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Qingyi decoction attenuates severe acute pancreatitis in rats via inhibition of inflammation and protection of the intestinal barrier

Song Su[1,*], Tiancheng Liang[2,*], Xiang Zhou[1], Kai He[1], Bo Li[1] and Xianming Xia[1]

Abstract

Objective: Qingyi decoction (QYD) has beneficial effects in severe acute pancreatitis (SAP). We assessed the therapeutic effect and mechanisms of QYD in SAP.

Methods: A rat model of SAP was induced by pancreatic ductal injection of sodium taurocholate. QYD was administered intragastrically immediately postoperatively and once every 12 hours. Serum amylase, endotoxin, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and D-lactate levels were measured at 12, 24, and 48 hours. Histological changes in the pancreas and ileum were analyzed. Expression of nuclear factor kappa-light-chain-enhancer of activated B cells p65 (NF-κB p65), Toll-like receptor 4 (TLR4), and zonula occludens-1 (ZO-1) in the small intestinal mucosa was also assessed.

Results: Pancreatic tissue showed extracellular space expansion, inflammatory infiltration, vessels with necrotic walls, and hemorrhage. Ileal tissue showed hemorrhage, inflammatory infiltration, and ileal mucosa destruction. These histological features were dramatically improved by QYD. Increased serum levels of amylase, endotoxin, TNF-α, IL-6, and D-lactic acid were significantly decreased by QYD administration. Increased expression of NF-κB p65 and TLR4 and decreased expression of ZO-1 in the ileal mucosa were also restored to normal levels by QYD treatment.

Conclusion: QYD alleviates SAP by reducing intestinal barrier dysfunction, inhibiting intestinal bacteria and endotoxin translocation, and preventing NF-κB activation.

[1]Department of Hepatobiliary Surgery, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan, P.R. China
[2]Department of First Surgery, Luzhou Traditional Chinese Medicine Hospital, Luzhou, Sichuan, P.R. China

*These authors contributed equally to this work.

Corresponding author:
Xianming Xia, Department of Hepatobiliary Surgery, The Affiliated Hospital of Southwest Medical University, 25 Taiping Street, Luzhou, Sichuan 646000, P.R. China.
Email: drxiaxianming@163.com

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Introduction

Severe acute pancreatitis (SAP) is characterized by a complicated cascade of inflammatory responses and activation of digestive enzymes in pancreatic acinar cells.[1] Early management is considered indispensable for cure of patients who show symptoms of SAP.[2] Although our knowledge and critical care of patients with SAP have improved, the mortality rate remains considerably high.[3] The underlying mechanisms of SAP have been extensively studied but remain unresolved. One of the most persuasive explanations of the underlying mechanism of SAP is that dysfunction of the intestinal barrier allows bacterial translocation from the intestinal lumen to distant organs.[4] In recent years, several standard therapeutic strategies including prophylactic antibiotics, aggressive fluid resuscitation, and early debridement for patients with SAP who do not show improvement have become obsolete because these methods are associated with worse rather than improved outcomes.[5] Therefore, a novel therapeutic intervention with high efficacy and fewer side effects is needed to improve the prognostic outcomes of patients with SAP.

The use of traditional Chinese medicine, specifically the Qingyi decoction (QYD), shows good prognostic value in the therapy of SAP because it has more extensive pharmacological effects and fewer adverse effects than other treatments.[6,7] QYD is generally well tolerated by patients, induces purgation, promotes blood circulation, eliminates blood stasis, and reduces inflammation.[8] The active components of QYD exhibit significant biological functions such as anti-inflammatory activity, scavenging of oxygen free radicals, promotion of microcirculation, and inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). QYD has recently been found to inhibit lung inflammatory responses and excessive lung tissue apoptosis.[9] Additionally, QYD has been shown to protect the intestinal barrier by reducing endotoxin generation, inhibiting excessive neutrophil activation, and minimizing the release of inflammatory cytokines.[10] However, the mechanisms underlying the anti-pancreatitis activity of QYD are not fully understood. In this study, we evaluated the therapeutic effect of QYD on SAP in rats and studied its potential mechanisms.

Materials and methods

Animals

In total, 54 male specific pathogen-free Sprague–Dawley rats (280 ± 20 g) were provided by the Experimental Animal Center of Southwest Medical University, Sichuan, China. All animals were housed for 1 week under sterile conditions and fed laboratory chow and water. All experimental procedures were performed in accordance with international guidelines for the care and use of laboratory animals and approved by the Ethics Committee of Southwest Medical University (Sichuan, China).

