Qingchang Mixture Prevents the Intestinal Ischemia-reperfusion Injury Through TLR4/NF-kB Pathway

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Research

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Abstract

**Background:** This study was performed to determine the protection and molecular responses of Chinese medicine Qingchang mixture on intestinal ischemia-reperfusion (IR) injury.

**Methods:** The rat intestinal IR model was prepared. Intestinal ischemic injury was evaluated by the HE staining, biochemical assay and western blot. In addition, human intestinal epithelial cells (IEC-6) hypoxia-reoxygenation (HR) *in vitro* model was prepared. The viability and apoptosis of IEC-6 cells were measured by CCK8 and apoptosis detection. TAK242 or PDTC was used as the small molecule inhibitor of TLR4 or NF-κB.

**Results:** Compared with the IR group, the pretreatment of Qingchang mixture relieved the morphological damage, oxidative stress, inflammatory response and barrier function damage of small intestine tissue. IR significantly increased the expression of TLR4 and NF-κB, while the pretreatment of Qingchang mixture inhibited the expression of TLR4 and NF-κB. Furthermore, the pretreatment of Qingchang mixture, TAK242 or PDTC effectively improved the viability, and hindered apoptosis of the HR-induced IEC-6 cells.

**Conclusions:** Chinese medicine Qingchang mixture prevents the intestinal IR injury through TLR4/NF-kB pathway.

**Background**

Intestinal ischemia-reperfusion (IR) injury is an important issue in patients with inadequate perfusion of intestines caused by necrotizing enterocolitis, midgut volvulus, hernia incarceration, abdominal aortic aneurysm surgery, small bowel transplantation, and in patients with circulatory failure due to post-traumatic hemorrhagic shock, septic shock, or heat stress\(^1\)\(^{−}\)\(^4\). Intestinal ischemia reduces mitochondrial ATP production, activates hydrolases, reduces cell membrane selective permeability, and increases calcium influx to ischemic cells, leading to cell death. Reperfusion activates a strong local or systemic inflammatory response\(^5\), such as the release of pro-inflammatory cytokines (TNF-α, IL-1 and IL-6), production of reactive oxygen species (ROS), increased expression of nitric oxide (NO), activation of Toll-like receptors (TLR) − 4 signaling, and other pro-inflammatory mechanisms\(^6\). The circulation of inflammatory factors can severely damage distant organs, such as the lungs, heart, kidneys and liver\(^7\). The local intestinal tissue damage, systemic inflammatory response, and multiple organ failure are mainly caused by IR, not due to the initial ischemic injury or reperfusion injury caused by oxygen free radicals. Therefore, IR injury is difficult to manage clinically with high morbidity and mortality\(^8\).

Our previous research found that the Chinese medicine Qingchang mixture can promote the recovery of gastrointestinal function after abdominal surgery and prevent the occurrence of intestinal adhesions\(^9\). In addition, Qingchang mixture increased plasma motilin levels and decreased IL-6 levels in patients with adhesive intestinal obstruction\(^10\). Based on these results, we speculate that Qingchang mixture could prevent the damage caused by intestinal IR.
Toll-like receptors (TLRs) are a family of pattern recognition receptors that play a key role in innate and adaptive immunity\(^\text{11}\). Currently, researchers have identified 10 and 12 TLRs in humans and mice, respectively. Among these TLRs, previous studies have shown that TLR4 plays an important role in intestinal IR damage\(^\text{12-17}\). However, the role of TLR4 in intestinal IR injury is controversial.

In this study, we aimed to examine the role of Qingchang mixture in the rat severe intestinal IR model and IEC-6 cells hypoxia-reoxygenation (HR) model in vitro. We evaluated intestinal IR damage by evaluating some parameters such as Chiu score, malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH)-Px, IL-6, IL-1β, TNF-α, and tight junction (TJ) proteins expression in the intestine. Our specific hypothesis is that Qingchang mixture prevents the intestinal IR injury and protects intestinal mucosal barrier function of rats through TLR4/NF-κB pathway.

