure (18). Further studies have revealed that the apoptotic degree of renal tubular epithelial cells is closely associated with the recovery time of RIRI (19). To the best of our knowledge, the present study is the first to report that magnesium isoglycrrhizinate significantly inhibited RIRI-induced BUN and Cr levels in the blood of RIRI model rats.

Ischemia causes hypoxemia, so as to generate an inflammatory response by the kidney (11). However, it is difficult to precisely establish factors generated by the kidney that affect hypoxemia, namely the downstream inflammatory effects, and trigger and response factors (20). For example, after generating the inflammatory factor IL-1, the epithelial cells of the kidney are stimulated to generate downstream the inflammatory factors TNF- α and IL-6 by the epithelial cells of kidney (21). An animal model study on rat kidneys demonstrated that hypoxemia may rapidly activate the nuclear factor- κ B signaling pathway, and cause transcription and synthesis of proinflammatory factors (22). The present study demonstrated that magnesium isoglycrrhizinate significantly recovered RIRI-induced upregulation of TNF- α , IL-1 β and IL-6, and inhibited SOD and GSH-Px activities in RIRI rats. Furthermore, Xie *et al* (14) suggested that magnesium isoglycrrhizinate suppressed inflammation via inhibition of the phospholipase A2/Arachidonic acid signaling pathway.

Ischemia may induce the kidney to generate iNOS. Furthermore, experimental evidence has indicated that iNOS

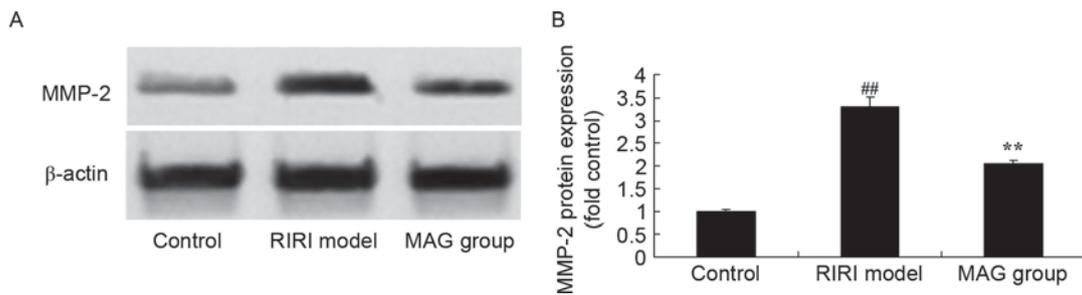


Figure 7. Magnesium isoglycyrrhizinate protects the protein expression levels of MMP-2 in the renal tissue of RIRI model rats. (A) Representative western blot images and (B) quantification of MMP-2 protein expression levels in the three groups. β -actin served as an internal control. Data are expressed as the mean \pm standard deviation. ^{##} $P < 0.01$ vs. control; ^{**} $P < 0.01$ vs. RIRI model. RIRI, renal ischemia-reperfusion injury; MAG, magnesium isoglycyrrhizinate; MMP-2, matrix metalloproteinase 2.

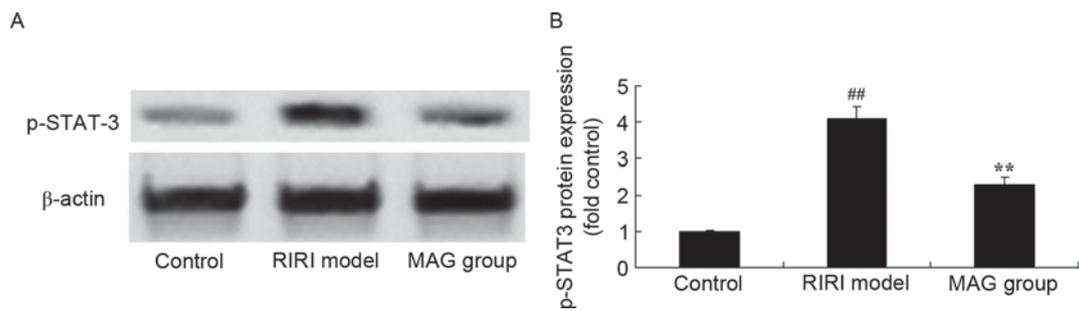


Figure 8. Magnesium isoglycyrrhizinate protects the protein expression levels of p-STAT3 in the renal tissue of RIRI model rats. (A) Representative western blot images and (B) quantification of p-STAT3 protein expression levels in the three groups. β -actin served as an internal control. Data are expressed as the mean \pm standard deviation. ^{##} $P < 0.01$ vs. control; ^{**} $P < 0.01$ vs. RIRI model. RIRI, renal ischemia-reperfusion injury; MAG, magnesium isoglycyrrhizinate; p-STAT3, phosphorylated signal transducers and activators of transcription 3.