Preparation of QYD

An herbal mixture of Radix Bupleuri (15 g), *Scutellaria baicalensis* (15 g), Fructus Aurantii Immaturus (15 g), Radix Paeoniae Rubra (12 g), Cortex Moutan (12 g), Rhizoma Corydalis (12 g), Cortex *Magnoliae officinalis* (15 g), Guang Mu Xiang (12 g), *Artemisia capillaris* (15 g), *Gardenia* (12 g), and *Houttuynia cordata* (30 g) was submerged in 500 mL of water and cooked for 1 hour at 100°C. The supernatant was then concentrated to 85 mL followed by the addition of 15 g of *Rheum officinale* and 10 g of mirabilite. After mixing for 5 to 10 minutes, the solution was kept at 4°C for subsequent experiments. All medicinal materials were purchased from Sichuan Luzhou Hospital (Luzhou, China), the products of which meet commercial quality control according to the China Pharmacopoeia 2010.

Induction of SAP and collection of samples

All animals were divided into 3 groups of 18 rats each: a sham control group, an SAP group, and a QYD treatment group. SAP was induced by administration of 3% sodium taurocholate (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 1 mL/kg by retrograde injection directly into the biliopancreatic duct at a constant infusion rate using a pump.[11] Rats were fasted for 12 hours prior to anesthesia, which was induced by intraperitoneal injection of 3% pentobarbital sodium (10 mg/kg), and the operation was performed under aseptic conditions. Two microvascular clamps were used to nip both ends of the biliopancreatic duct to prevent reflux of the infused material into the liver. Sodium taurocholate was administered, and the clamp was removed 5 minutes after injection. The sham controls underwent the same procedure with no sodium taurocholate injection. The abdomen was closed after ensuring that no active bleeding was present in the abdominal cavity. Immediately after the surgery, rats in the QYD group received 10 mL/kg of QYD intragastrically once every 12 hours, while the rats in the other two groups were treated with water. Six of the 18 rats in each group were killed at 12, 48, and 72 hours postoperatively. Whole blood, the pancreatic tail, and 5 cm of the distal ileum (10 cm from the ileocecal part) were collected for detection of the serum levels of amylase, endotoxin, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and D-lactate; observation of pathological changes of the pancreas and ileum; and determination of the expression of NF-κB p65, Toll-like receptor 4 (TLR4), and zonula occludens-1 (ZO-1) in the ileal mucosa, as described below.

Biochemical analysis

At indicated time points, the rats were killed and blood samples were collected from the inferior vena cava. Samples were kept at room temperature for 10 minutes and then centrifuged at $3{,}000 \times g$ for 10 minutes at 4°C, and the serum was stored at −70°C until measurement. Tissue samples from the distal ileum and pancreas were collected for histopathological examination. The serum amylase activity was determined using an amylase kit and automated clinical biochemistry analysis equipment (Hitachi Co., Tokyo, Japan). The serum endotoxin levels were measured using a commercially available detection kit (GenScript, Shanghai, China). The serum levels of TNF-α, IL-6, and D-lactic acid were determined using enzyme-linked immunosorbent assays (Goybio, Shanghai, China), according to the manufacturer's instructions.

Histopathological analysis

Pancreatic and ileal tissue samples were fixed in 10% buffered formalin overnight

and subsequently dehydrated through a graded ethanol series. Tissues were cut into 4-μm slices and placed on slides after embedding them into paraffin blocks. The tissue samples were stained with hematoxylin and eosin and examined by light microscopy. Pathological scoring was performed by experts in the Department of Pathology according to reference methods. Pathological changes of the pancreas were evaluated according to the standard established by Kusske et al.,[12] while intestinal pathological changes were evaluated according to the criteria established by Chiu.[13]

Immunohistochemical analysis

Immunohistochemistry was performed to determine the protein expression of NF-κB p65, TLR4, and ZO-1 in the small intestinal mucosa. Briefly, formalin-fixed, paraffin-embedded tissue sections (4 μm) were blocked with 10% normal rabbit serum in Tris-buffered saline for 20 minutes followed by 15 minutes of incubation with avidin and then biotin using an avidin-biotin blocking kit. For the detection of target proteins, blocked sections were then incubated with goat anti-rat polyclonal anti-NF-κB p65, anti-TLR4, and anti-ZO-1 primary antibody at 37°C for 2 hours. The sections were then washed and incubated with anti-goat secondary antibody at room temperature for 20 minutes and then incubated with freshly prepared 0.1% 3,3-diaminobenzidine tetrahydrochloride containing 0.02% hydrogen peroxidase in phosphate-buffered saline. Finally, the sections were stained with hematoxylin, dehydrated, mounted, and covered with coverslips. Normal blocking serum without primary antibody was used for the negative control.