**Materials And Methods**

**Animals and groups**

Male SD rats (weighing 220–250 g) were obtained from the Beijing Vital River Laboratory Animal Technology Co., Ltd. and were randomly divided into three or five groups (n = 8 in each group):

Sham group: Rats were treated with same dose of saline by gastric gavage 1 h before surgery, and were anesthetized for laparotomy. The superior mesenteric artery (SMA) was isolated but not clamped. During separation, saline was injected into the abdominal cavity.

IR group: Rats were treated with same dose of saline by gastric gavage 1 h before surgery. The SMA was clamped for 60 minutes and then reperfused for 120 minutes. During the closure period, saline was injected into the abdominal cavity.

QIR group: Rats were treated with 5 ml/kg Qingchang mixture by gastric gavage 1 h before surgery, and subjected to IR. Qingchang mixture (Lu Pharmaceutical Z01080527) came from the Affiliated Hospital of Shandong University of Traditional Chinese Medicine.

IR + TAK or IR + PDTC group: Rats were treated with same dose of saline by gastric gavage 1 h before surgery. The SMA was clamped for 60 minutes and then reperfused for 120 minutes. During the closure period, the small molecule inhibitors of TLR4, TAK242 (3 mg/kg) (HY-11109, MedChemExpress), or the small molecule inhibitors of NF-κB, PDTC (60 mg/kg) (HY-18738, MedChemExpress), was injected into the abdominal cavity.

Animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Institute for Laboratory Animal Research and were approved by the Animal Ethics Committee Affiliated Hospital of Shandong University of Traditional Chinese Medicine (AWE-2019-022).

**Surgical procedure**
The rat intestinal ischemia-reperfusion model was prepared according to reference\(^{18}\). The experimental animals were fasted and received only water 12 hours before the operation. One hour before the operation, rats in the QIR group were treated with Qingchang mixture by gastric gavage, and which in the other groups were treated with the same volume of saline. Under sterile conditions, rats were anesthetized by intraperitoneal injection of chloral hydrate (3 ml/kg). A midline incision opens the abdomen, exposing SMA. Except for the Sham group, the SMA of rats in other groups was clamped with an atraumatic microvascular artery clamp. Intestinal ischemia developed within 60 minutes. Pale coloring of the intestines and the absence of a pulse indicated ischemia. During occluding of SMA, saline was intermittently dripped into the abdominal cavity of rats. However, the dripped solution was replaced with TAK242 in the IR + TAK group, and was replaced with PDTC in the IR + PDTC group, respectively. After ischemia, the clip was removed and reperfusion was induced for 120 minutes. The re-establishment of a pulse and of pink coloring indicated reperfusion of the intestines.

Sample Collection

At the end of reperfusion, blood samples were taken from the heart, left at room temperature for 30 minutes. The supernatant was collected by centrifugation and placed at -80 °C. Then, all rats were sacrificed, and their small intestine segment about 10 cm away from the ileocecal part was separated. After rinsing with sterile PBS, it was divided into two parts. The first part was used for histopathological evaluations. The second part was quickly frozen in liquid nitrogen and transferred to -80 °C.

Microscopic examination of intestinal tissue

1 cm of the small intestine tissue was immersed in 10% formaldehyde for 24 h and was embedded in paraffin. Five micrometer thick sections were taken from the paraffin-embedded tissues and stained using the hematoxylin and eosin (HE) method. Two observers conducted histological evaluations without knowing the source of the specimens on two separate circumstance. The entire cross-section for each specimen was visually split into quarters and scored 0–5. 0 point represented normal mucosal villi without damage. 1 point represented enlarged subepithelial space at the villous tips, with capillary congestion. 2 point represented a further expansion of subepithelial space with a moderate separation of the epithelial layer from the lamina propria. 3 point represented the destruction of the villous tips. 4 point represented denuded villi with partial mucosal necrosis of the lamina propria. 5 point represented the digestion and breakdown of the lamina propria with bleeding and ulcers. The histological damage grade for each quarter was averaged to represent the grade of each section, and grades for each of the five sections were averaged in each animal. Stained specimens were studied under an Olympus BH2 light microscope.