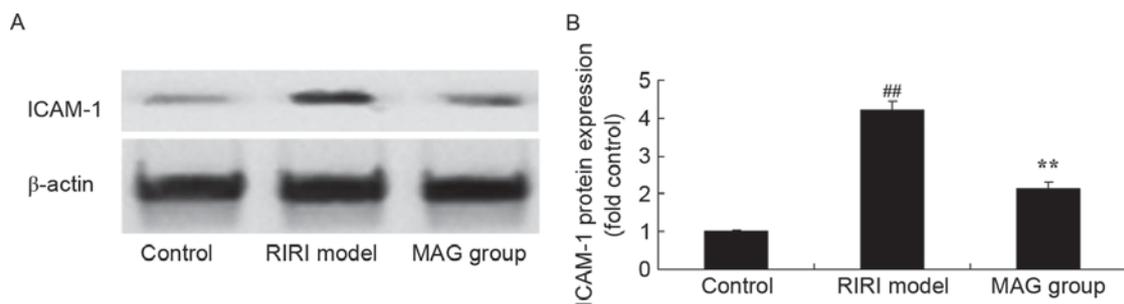


Figure 9. Magnesium isoglycyrrhizinate protects the protein expression levels of ICAM-1 in the renal tissue of RIRI model rats. (A) Representative western blot images and (B) quantification of ICAM-1 protein expression levels in the three groups. β -actin served as an internal control. Data are expressed as the mean \pm standard deviation. ^{##} $P < 0.01$ vs. control; ^{**} $P < 0.01$ vs. RIRI model. RIRI, renal ischemia-reperfusion injury; MAG, magnesium isoglycyrrhizinate; ICAM-1, intercellular adhesion molecule-1.

may mediate the damage process of renal tubular epithelial cells for ischemic renal function failure (23). The experimental study *in vitro* indicated that when adopting dissociative cells of kidney tubules to cultivate *in vitro*, anoxia may result in increasing synthesis levels of nitric oxide for renal tubular epithelial cells (24). This phenomenon implies that increasing synthesis levels of nitric oxide may be a direct response generated by renal tubular epithelial cells for stimulation of anoxia (25). The results of the present study demonstrated that magnesium isoglycyrrhizinate pretreatment significantly protects against RIRI-induced iNOS activity and protein expression levels in RIRI rats. Tang *et al* (13) demonstrated that magnesium isoglycyrrhizinate protects remnant liver function via the STAT3 and iNOS signaling pathways.

RIRI is primarily characterized by increased oxygen radicals, intracellular calcium ion concentration, endocrine hormones, cellular immunity and apoptosis (26). Numerous cell factors have been implicated to be involved in RIRI, including IL-1 β , IL-6, ICAM-1, VCAM-1 and TNF- α (27). Inflammatory factors induce an inflammatory cascade, magnify inflammatory responses, and induce white blood cells (WBCs) to concentrate on the external vessel lumen of marrow (28). ICAM-1 is a cell surface glycoprotein expressed on endothelial cells. Following RIRI, the stress reaction of the human body generates lots of inflammatory mediators and cellular factors. These substances also stimulate vascular endothelial cells, while stimulating WBCs (28). Vascular endothelial cells express cluster of differentiation (CD)11/18 glycoprotein

adhesive compounds on the cell surface, while WBCs express ICAM-1 (29). Adhesion occurs between WBC and endothelial cells, which promotes activation of circulating WBCs, releases inflammatory mediators, and serves a role in RIRI. IR results in upregulated expression of ICAM-1, resulting in local adhesion of circulating WBCs, forming an external medulla and increasing the endothelium permeability of WBC mediators. Cohesion of red blood cells results in external marrow losing perfusion following reperfusion. Furthermore, infiltrated and activated WBCs may result in direct tissue damage (29). In the present study, magnesium isoglycrrhizinate treatment significantly reduced ICAM protein expression levels in RIRI rats.

MMPs are a family of 23 molecules that are zinc-dependent peptide chain incision and proteolytic enzymes (8). They are involved in degradation and reconstruction of the extracellular matrix, inflammatory responses and ischemia hypoxic-ischemic damage (30). MMP-2 degrades main components of the extracellular matrix, including type IV collagen proteins (30). A previous study demonstrated that healthy expression of MMP-2 serves important roles in the development and progression of IRI (31). The present study demonstrated that pretreatment with magnesium isoglycrrhizinate significantly protects MMP-2 protein expression in the renal tissue of RIRI rats. Xiao *et al* (32) reported that magnesium isoglycrrhizinate decreased lung injury via downregulation of ICAM-1 and MMP-9.

The STAT channel may regulate proliferation, apoptosis, invasion and metastasis and angiogenesis of cells. In the STAT signaling pathway, for extracellular cells and cells on the cytomembrane, STAT3 may be activated via reversible acetylation (12). Activated STAT3 may enter the nucleus and combine with genomic DNA to induce transcription and regulatory effects (33). The signal transduction and transcriptional activation of STAT3 maintains and regulates a series of biological behaviors in the healthy body, including embryonic development, programmed cell death, organogenesis, congenital immunity, adaptive immunity and cell growth, whereas abnormal activation of STAT3 may result in occurrence of multiple diseases (34). In the present study, magnesium isoglycrrhizinate significantly suppressed p-STAT3 protein expression levels in the renal tissue of RIRI rats. Tang *et al* (13) demonstrated that magnesium isoglycrrhizinate protects remnant liver function via the STAT3 signaling pathway (13).

In conclusion, the present study demonstrated that magnesium isoglycrrhizinate significantly inhibits RIRI-induced BUN and Cr levels, recovers the RIRI-induction of TNF- α , IL-1 β and IL-6, and inhibits SOD and GSH-Px activities in RIRI rats. These results implicate magnesium isoglycrrhizinate pretreatment as a potential approach to protect against RIRI via suppression of the iNOS, ICAM-1, MMP-2 and STAT3 signaling pathways.

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