Western blotting

The tissue was ground in liquid nitrogen and lysed in RIPA buffer containing 1% cocktail and 1% phenylmethylsulfonyl fluoride. Protein concentrations were determined using a bicinchoninic acid kit according to the manufacturer's protocol. Equal amounts of protein samples were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Burlington, MA, USA). After blocking with 5% nonfat dry milk, the blots were probed with primary antibodies to NF-κB p65, TLR4, ZO-1, and glyceraldehyde 3-phosphate dehydrogenase overnight at 4°C. The membranes were washed with tris-buffered saline + Tween 20 five times and incubated for 2 hours with a conjugated horseradish peroxidase secondary antibody. Bands were visualized using enhanced chemiluminescence reagent (GE Healthcare, Chicago, IL, USA) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Statistical analysis was conducted using SPSS Statistics for Windows, Version 17.0 (SPSS, Chicago, IL, USA). Data are presented as mean ± standard deviation. Student's *t*-test or one-way analysis of variance was used for the normally distributed data, while the Mann–Whitney U or Kruskal–Wallis test was used for nonparametric data. A P value of <0.05 was considered statistically significant.

Results

Successful establishment of an SAP animal model and alleviation of the pathogenetic condition with QYD treatment

We examined the serum amylase levels in different groups to determine whether the SAP animal model was successfully

established. Compared with the sham control group, the serum amylase levels were significantly higher in the SAP group than sham control group at multiple time points (12, 24, and 48 hours) ($P < 0.05$). This increase in the SAP group decreased after treatment with QYD ($P < 0.05$) (Figure 1(a)).

The pancreatic tissues of the sham control rats exhibited intact pancreatic characteristics with mild pancreatic acinar cell edema, low numbers of inflammatory cells, and no evidence of hemorrhage or necrosis. However, tissues from the SAP group 12 hours after induction showed significant expansion of the extracellular space, inflammatory cell infiltration, vessels with necrotic walls, and hemorrhage. These histological features were further exacerbated 24 and 48 hours after pancreatic induction, and severe inflammation and necrosis were observed. Tissues from the QYD treatment group at 12 hours exhibited fewer inflammatory cells and vessels with necrosis and hemorrhage than the tissues from the SAP group, and this alleviation was more obvious after 24 and 48 hours of treatment (Figure 1(b)). The histological scores were significantly higher in the SAP and QYD groups than in the sham control group at corresponding time points ($P < 0.05$). There was a significant reduction in the histological scores in the QYD group compared with the SAP group ($P < 0.05$) (Figure 1(c)).

Histological sections of ileal tissue from rats with SAP showed large areas of hemorrhage and infiltration of inflammatory cells into the lamina propria. In addition, massive destruction of ileal mucosa with shorter, atrophic, and fractured villi was observed at 24 and 48 hours after induction of SAP, and these features were less severe at the 12-hour time point. In the sham control group, the ileal mucosa and lamina propria were normal and intact, with only mild hemorrhage and small amounts of inflammatory cells in some sections. In the QYD treatment group, the order of the intestinal mucosal villi was restored compared with the SAP group, with only slight edema and improved integrity of the mucous membranes (Figure 1(d)). The histological scores were significantly higher in the SAP and QYD groups than in the sham control group at different time points ($P < 0.05$). There was a significant reduction in the histological scores in the QYD group compared with the SAP group ($P < 0.05$) (Figure 1(e)).

Significant reduction of serum endotoxin and cytokine levels with QYD treatment

In the SAP group, the serum endotoxin levels increased at 12 hours postoperatively, peaked at 24 hours, and decreased at 48 hours. QYD treatment significantly reduced the serum endotoxin levels ($P < 0.05$) (Figure 2(a)). The serum IL-6, TNF-α, and D-lactic acid levels were significantly higher than in the sham controls at different time points ($P < 0.05$), and a significant reduction was observed with QYD treatment (Figure 2(b)–(d)). The kinetics of TNF-α and IL-6 were similar to those of endotoxin, with both peaking at 24 hours postoperatively, although they did not differ significantly between different time points. However, the serum D-lactic acid level peaked at 12 hours postoperatively and gradually decreased at the 24- and 48-hour time points (Figure 2(d)).