Cell culture and hypoxia-reoxygenation (HR) in vitro model

Human intestinal epithelial cells (IEC-6) were cultured in serum-free DMEM, and maintained in a humidified atmosphere containing 5% CO\(_2\) at 37°C. To HR group, cells were incubated in a microaerophilic system (Thermo, Waltham, MA) with 5% CO\(_2\), 1% O\(_2\) and 94% N\(_2\) for 12 h, and then were
transferred to normoxic conditions for 6 h to achieve reoxygenation. To QHR group, cells were incubated in medium containing Qingchang mixture (200 µL) for 2 h before suffering from HR. To IR + TAK or IR + PDTC group, cells were incubated in a medium containing TAK242 (3 µg) or PDTC (60 µg) for 2 h, and then subjected to HR.

Biochemical assay

100 mg of small intestine tissue was washed with PBS. Subsequently, the tissue was cut and placed in a variable speed homogenizer to fully grind. Tissue homogenate was centrifuged at 4 °C, 5000 g for 5 minutes. Take an appropriate amount of supernatant for subsequent experiments. 100 mg of small intestine tissue was washed with PBS. Subsequently, the tissue was cut and placed in a variable speed homogenizer to fully grind. Tissue homogenate was centrifuged at 4 °C, 5000 g for 5 minutes. Take an appropriate amount of supernatant for subsequent experiments. The contents of MDA, SOD and GSH-Px in small intestine tissue, and the contents of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) on serum were measured using commercial kits (Nanjing Jiancheng, China) according to the manufacturer’s instructions.

IL-6, IL-1β and TNF-α concentration detection

The IL-6, IL-1β and TNF-α concentration in intestinal tissues were measured by ELISA (CSB-E04640r, CSB-E08055r and CSB-E11987r, CUSABIO) according to the manufacturer’s instructions.

Western blot

Proteins from the tissues and cells were separated by SDS-PAGE and were transferred to PVDF membranes. Western blotting was performed using antibodies (Abcam, Cambridge, UK). Protein quantification was measured in optical density units using Image Lab software (Bio-Rad, CA, USA) and was normalized to the corresponding sample expression of GAPDH.

CCK8 assay

The viability of IEC-6 cells was measured by CCK8 assay. After HR, 10% CCK8 solution was added to each well, and the cells were incubated at 37 °C for 2 h. The optical density was measured at 450 nm using a microplate reader (Biotec, USA).

Apoptosis detection

Apoptosis in IEC-6 cells was identified using the FITC-labeled AnnexinV/propidium iodide (PI) Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ) following the manufacturer’s instructions. After HR, the IEC-6 cells were suspended in 200 ml of 1 × binding buffer (1 × 10⁶ cells/ml). The cells were incubated with AnnexinV (1:20) for 3 min followed by PI for 15 min in the dark at room temperature. The cells were subjected to flow cytometry using the BD FACS Calibur system, and the data were analyzed using FlowJo software.

Statistical analysis
All analyses were completed using SPSS 22.0 software (SPSS Inc. Chicago, IL, USA). Comparisons between the groups were performed by one-way analyses of variance (ANOVA). Data is presented as mean ± SD. P < 0.05 was considered statistically significant.

Results

Prevention of Qingchang mixture against intestinal IR-induced morphological injury

IR damage destroys the structure of the small intestinal mucosa. HE staining showed that the intestinal mucosa of rats in the Sham group was intact, the villi were neat, and the structure of each layer was intact (Fig. 1A). The intestinal mucosa of the rats in the IR group was severely damaged, the lamina propria was damaged, the villous structure was blurred, accompanied by ulcers and bleeding (Fig. 1A). The intestinal mucosal damage was reduced, subepithelial space was enlarged, and the villi tip was partially damaged in the QIR group (Fig. 1A). Compared with the Sham group, the Chiu score of IR group were significantly increased (Fig. 1A, P < 0.05). Compared with the IR group, the Chiu score of QIR group were decreased (Fig. 1A, P < 0.05). The intestinal morphologic alterations were markedly improved in QIR group. These results suggested the prevention of Qingchang mixture against intestinal IR-induced morphological injury.

