

Isoacteoside attenuates acute kidney injury induced by severe acute pancreatitis

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Abstract. Severe acute pancreatitis (SAP) is a common acute abdominal disease accompanied by systemic inflammatory response syndrome, which may be complicated by acute kidney injury (AKI). Isoacteoside (ISO) is the active ingredient of *Monochasma savatieri* Franch. ex Maxim and has been reported to have anti-inflammatory activities. The present study detected the effects of ISO on AKI induced by SAP in rat models, and the underlying mechanism. The optimum dose of ISO for treatment of AKI induced by SAP was determined. The serum levels of TNF- α and IL-6 were estimated using an ELISA. Kidney injury was evaluated by histopathological examination, and the expression levels of nitric oxide were also detected. The expression levels of Toll-like receptor 4 (TLR4) and NF- κ B p65 were measured by immunohistochemistry and western blotting. The results revealed that ISO may serve a critical role in ameliorating AKI induced by SAP. These effects may be associated with the TLR4/NF- κ B signaling pathway.

Introduction

Severe acute pancreatitis (SAP) is a common gastrointestinal inflammatory disease with high morbidity and mortality. It can induce overwhelming loads of inflammatory mediators, which leads to systemic inflammatory response syndrome (SIRS), and even multiple organ dysfunction syndrome (1-3). Acute kidney injury (AKI) is a serious complication of SAP, and the morbidity of AKI induced by SAP is 74.7% and its mortality is 75-81% (4,5). Usually, AKI occurs in the early

stage of SAP and may predict the prognosis of this disease. Additionally, it may induce the failure of other organs and promote disease progression, eventually resulting in death. SAP-induced AKI is associated with oxidative stress and activation of inflammatory cytokines, including TNF- α and IL-6 (6,7). Therefore, reduction of inflammatory cytokines may be the key treatment strategy to attenuate AKI induced by SAP.

Isoacteoside (ISO) is a major compound separated from *Monochasma savatieri* Franch. ex Maxim, which is a Chinese herbal medicine that is a composition of Yanning particles. A number of inflammatory diseases may be treated using Yanning particles (8). ISO may inhibit the production of pro-inflammatory cytokines via blocking of the caspase-1, MAPK and NF- κ B signaling pathway *in vitro* (9). ISO exerts anti-inflammatory effects that may be mediated through the NF- κ B and MAPK signaling pathways via blocking of Toll-like receptor 4 (TLR4) dimerization. TLR4 has been reported to be involved in the myeloid differentiation factor 88 and TIR-domain-containing adaptor-inducing interferon signaling pathways in an AKI mouse model (10). A number of studies have indicated that TLR4 and NF- κ B are involved in the activation of cytokines in SAP (11-13). Toll-like receptors are transmembrane proteins of cytokines that recognize extracellular antigens and induce inflammation (14,15). TLR4 was the first identified member, and also recognizes certain endogenous ligands. Activation of TLR4 triggers intracellular signal transduction cascades, leading to the activation of NF- κ B, thereby regulating the release of inflammatory cytokines (16,17). Our previous study has demonstrated that the inflammatory response in SAP may be attenuated via inhibition of the TLR4/NF- κ B signaling pathway (18). However, the pharmacological effects of ISO on SAP-induced AKI remain unclear.

The present study established SAP rat models to investigate the anti-inflammatory effects of ISO on SAP-induced AKI. Furthermore, the present study investigated the role of the TLR4/NF- κ B signaling pathway during the course of this treatment. The present study aimed to determine the optimal dose of ISO for treatment of SAP-induced AKI, and to further investigate the underlying mechanisms of ISO treatment in AKI induced by SAP.

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Materials and methods

Materials. Sodium taurocholate was purchased from Sigma-Aldrich; Merck KGaA. ISO ($C_{29}H_{36}O_{15}$; FW, 624.59; purity, $\geq 98\%$) was obtained from Nanjing TCM Institute of Chinese Materia Medica. ELISA kits for rat TNF- α and IL-6, goat anti-rat TLR4 antibody, anti-rat NF- κ B p65 antibody and secondary antibodies were obtained from Abcam. Nuclear-cytosol extraction kits were purchased from Beyotime Institute of Biotechnology. The nitric oxide (NO) assay kit was purchased from AmyJet Scientific, Inc.

Animals. A total of 120 male Sprague Dawley (SD) rats (weight, 230–250 g; age, 8 weeks) were obtained from the Experimental Animal Center of Jinling Hospital (Nanjing, China). The rats were housed at a consistent temperature of 23°C with a 12-h light-dark cycle and free access to food and water. The rats were fasted for 12 h prior to induction of SAP; however, the rats had free access to water. All experiments were performed according to protocols approved by the Animal Care Committee of Jinling Hospital (Nanjing, China) and were performed according to established international guiding principles for animal research.

Experimental design. In the first part of the present study, 48 SD rats were randomly divided into six groups (8 rats/group): Sham operation group (sham); SAP group (SAP); SAP+ISO-20 mg/kg treatment group (SAP+ISO20); SAP+ISO-40 mg/kg treatment group (SAP+ISO40); SAP+ISO-60 mg/kg treatment group (SAP+ISO60); and vehicle group (saline). Surgical anesthesia was accomplished by intraperitoneal injection of 10% chloral hydrate (300 mg/kg; Shanghai Shifeng Technology Co., Ltd.), and none of the rats exhibited any signs of peritonitis or discomfort. SAP was induced by retrograde infusion of 5% sodium taurocholate (0.1 ml/100 g body weight) into the pancreatic and biliary duct (19). Sham group animals were administered saline instead of sodium taurocholate into the pancreatic and biliary duct under the same conditions. For the ISO treatment group, ISO was diluted in dimethyl sulfoxide and was administered by intraperitoneal injection at 30 min after the SAP model establishment. Furthermore, the vehicle group was given the same volume of normal saline. The rats were euthanized by exsanguination under anesthesia at 12 h after the SAP model establishment, and anesthesia was accomplished by intraperitoneal injection of 10% chloral hydrate (300 mg/kg), then blood and pancreatic tissue samples were collected immediately. Furthermore, the pancreas samples were rapidly fixed in formalin at 35°C for 48 h for histological examination and scoring. The effect of ISO was determined by evaluating the pathological changes of the pancreas, and the serum levels of amylase (AMY), lipase (LIPA), aspartate-transaminase (AST) and alanine-aminotransferase (ALT).