Significant alteration of NF-κB p65, TLR4, and ZO-1 expression in the intestinal mucosa by QYD treatment

Immunohistochemical analysis showed that NF-κB p65 expression was significantly higher in the SAP than sham group at 12 hours postoperatively ($P < 0.05$). However, NF-κB p65 expression rapidly decreased to normal levels at 24 and 48 hours (Figure 3(a) and (b)). QYD treatment

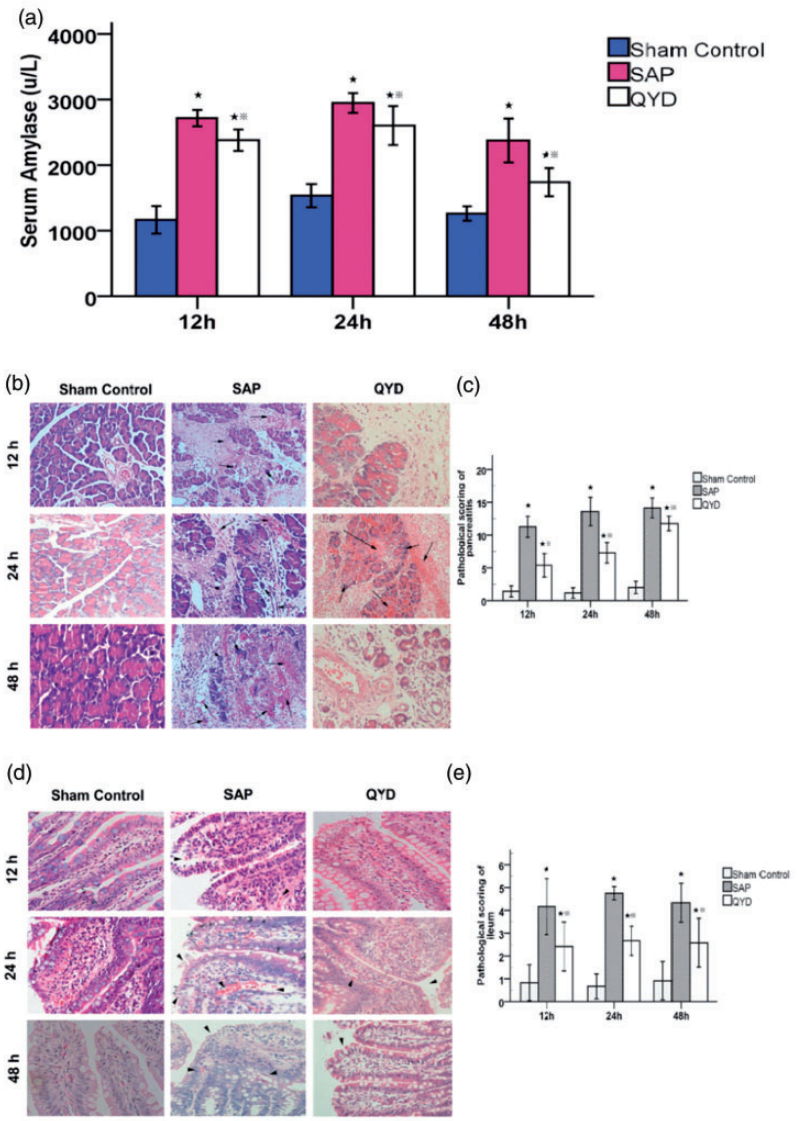

Figure 1. Establishment of SAP rat model and therapeutic effect of QYD. (a) Serum amylase level measured with an amylase kit at 12, 24, and 48 hours post-induction. Microscopic observation of (b) pancreatic and (d) ileal tissues from different groups at different time points and histological evaluation of (c) pancreatic and (e) ileal lesions. Scoring differences were evaluated using the Mann–Whitney U test. ($^{\bigstar}P < 0.05$ compared with sham controls; $^{\divideontimes}P < 0.05$ compared with SAP group; $^{\triangle}P < 0.05$ compared with 12 hours within the same group; $^{\blacktriangle}P < 0.05$ compared with 24 hours within the same group). The black arrow indicates typical signs of pancreatic inflammatory pathology, including congestion and edema, cell death, and leukocyte infiltration. The arrowhead indicates hemorrhage, inflammatory cell infiltration, and massive destruction of ileal mucosa. SAP, severe acute pancreatitis; QYD, Qingyi decoction.