Prevention of Qingchang mixture against intestinal IR-induced oxidative stress and inflammatory response

Excessive oxidative stress and inflammation response are two important features of intestinal IR injuries. Compared with the Sham group, the IR group showed up-regulation of MDA (a biomarker of oxidative damage), and down-regulation of SOD and GSH (biomarkers of antioxidant capacity) (Fig. 1B). Pretreatment of Qingchang mixture reversed these parameters (Fig. 1B). In addition, compared with the Sham group, the level of proinflammatory cytokines including IL-6, IL-1β and TNF-α in the IR group increased (Fig. 1B and C). Pretreatment of Qingchang mixture significantly reduced the levels of these proinflammatory cytokines (Fig. 1B and C). Moreover, the pretreatment of Qingchang mixture also significantly reduced the levels of ALT and AST (Fig. 2A), which are the serum biochemical indicators of remote organ injury, indicating that Qingchang mixture also protect remote organs. These results indicated that Qingchang mixture pretreatment selectively alleviated the intestinal IR injuries by suppressing the oxidative stress and proinflammatory cytokines release.

Protection of Qingchang mixture against intestinal mucosal barrier function

IR injury results in the rupture of tight junctions (TJ), located at the most apical part of the intestinal epithelium, and the dysfunction of intestinal barrier, which is characterized by altered expression and
redistribution of TJ proteins\textsuperscript{20,21}. Our results found that compared with the Sham group, the expression levels of claudin-1, claudin-5, ZO-1, and occludin in the IR group were significantly reduced (Fig. 2B and C, \( P < 0.05 \)). Compared with the IR group, the expression levels of these proteins in the QIR group were significantly increased (Fig. 2B and C, \( P < 0.05 \)). These results suggested the protection of Qingchang mixture against intestinal mucosal barrier function.

**Qingchang mixture prevents the intestinal IR injury and protects intestinal mucosal barrier function of rats through TLR4/NF-κB pathway**

The mechanisms underlying intestinal IR damage are likely to be complex and multifactorial, although the TLR4/NF-κB/proinflammatory factor pathway has been considered to play a major role in it\textsuperscript{22}. TLR4 is most closely related to IR damage in the small intestine. A variety of inflammatory factors, such as IL-6, IL-1\( \beta \) and TNF-\( \alpha \), are released through the activation of NF-κB effected by TLR4 complex\textsuperscript{23}. We found that IR significantly increased the expression of TLR4 and NF-κB, while the pretreatment of Qingchang mixture inhibited the expression of TLR4 and NF-κB (Fig. 3A, \( P < 0.05 \)). Furthermore, we analyzed whether Qingchang mixture protects IR damage and intestinal mucosal barrier function in rats through the TLR4/NF-κB pathway using specific small molecule inhibitors of TLR4 and NF-κB. As shown in Fig. 3B, the treatment of TAK242 and PDTC decreased the level of TLR4 and NF-κB (Fig. 3B). In addition, compared to the IR group, the treatment of TAK242 and PDTC decreased the Chiu score, inhibited the release of MDA, IL-6, IL-1\( \beta \) and TNF-\( \alpha \), and increased the expression of SOD, GSH and TJ proteins, which was not significantly different from the role of QIR group (Fig. 3C-F, \( P < 0.05 \)).