Based on the first part of the present experiment, the optimal dose of ISO was fixed as 40 mg/kg. Subsequently, 72 SD rats were randomly divided into three groups (24 rats/group): Sham operation group (Sham); SAP group (SAP); and SAP+ISO-40 mg/kg treatment group (SAP+ISO40). Furthermore, 8 rats in each group were sacrificed by exsanguination under anesthesia at 3, 6 and 12 h after SAP model establishment. Subsequently, the blood, pancreas and kidney

tissue samples were collected immediately. The collected blood samples were centrifuged at 12,000 \times g for 10 min at 4°C, and then the serum was collected. The pancreas and kidney samples were rapidly fixed in formalin at 35°C for 48 h for histological examination and scoring. For western blot analysis, three portions of each kidney sample were stored at -80°C.

Enzyme assay. The AMY, LIPA, AST, ALT, blood urea nitrogen (BUN) and creatinine (Cr) levels in the serum were measured using an automated biochemical analyzer (Hitachi 7600; Hitachi, Ltd.).

ELISA. Serum concentrations of TNF- α (ab236712) and IL-6 (ab234570) were measured using commercially available ELISA kits according to the manufacturer's protocols (R&D Systems, Inc.). All samples were assayed three times.

Histopathological examination. The pancreas and kidney tissue samples were fixed in 4% formalin at 35°C for at least 48 h. The tissues were cut into sections (thickness, 5 μ m), and stained with hematoxylin and eosin at 23°C for 2 h. Histopathological evaluation was performed under a light microscope (magnification, $\times 100$) by two experienced laboratory pathologists in a blinded manner. The pathological scores of the pancreas samples were evaluated according to the extent of edema, inflammation, vacuolization and necrosis (20). Histological scores of the kidney were assessed according to the point-counting method described by Paller *et al.* (21). For each kidney, 100 cortical tubules from at least 10 different regions were scored, and repeated scoring of different convolutions of the same tubule was avoided. Higher scores represented more severe damage (maximum score per tubule, 10), with points given for the presence and degree of tubular epithelial cell flattening (1 point), brush border loss (1 point), cell membrane bleb formation (1 or 2 points), interstitial edema (1 point), cytoplasmic vacuolization (1 point), cell necrosis (1 or 2 points) and tubular lumen obstruction (1 or 2 points).

Immunohistochemistry. Kidney tissues were fixed in 4% formalin at 35°C for 12 h and embedded in paraffin. Subsequently, kidney tissues were harvested to prepare frozen sections of 6- μ m thickness. Following deparaffinization, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 10 min at room temperature. Non-specific adsorption was minimized following incubation of the sections in 5% normal goat serum in PBS (Nanjing Jiancheng Bioengineering Institute) at 37°C for 1 h. Avidin and biotin were used to block endogenous biotin and avidin binding sites, respectively. The sections were incubated overnight with goat anti-rat TLR4 (cat. no. ab22048) and anti-rat NF- κ B p65 (cat. no. ab16502) monoclonal antibodies (both 1:100; Abcam) in a humidified incubator at 4°C. Hematoxylin was subsequently used to counterstain the sections for 2 min at room temperature. Stained sections were visualized using a light microscope. PBS was used instead of the primary antibody as a negative control.

NO assay. Kidney tissues were thawed and homogenized in phosphate buffer containing 0.5% hexadecyltrimethylammonium

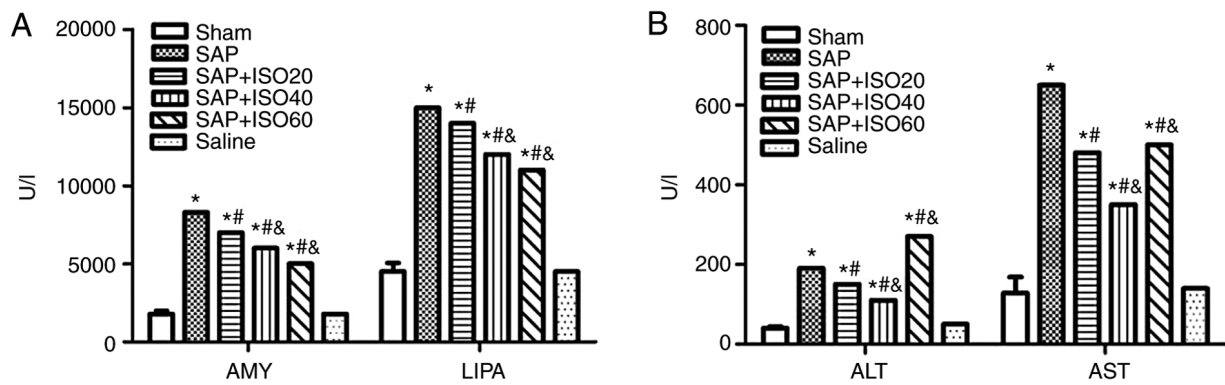


Figure 1. AMY, LIPA, ALT, AST activity. Effect of Isoocteoxide on (A) AMY and LIPA, and (B) ALT and AST levels. Data are expressed as the mean \pm standard deviation; n=8 in each group. *P<0.05 vs. the Sham group; #P<0.05 vs. the SAP group; &P<0.05 vs. the SAP+ISO20 group. AMY, amylase; LIPA, lipase; AST, aspartate-transaminase; ALT, alanine-aminotransferase; SAP, severe acute pancreatitis.

bromide. NO production was determined using a commercial assay kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocols.