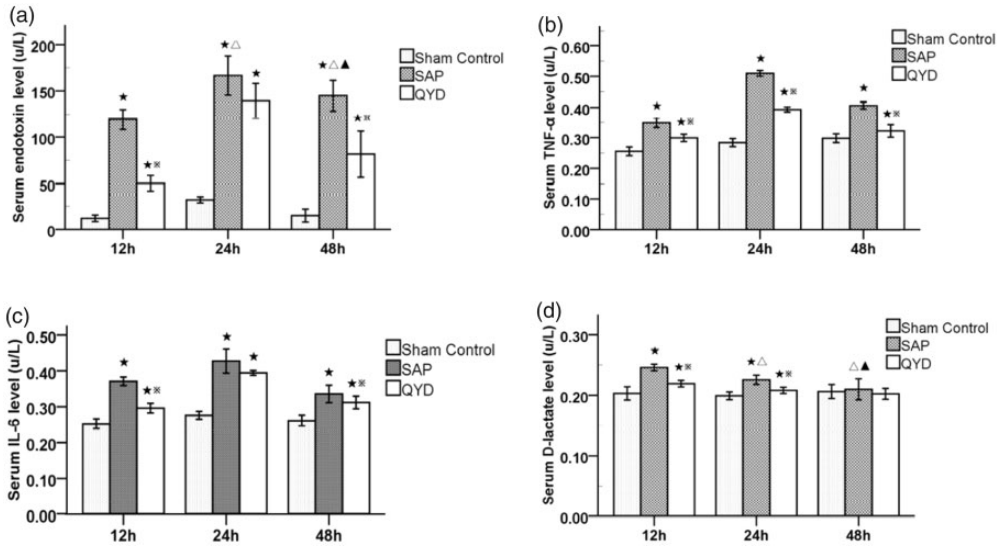

Figure 2. Expression levels of inflammatory molecules. Serum levels of (a) endotoxin, (b) TNF-α, (c) IL-6, and (d) D-lactate were measured using an enzyme-linked immunosorbent assay kit (mean ± standard deviation). ($^{\bigstar}P < 0.05$ compared with sham controls; $^{\divideontimes}P < 0.05$ compared with the SAP group; $^{\triangle}P < 0.05$ compared with 12 hours within the same group; $^{\blacktriangle}P < 0.05$ compared with 24 hours within the same group). SAP, severe acute pancreatitis; QYD, Qingyi decoction; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6.

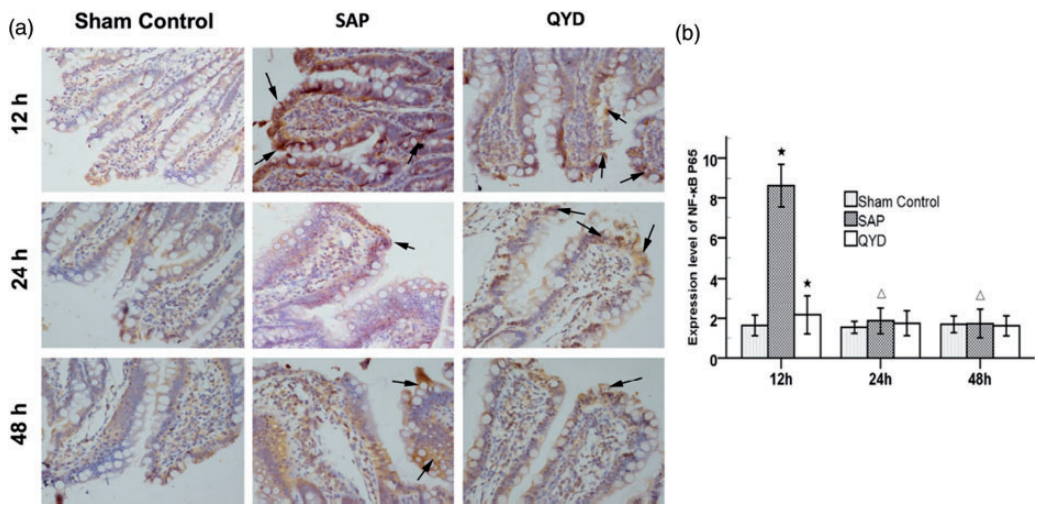

Figure 3. Immunohistochemical staining of NF-κB p56 protein. (a) Immunohistochemistry showing NF-κB p65 expression (black arrows) in intestinal mucosa of rats in each group at different time points and (b) the number of NF-κB p65-positive cells (magnification, ×40). ($^{\bigstar}P < 0.05$ compared with sham controls; $^{\divideontimes}P < 0.05$ compared with the SAP group; $^{\triangle}P < 0.05$ compared with 12 hours within the same group; $^{\blacktriangle}P < 0.05$ compared with 24 hours within the same group).
SAP, severe acute pancreatitis; QYD, Qingyi decoction; NF-κB p65, nuclear factor kappa-light-chain-enhancer of activated B cells p65.