**Protection of Qingchang mixture against HR-induced apoptosis through TLR4/NF-κB pathway**

We also studied the protective mechanism of Qingchang mixture in an HR model *in vitro*. As shown in Fig. 1A, HR induced the enhanced expression of TLR4 and NF-κB in IEC-6 cells; while the pretreatment of Qingchang mixture effectively suppressed the enhanced expression of TLR4 and NF-κB. The addition of TAK242 and PDTC also effectively inhibited the enhanced expression of TLR4 and NF-κB (Fig. 4A). CCK-8 assay showed that HR impaired the cell viability of IEC-6 cells, and the pretreatment of Qingchang mixture significantly alleviated the HR injury on cell viability (Fig. 4B). The addition of TAK242 and PDTC also effectively relieved the HR injury on cell viability (Fig. 4B). The results of Annexin V/PI analysis using flow cytometry confirmed that HR induced apoptosis (Fig. 4C and D), and pretreatment with Qingchang mixture effectively inhibited HR-induced apoptosis. The addition of TAK242 and PDTC also had a consistent effect (Fig. 4C and D). Western blot analysis showed that in cells subjected to HR, the expression of apoptotic proteins Bax and Caspase-3 was up-regulated, and the expression of anti-apoptotic protein Bcl2 was down-regulated (Fig. 4E). Compared with IEC-6 cells injured by HR, the pretreatment of Qingchang mixture, the addition of TAK242 and PDTC can significantly increase the expression of Bcl2, and significantly reduce the level of Bax and Caspase-3 (Fig. 4E). These results
indicated the protection of Qingchang mixture against HR injury on cell viability and HR-induced apoptosis through TLR4/NF-kB pathway.

Discussion

The small intestine is made up of labile cells which are particularly sensitive to IR damage, and intestinal IR injury is a common event under various clinical conditions. Intestinal IR can cause epithelial cell damage, disrupt mucosal integrity and small intestinal function, and subsequently increase mucosal and vascular permeability, systemic inflammation, multiple organ dysfunction, and even death. There is currently no effective treatment for intestinal IR injury. In this study, we investigated the protection of Qingchang mixture on the integrity and function of the small intestinal mucosa using the rat intestinal IR model and IEC-6 cells HR model in vitro, providing an effective clinical treatment for intestinal IR injury.

The HE staining results showed obvious intestinal histopathological damage in the rat intestinal IR model. The changed expression of MDA, SOD, and GSH-Px indicated the oxidative stress state of the rat intestinal. The secretion of pro-inflammatory factors (IL-6, IL-1β and TNF-α) suggested the occurrence of local inflammation. The expression levels of ALT and AST indicated the damage to distant organs. Down-regulation of TJ proteins (such as ZO-1) further supported the TJ destruction and intestinal barrier dysfunction. TJ consists of a group of transmembrane proteins, including occludin, claudins, and ZO-1, which acts as a closed barrier by maintaining cell polarity and internal environment balance and regulating the permeability of the epithelial cell space. IR injury results in the destruction of TJ, which is characterized by altered and redistributed TJ protein expression.

Importantly, our research found that Qingchang mixture effectively prevented the pathological damage and permeability changes caused by IR. Compared with the IR group rat model, the administration of Qingchang mixture significantly reduced the Chiu score, the expression of MDA, and the secretion of pro-inflammatory factors, ALT and AST, and significantly increased the expression of SOD, GSH-Px and TJ proteins. This is the first report that Qingchang mixture can prevent intestinal pathological damage and barrier dysfunction caused by IR. In addition, studies have shown that changes in TJ protein expression are partially mediated by TNF-α. One research suggests that Qingchang mixture can significantly promote the intestinal motility of mice, has no obvious stimulating effect on gastric mucosa. The conventional dosage is safe, and the lethal dose of the acute toxicity experiment mouse was 13.95 g/kg. Regarding the dosage and mode of Qingchang mixture, more research is needed. In addition to the rat intestinal IR model, we also used the IEC-6 cell HR model in vitro to investigate the protective effect of Qingchang mixture on the intestinal epithelial cells. The pretreatment of Qingchang mixture improved the viability, and hindered apoptosis of the HR-induced IEC-6 cells.

The intestinal epithelium is constantly exposed to various bacteria and bacterial products. The biological response to endotoxin is mediated by the TLR4 complex, which leads to the activation of NF-κB and the release of cytokines, including IL-6, IL-1β and TNF-α. One research reveals that the expression of TLR4 and NF-κB is increase after intestinal IR in mice. One report shows that TLR4 has a protective
effect on intestinal IR damage using TLR4 knockout mice\textsuperscript{13}. Chen \textit{et al.} also suggest that administration of the representative TLR4 ligand LPS, reduces the incidence of intestinal IR damage\textsuperscript{30}.