Western blot analysis. Kidney tissue samples were homogenized, and the lysate was boiled in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 20% glycerol and 10% 2-mercaptoethanol). The protein concentration was determined using a Bradford protein assay kit (Thermo Fisher Scientific, Inc.) with bovine serum albumin (Nanjing Jiancheng Bioengineering Institute) as the standard. Proteins (50 mg) were separated via 10% SDS-PAGE and transferred to PVDF membranes. Following the transfer of proteins, the membrane was blocked with 5% skimmed milk in PBS with 0.1% Tween-20 (PBST) for 2 h at room temperature, and then incubated overnight with antibodies (all 1:1,000; Abcam) against TLR4 (ab22048), NF- κ B p65 (ab16502) or β -actin (cat. no. ab8227) at 4°C. Subsequently, the membranes were washed with PBST containing 0.1% Tween. Following three washes in PBST, each membrane was incubated with peroxidase-conjugated secondary antibody (anti-mouse IgG; 1:5,000; cat. no. ab131368; Abcam) for 1 h at 37°C. Labeled proteins were visualized using an Odyssey infra-red scanner (LI-COR Biosciences). Signals were assessed by densitometry and normalized to the β -actin signals. An enhanced chemiluminescence detection system (Amersham; Cytiva) was used to visualize the antibody-specific proteins according to the manufacturer's recommended protocol.

Statistical analysis. Data are presented as the mean \pm standard deviation. One-way analysis of variance with Bonferroni's test was used to analyze the differences among multiple groups. Statistical analysis was performed using SPSS v19.0 software (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

Surgical outcomes. The operation was completed successfully on all rats, and no rats died during the experimental period between the first injection and the time of euthanasia in the present study.

Optimum dose of ISO. The present study evaluated the serum levels of AMY, LIPA, ALT and AST in SAP rats that were treated with multiple doses of ISO (20, 40 and 60 mg/kg) in order to determine the optimum dose of ISO. The three doses of ISO significantly decreased the levels of AMY and LIPA compared with those in the SAP group (P<0.05; Fig. 1A). However, the SAP group treated with ISO was the only ISO-treated group in which both AST and ALT levels were decreased significantly compared with those in the SAP group (P<0.05; Fig. 1B). Therefore, 40 mg/kg ISO was determined to be the optimum dose for treatment of SAP. Additionally, 3 h following induction of the SAP model was identified to be the best time of medication to reduce inflammation.

Analysis of serum levels of AMY, LIPA, BUN and Cr. Compared with those in the sham group, the serum levels of AMY, LIPA, BUN and Cr in the SAP group were significantly increased at either designated time point (P<0.05; Fig. 2). However, the serum levels of AMY, LIPA, BUN and Cr in the ISO-treated group were markedly decreased compared with those in the SAP group (P<0.05; Fig. 2). Furthermore, the serum levels of AMY peaked at the 6 h time point in the SAP group (Fig. 2A), and the serum levels of LIPA, BUN and Cr peaked at the 12 h time point (Fig. 2B-D).

ELISA. As shown in Fig. 3, a significant increase in serum levels of TNF- α and IL-6 was observed in the SAP group compared with the sham group (P<0.05), and the levels of these cytokines were progressively upregulated between 3 and 12 h. Furthermore, the serum levels of TNF- α and IL-6 were decreased following treatment with ISO (P<0.05). However, the levels of these cytokines in the ISO group were still higher than those in the sham group at each time point (P<0.05).

Histopathological examination. Representative histological sections of the pancreas and kidney are shown in Figs. 4 and 5. The pancreas tissues of the sham group exhibited a normal histological structure (Fig. 4A). Samples in the SAP group were characterized by typical histological signs of pancreatic edema, inflammatory leukocyte infiltration, hemorrhage and necrosis (Fig. 4B). However, there was a significant decrease