significantly inhibited NF-κB p65 expression at the 12-hour time point and showed no significant effect on NF-κB p65 expression at the 24- and 48-hour time points (Figure 3(a) and (b)). In contrast to NF-κB p65, TLR4 expression in the SAP group peaked at 12 hours postoperatively and gradually decreased at 24 and 48 hours, but all three levels were significantly higher than those in the sham group. TLR4 expression was significantly lower in the QYD than SAP group at all time points, but only significantly so at the 12-hour time point ($P < 0.05$), not at the 24- and 48-hour time points (Figure 4(a) and (b)). ZO-1 expression was significantly lower in the SAP than sham control group at all time points, and this reduction was markedly pronounced in the QYD treatment group compared with the SAP group ($P < 0.05$). However, no significant difference in ZO-1 expression was observed among the different time points of each group (Figure 5(a) and (b)). Western blotting for NF-κB p65, TLR4, and ZO-1 expression in the intestinal mucosa was performed to confirm these results. NF-κB p65 and TLR4 expression peaked at 12 hours after SAP induction and gradually decreased at 24 and 48 hours (Figure 6). QYD treatment significantly restored NF-κB p65 expression to basal levels at 12 hours and TLR4 expression at 24 hours (Figure 6). ZO-1 expression was markedly suppressed in rats with SAP throughout the experimental period and returned to normal in QYD-treated rats as early as 12 hours (Figure 6).

Discussion

SAP is a devastating disease, and its detrimental effects can extend beyond the pancreas, resulting in multiple organ failure. In the present study, we successfully established a rat model of SAP and showed that intestinal pathological changes cause damage to the intestinal barrier. In

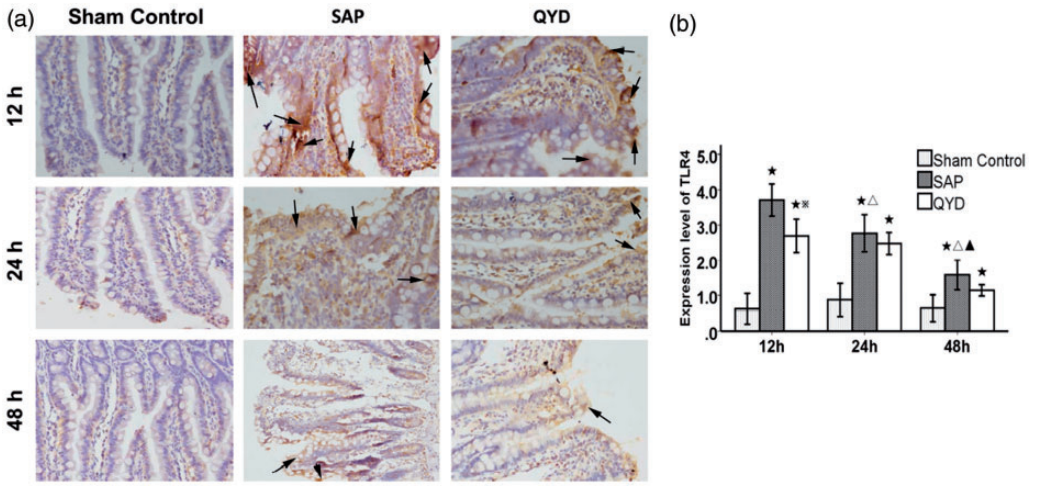

Figure 4. Immunohistochemical staining of TLR4 protein. (a) Immunohistochemistry showing TLR4 expression (black arrows) in intestinal mucosa of rats in each group at different time points and (b) the number of TLR4-positive cells (magnification, ×40). ($^{\bigstar}P < 0.05$ compared with sham controls; $^{\divideontimes}P < 0.05$ compared with the SAP group; $^{\triangle}P < 0.05$ compared with 12 hours within the same group; $^{\blacktriangle}P < 0.05$ compared with 24 hours within the same group).
SAP, severe acute pancreatitis; QYD, Qingyi decoction; TLR4, Toll-like receptor 4.