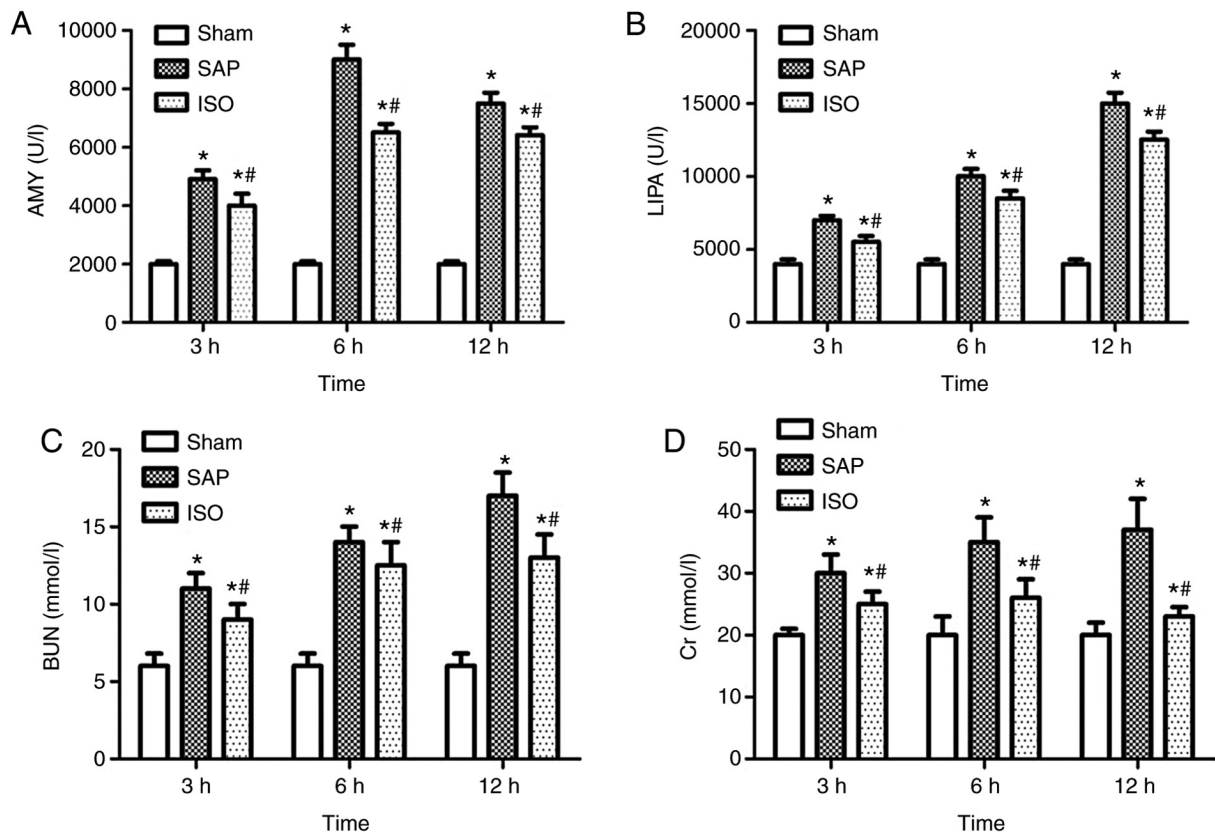


Figure 2. AMY, LIPA, BUN and Cr activity. Serum levels of AMY, LIPA, BUN and Cr in all groups. Serum levels of (A) AMY, (B) LIPA, (C) BUN and (D) Cr. Data are expressed as the mean \pm standard deviation; n=8 in each group. *P<0.05 vs. the Sham group; #P<0.05 vs. the SAP group. AMY, amylase; LIPA, lipase; BUN, blood urea nitrogen; Cr, creatinine; SAP, severe acute pancreatitis; ISO, Isoacteoside.

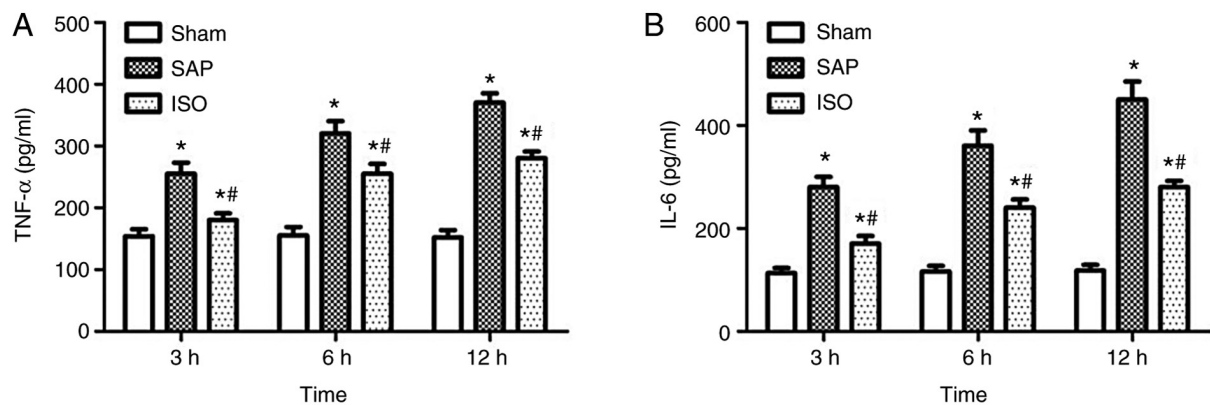


Figure 3. TNF-α and IL-6 activity. Serum levels of TNF-α and IL-6. Serum levels of (A) TNF-α and (B) IL-6 were measured by enzyme-linked immunosorbent assay kits. Data are expressed as the mean \pm standard deviation; n=8 in each group. *P<0.05 vs. the sham group; #P<0.05 vs. the SAP group. TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; SAP, severe acute pancreatitis; ISO, Isoacteoside.

in the severity of the pancreatic histological injuries in the ISO-treated group (P<0.05; Fig. 4C and D).

The pathological changes in kidney samples were analyzed to examine the effect of ISO on AKI induced by SAP (Fig. 5). The kidney samples of the sham group exhibited a normal histological structure of the renal glomerulus, tubules and interstitium (Fig. 5A). By contrast, samples in the SAP group were characterized by typical histological signs of glomerular and tubular damage, and the most severe kidney injury was detected at the 12 h time point (Fig. 5B and D). ISO treatment

attenuated the severity of kidney injury, as demonstrated by decreased glomerular and tubular damage and decreased inflammatory cell infiltration (Fig. 5C). Additionally, the kidney pathohistological scores were significantly decreased in the ISO-treated group compared with the SAP group at each time point (P<0.05; Fig. 5D).

Immunohistochemical analysis of TLR4 and NF-κB p65 expression. Localization of TLR4 and NF-κB p65 in the kidney is shown in Fig. 6. There was almost no TLR4

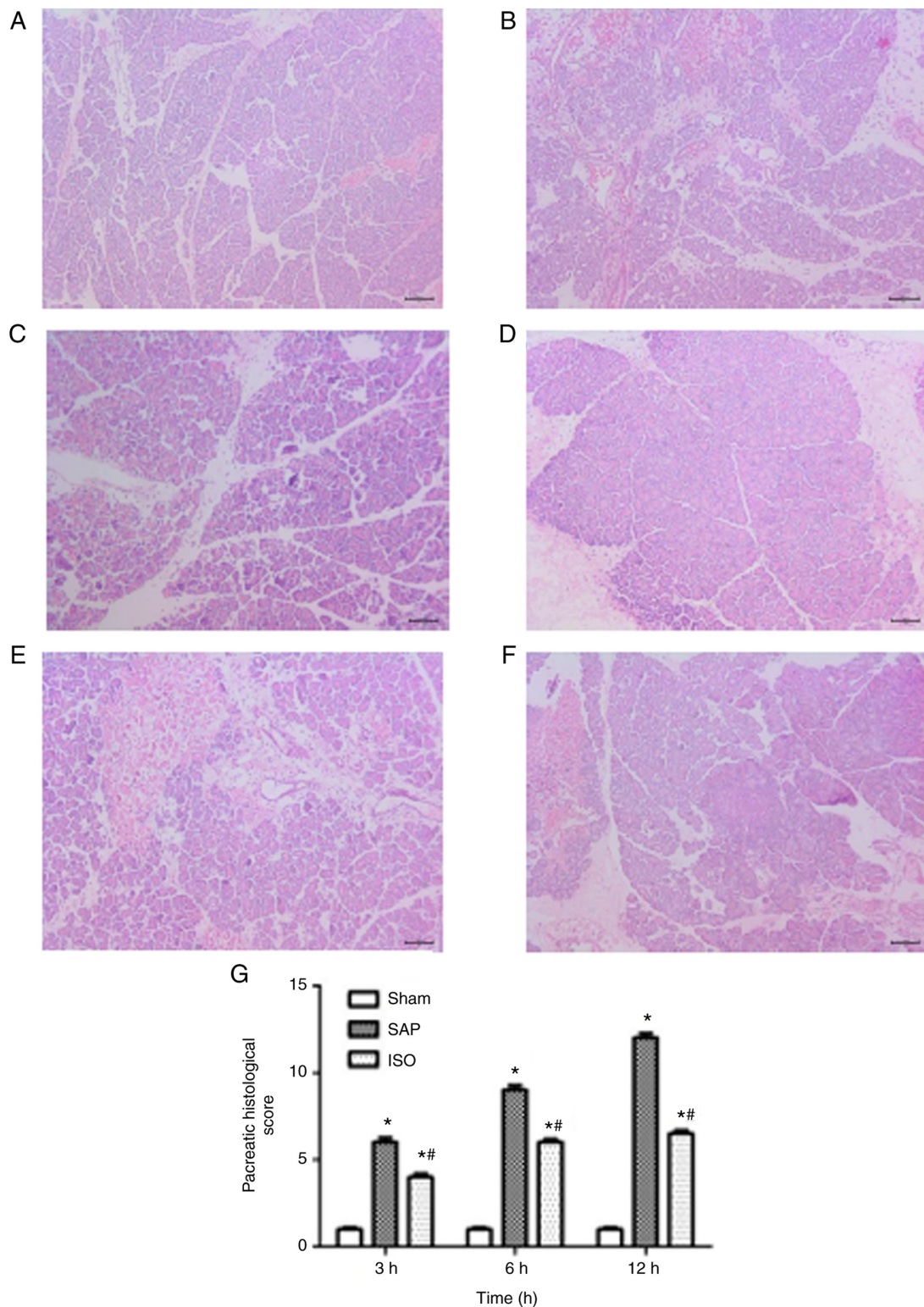


Figure 4. Pancreatic histological changes. Morphological changes in pancreatic tissue at (A-C) 6 h and (D-F) 12 h and (G) the pancreatic histological score at different time points. Hematoxylin and eosin staining was used to investigate the pathological changes (original magnification, x100). (A and D) The sham group, (B and E) the SAP group, (C and F) the ISO group and (G) the pancreatic histological score at corresponding times. * $P < 0.05$ vs. the sham group; # $P < 0.05$ vs. the SAP group. SAP, severe acute pancreatitis; ISO, Isoocteside.

and NF- κ B p65 expression in the sham group. In the SAP group, marked positive TLR4 and NF- κ B p65 expression was observed compared with the sham group. However, following treatment with ISO, the expression levels of TLR4 and NF- κ B p65 were markedly decreased compared with

those in the SAP group at the corresponding time points ($P < 0.05$).

ISO treatment decreases renal NO production. NO activity in the kidney was detected to examine the infiltration of