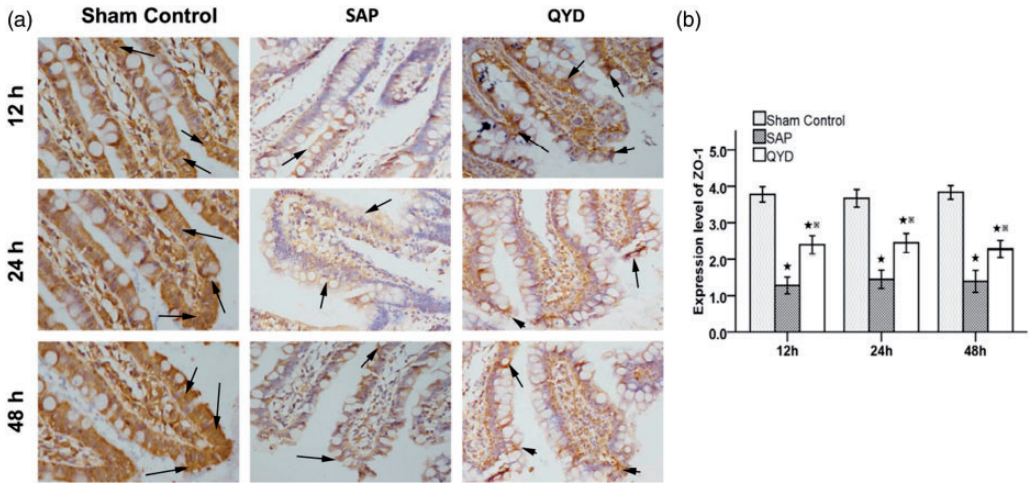

Figure 5. Immunohistochemical staining of ZO-1 protein. (a) Immunohistochemistry showing ZO-1 expression (black arrows) in intestinal mucosa of rats in each group at different time points and (b) the number of ZO-1-positive cells (magnification, ×40). ($^{\bigstar}P < 0.05$ compared with sham controls; $^{\divideontimes}P < 0.05$ compared with the SAP group; $^{\triangle}P < 0.05$ compared with 12 hours within the same group; $^{\blacktriangle}P < 0.05$ compared with 24 hours within the same group).
SAP, severe acute pancreatitis; QYD, Qingyi decoction; ZO-1, zona occludens-1.

addition, the expression of TLR4 and NF-κB in the intestinal mucosa of rats with SAP was distinctly upregulated, while the expression of ZO-1 was significantly downregulated. QYD treatment can reduce SAP-induced damage to the pancreas and small intestine by inhibiting the translocation of intestinal bacteria, reducing the release of inflammatory factors, preventing destruction of the intestinal mucosa, and protecting the intestinal barrier function. The protective function of QYD may be due to its inhibitory effect on the TLR4/NF-κB signaling pathway in intestinal mucosal cells and promotive effect on ZO-1 expression.

Necrosis has been recognized as an initiator of inflammation in the pathogenesis of SAP. In the early stages of SAP, pancreatic acinar cell death is induced by various inflammatory factors. As SAP progresses, acinar cell necrosis causes intrapancreatic activation of digestive enzymes, which leads to autodigestion as well as further tissue necrosis.[14,15] Methods used for induction of SAP in model organisms vary. Some experiments have employed cerulein alone to induce mild acute pancreatitis with moderate inflammatory infiltration in lung tissue. In contrast, a combination of cerulein and lipopolysaccharide has been employed to induce SAP with drastic systemic inflammatory responses in lung injury.[16] In the present study, SAP was induced with sodium taurocholate alone, and the severity was determined by controlling the induction and treatment times. In tissue sections from rats with SAP, severe inflammation and necrosis were observed in the later stages of SAP with significant scoring differences compared with sham controls. These inflammatory and necrotic histological features were ameliorated with QYD treatment in both pancreatic and ileal tissues, which is consistent with a previous report.[17]

The intestinal mucosa is an effective protective barrier against the spread of toxins.