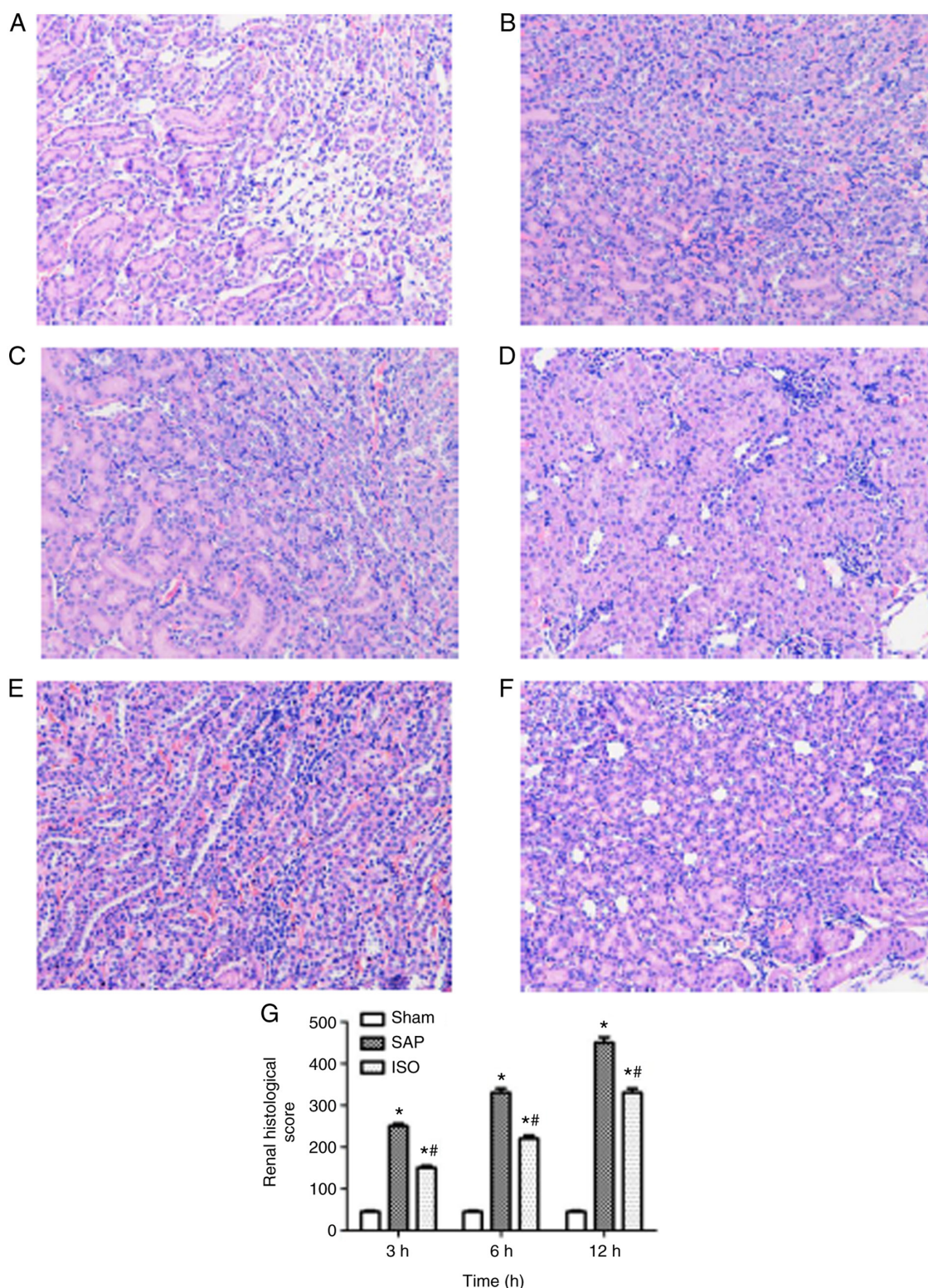


Figure 5. Kidney histological changes. Morphological changes in kidney tissues at (A-C) 6 h and (D-F) 12 h and (G) the kidney pathological scores at different time points. Hematoxylin and eosin staining was used to investigate the pathological changes (original magnification, x200). (A and D) The sham group, (B and E) the SAP group, (C and F) the ISO group and (G) the renal histological score at corresponding times. * $P < 0.05$ vs. the sham group; # $P < 0.05$ vs. the SAP group. SAP, severe acute pancreatitis; ISO, Isoacteoside.

neutrophils (Fig. 7). NO activity was markedly elevated in the SAP group compared with the sham group ($P < 0.05$). However, treatment with ISO significantly decreased NO activity in the kidney compared with the SAP group at each time point ($P < 0.05$).

Role of the TLR4/NF- κ B p65 signaling pathway in the effects of ISO in AKI induced by SAP. Western blotting was used to determine the effects of ISO on the expression levels of TLR4 and NF- κ B p65 in the kidney (Fig. 8). Compared with those in the sham group, the expression levels of TLR4 and NF- κ B p65

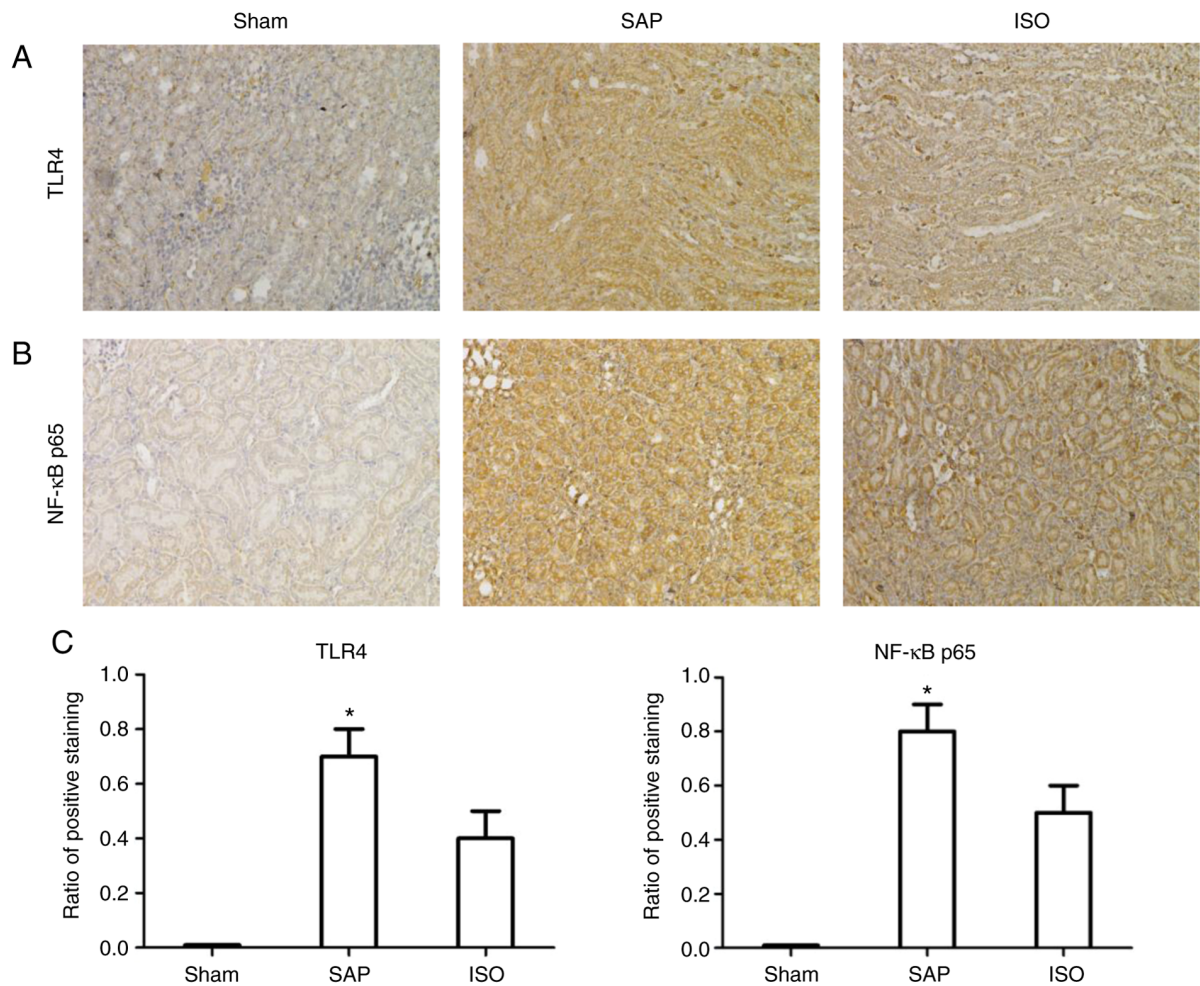


Figure 6. Immunohistochemical analysis of TLR4 and NF-κB p65 expression. Immunohistochemical analysis of TLR4 and NF-κB p65 staining at the 12 h time point. The expression of (A) TLR4 and (B) NF-κB p65 were analyzed by immunohistochemistry (magnification, x100). (C) The ratio of positively-stained cells was determined. *P<0.05 vs. the SAP group. TLR4, Toll-like receptor 4; SAP, severe acute pancreatitis; ISO, Isoocteoxide.

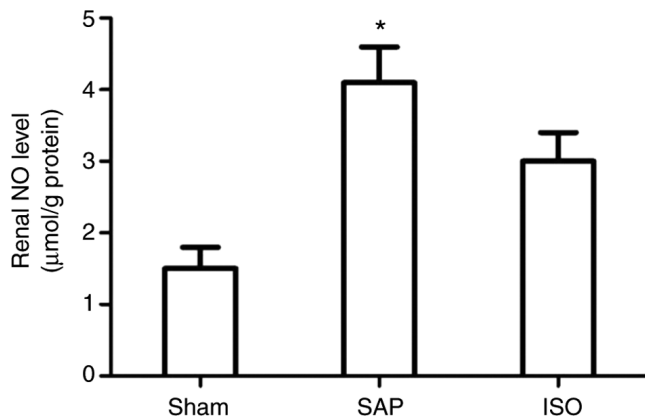


Figure 7. NO production. ISO decreases renal NO production in AKI induced by SAP at the 12 h time point. Data are expressed as the mean ± standard deviation. *P<0.05 vs. the SAP group. SAP, severe acute pancreatitis; ISO, Isoocteoxide; NO, nitric oxide; AKI, acute kidney injury.

were upregulated in the SAP group (P<0.05). Following treatment with ISO, the expression levels of TLR4 and NF-κB p65 were downregulated compared with those in the SAP group at each time point (P<0.05).

Discussion

The present study investigated the effects of treatment with ISO on SAP-induced AKI rat models. ISO is a major ingredient of Yanning particles, and it has been used in clinical settings for numerous years in China. It has been reported that ISO may be a potential anti-dementia phenylethanoid glycoside and has various memory- and behavior-improving effects against Aβ1-42-induced behavioral dysfunction (22). Furthermore, studies have revealed that ISO suppresses lipopolysaccharide-induced phosphorylation of TGF-β activated kinase 1 (TAK1) while having no effects on total TAK1 in RAW264.7 cells. ISO may attenuate inflammation by inhibiting the production of pro-inflammatory cytokines via the NF-κB and MAPK signaling pathway, and treatment of mice with ISO may protect them against AKI induced by lipopolysaccharides (10). The results of the present study revealed that ISO may relieve SAP and AKI induced by SAP, as demonstrated by decreased serum levels of AMY, LIPA, BUN and Cr, and amelioration of pancreas and kidney injury. AKI is one of the most common systemic complications, and it has been demonstrated to have a marked impact on the clinical outcome (5,23,24). However, the mechanism of AKI induced by SAP is complicated.