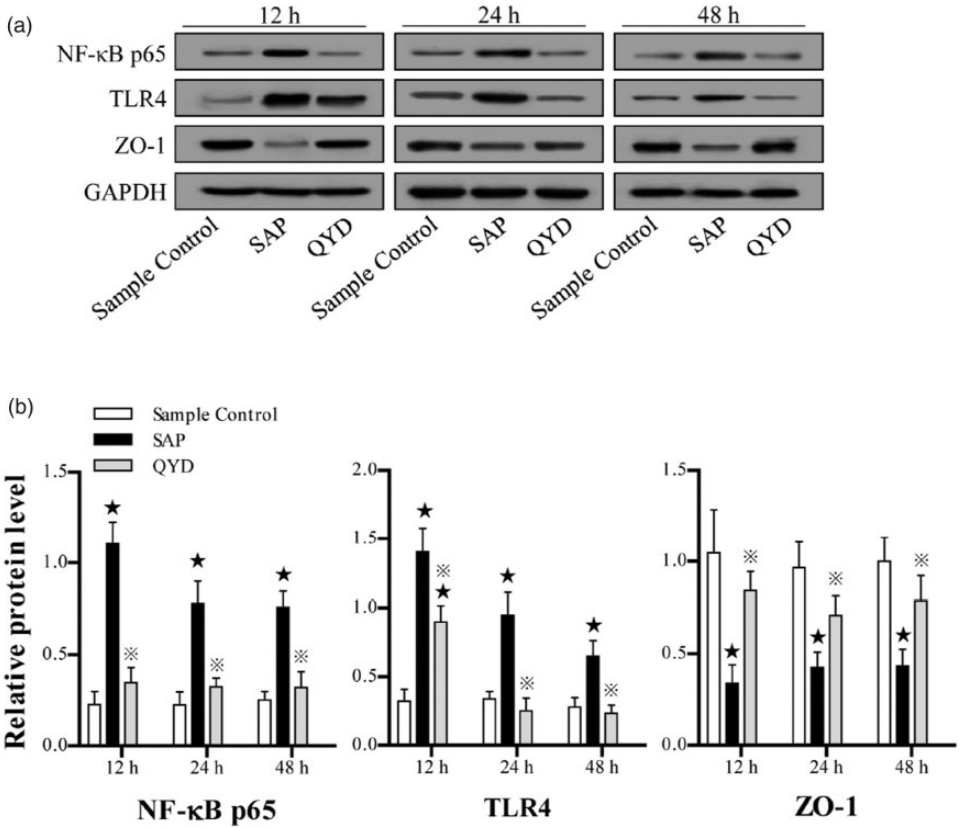

Figure 6. Western blot analysis of NF-κB p56, TLR4, and ZO-1 protein expression. (a) Representative results of western blotting. (b) Statistical analysis of western blotting using Image J software. ($^{★}P < 0.05$ compared with sham controls; $^{※}P < 0.05$ compared with the SAP group; $^{△}P < 0.05$ compared with 12 hours within the same group; $^{▲}P < 0.05$ compared with 24 hours within the same group).
SAP, severe acute pancreatitis; QYD, Qingyi decoction; NF-κB p65, nuclear factor kappa-light-chain-enhancer of activated B cells p65; TLR4, Toll-like receptor 4; ZO-1, zona occludens-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

The recession of the intestinal barrier in the early phase of SAP usually results in bacterial translocation across the gut.[18,19] Previous studies have shown that increased endotoxin is associated with a systemic inflammatory response in patients with acute pancreatitis.[20,21] D-lactic acid is another important indicator of SAP with substantial diagnostic and prognostic value in the clinical setting.[22] It has also been suggested that SAP leads to excessive activation of leukocytes,[23] which in turn results in the release of secondary proinflammatory cytokines, including TNF-α and IL-6, that play crucial roles in the pathogenesis of SAP.[24] In the present study, increased serum levels of endotoxin, D-lactic acid, TNF-α, and IL-6 were observed in rats with SAP and significantly reduced by QYD treatment, which is also consistent with previous reports.[7,10,25] The potential therapeutic effects of QYD are believed to include promotion of blood circulation, clearance of toxic factors, and

stabilization of the function of cell membranes in SAP.[25–28] On the basis of our results, we postulate that the therapeutic effect of QYD may be associated with a reduction of toxins and proinflammatory cytokines.

TLRs activate NF-κB by stimulating IκB phosphorylation, leading to p65 translocation to the nucleus,[29] and this regulates production of downstream proinflammatory cytokines.[30] Upregulation of TLR4 and NF-κB is associated with large amounts of neutrophils and inflammatory mediators in SAP.[31] TLR4 mostly recognizes lipopolysaccharide and a few gram-negative bacterial pathogen-associated molecular patterns by forming homologous dimers.[32] In normal rat pancreatic cells, activation of NF-κB is not obvious;[31] however, in the SAP rat model, activation of NF-κB is more pronounced, suggesting that NF-κB plays a crucial role in the development and prognosis of SAP.[33] Suppression of the TLR4/NF-κB pathway is reportedly helpful in reducing overproduced proinflammatory cytokines, suggesting that it could be an effective therapeutic target in SAP.[34] Our results demonstrated that the protein expression of TLR4 and NF-κB p56 were significantly higher in the SAP model group than in the normal controls and that this increase was reduced with QYD treatment. To the best of our knowledge, this is the first report to link the therapeutic effect of QYD to the TLR4/NF-κB pathway. ZO-1 is a tight junction protein that plays an important role in the maintenance of the intestinal barrier.[35] Recent studies have shown that dysregulation of ZO-1 results in disorganization of tight junctions, which causes disruption of the intestinal barrier.[36,37] Protein expression of ZO-1 was significantly lower in our SAP model than in the control group, and this decrease was reversed with QYD treatment, suggesting that QYD affects the tight junctions of the intestinal barrier to prevent continuous bacterial invasion.

In summary, this study demonstrated that QYD has therapeutic value as evidenced by reduced levels of endotoxin and proinflammatory cytokines through suppression of the TLR4/NF-κB pathway. In addition, QYD may play a protective role in maintaining the intestinal barrier in SAP. While these are significant findings, there are some limitations of the study. First, QYD is a complex herbal formula that contains diverse bioactive components, and we did not extract or study the active molecule (s). Second, other crucial molecules that we did not examine might be involved in the QYD therapeutic effect. Our next study will be conducted using a larger sample size and will consider the above-mentioned limitations to better understand the function of QYD in the treatment of SAP.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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