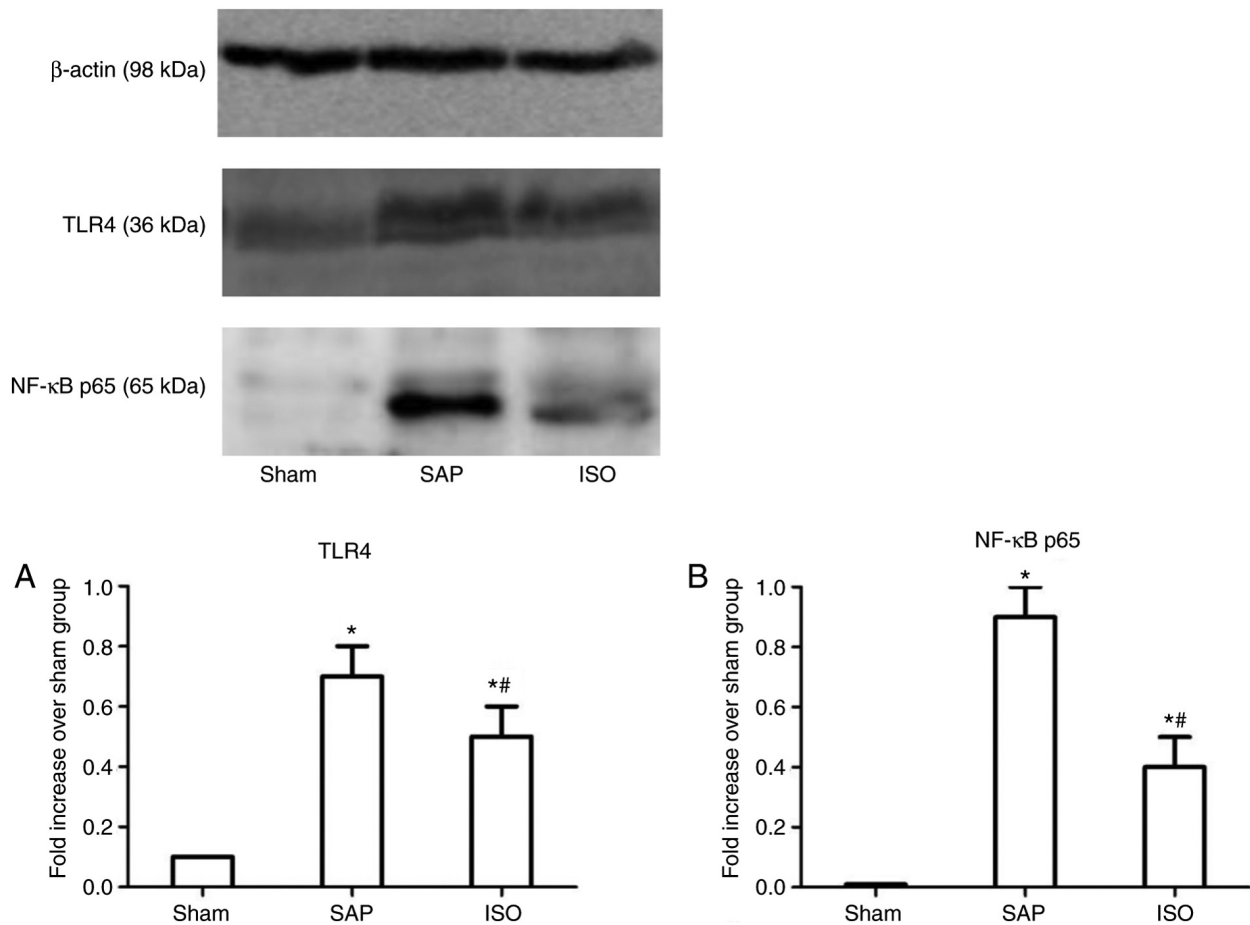


Figure 8. Western blot analysis of TLR4 and NF-κB p65 protein expression. Levels of (A) TLR4 and (B) NF-κB p65 in the kidney tissues at the 12 h time point. Western blot analysis was used to determine the protein levels. * $P < 0.05$ vs. the Sham group; ** $P < 0.05$ vs. the SAP group. TLR4, Toll-like receptor 4; SAP, severe acute pancreatitis; ISO, Isoacteoside.

A previous study demonstrated that the inflammatory response serves an important role in the development of SAP-induced AKI, and that inflammatory cytokines are the key factors (25). During the inflammatory response, numerous cytokines, including TNF- α and IL-6, are released into the blood. It has been revealed that TNF- α and IL-6 serve an important role in SAP and contribute toward the activation of SIRS and AKI. TNF- α acts on glomeruli and tubular capillaries directly resulting in ischemia and tubular necrosis of the kidney. Additionally, TNF- α stimulates the release of other cytokines, including IL-6, which acts on endothelial cells, resulting in ischemia, thrombosis of the kidney and the release of oxygen free radicals (26). A clinical study reported that patients who developed AKI had high serum levels of IL-6 and IL-8 (27). The cytokine-mediated inflammatory response serves an important role in the pathophysiology of AKI regardless of its cause (28). The present study demonstrated that the serum levels of TNF- α and IL-6 were lower in the ISO treatment group compared with the SAP group at each time point, indicating that treatment with ISO may inhibit inflammation in SAP-induced AKI.

A number of studies have indicated that NO may serve an important role during the inflammatory process of SAP (29,30). In the present study, upregulation of NO was detected in kidney tissues in the SAP group, which indicated that NO may

contribute toward the development of AKI induced by SAP. The upregulation of NO may lead to the production of oxygen free radicals, which increases cytotoxic effects and aggravates AKI. However, ISO decreased NO expression in the kidneys of SAP rats. Lower NO expression in the ISO treatment group may lead to the reduction of nitrotyrosine formation and lipid peroxidation in the kidneys of SAP rats. Therefore, these results demonstrated that ISO may ameliorate SAP-induced AKI by attenuating inflammation induced by NO in the kidney.

As a 'switch' of the inflammatory response, the TLR4/NF-κB p65 signaling pathway may be associated with the development and progression of SAP-induced AKI. The transcription factor NF-κB p65 serves a critical role in the regulation of numerous genes which are responsible for the generation of cytokines, including TNF- α and IL-6, in inflammatory diseases, including SAP (31). One study has demonstrated that the activation of NF-κB p65 is associated with TLR4 expression; following stimulation by TLR4, NF-κB is activated, phosphorylated and transferred into the nucleus (32). TLR4 may activate IκB and induce the NF-κB signal transduction pathway. Other studies have revealed that there are high expression levels of TLR4 in the kidney tissues of mice models of AKI (33,34). In the present study, immunohistochemistry revealed an increase in the expression levels of NF-κB p65 in the kidney samples from the

SAP group compared with the sham group. Additionally, a decrease in the expression levels of NF- κ B p65 in the ISO group compared with the SAP group was observed. Western blotting demonstrated that ISO treatment decreased the expression levels of TLR4 in the kidney. Therefore, ISO may attenuate the inflammatory response by inhibiting the TLR4/NF- κ B p65 signaling pathway. However, there was a limitation to the present study. A TLR4 signaling pathway inhibitor was not used in the present experiments. Furthermore, it remains unclear whether ISO only works through the TLR4/NF- κ Bp65 signaling pathway. Although the present study indicated that ISO protected the kidney from SAP, the precise mechanism underlying the effects of ISO requires further investigation.

In conclusion, ISO attenuated AKI induced by SAP by decreasing inflammation. Therefore, ISO may be a potential therapeutic agent for the treatment of SAP and SAP-induced AKI. The mechanism may involve inhibition of the TLR4/NF- κ B p65 signaling pathway.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BW and XPZ designed and performed the research and wrote the manuscript. XHL, MLL, XWW and XHZ performed the research. ZS and MXG collected and analyzed data. The authenticity and legitimacy of all the raw data have been assessed by XPZ and XHZ. All authors read and approved the final manuscript.

Ethics statement and consent to participate

The present study was approved by the Institutional Review Board of Jinling Hospital and the Institutional Animal Care and Use Committee of Jinling Hospital. All operations were performed according to National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications no. 8023, revised 1978) concerning the care and treatment of experimental animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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