Contrary to these studies, some reports indicate that the TLR4 signaling pathway is an exacerbating factor for intestinal IR damage. Pope \textit{et al.} demonstrate that TLR4 deficiency reduces intestinal IR damage by reducing complement activation\textsuperscript{14}. Zhu \textit{et al.} find that the TLR4 mutant significantly reduced intestinal IR damage partially by reducing inflammatory response and oxidative stress\textsuperscript{15}. Moses \textit{et al.} believe that TLR4-mediated COX-2 expression exacerbates intestinal IR damage\textsuperscript{16}. In addition, the interaction of TLR4 and endogenous TLR4 ligand high mobility group protein 1 (HMGB1) increases mouse intestinal IR damage\textsuperscript{17}. Other studies have revealed the role of the TLR4/NF-κB signaling pathway in non-human primates intestinal IR damage. Wu \textit{et al.} point out that macaques develop multiple organ dysfunction after intestinal IR injury, which is related to the expansion of the severe innate immune response\textsuperscript{31, 32}. This response is mediated by the general activation of the TLR/NF-κB/cytokine pathway throughout the small intestinal mucosal system.

Due to the wide variety of TLR4 ligands, the role of the TLR4 signaling pathway may vary depending on the activation ligand. In addition, unlike other organs, the gut has always coexisted with bacteria. The identification of intestinal bacteria by TLRs plays an important role in controlling intestinal homeostasis and preventing damage\textsuperscript{22}. Therefore, the balance between the beneficial and deleterious effects of the TLR pathway may be disturbed by intestinal flora colonies\textsuperscript{33}.

In this study, we found the activation of TLR4/NF-κB pathway in a rat intestinal IR injury model. The administration of Qingchang mixture inhibited the activation of TLR4/NF-κB pathway. In addition, treatment with TLR4 or NF-κB specific inhibitors effectively protected intestinal function. In other words, Qingchang mixture was likely to protect the intestinal function by inhibiting the activation of the TLR4/NF-κB pathway.

**Conclusion**

In conclusion, we found that the Chinese medicine Qingchang mixture effectively prevented the pathological damage of intestinal mucosa, oxidative stress, the release of proinflammatory factors and the destruction of TJ in rat intestinal IR model, which indicated that the Qingchang mixture might be a valuable agent for preventing and treating intestinal IR damage. Further research is needed on the function, safety, dosage and administration of Qingchang mixture.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.
Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Meng Wang have made substantial contributions to the conception and design of the work. Each author has made substantial contributions to the acquisition, analysis, and interpretation of data.

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Not applicable.

References


Figures

Figure 1

Prevention of Qingchang mixture against intestinal IR-induced morphological injury, oxidative stress and inflammatory response in rats. The HE staining and Chiu score (A), the levels of MDA, SOD and GSH-Px (B), the concentration of IL-6, TNF-α and IL-1β (C) in intestinal tissue contents of Sham, IR and QIR groups. *P<0.05, compared to Sham group; #P<0.05, compared to IR group.
Figure 2

Protection of Qingchang mixture against remote organ injury and intestinal mucosal barrier function. The concentration of ALT and AST (A), and the expression of tight junction (TJ) proteins (B and C) contents of Sham, IR and QIR groups. *P<0.05, compared to Sham group; #P<0.05, compared to IR group.
Figure 3

Qingchang mixture prevents the intestinal IR injury and protects intestinal mucosal barrier function of rats through TLR4/NF-kB pathway. (A) The expression of TLR4 and NF-kB in Sham, IR and QIR groups was detected by western blot. (B) The expression of TLR4 and NF-kB in Sham, IR, QIR, IR+TAK and IR+PDTC groups was measured by western blot. The HE staining and Chiu score (C), the levels of MDA, SOD and GSH-Px (D), the concentration of IL-6, TNF-α and IL-1β (E), and the expression of tight junction (TJ) proteins (F) contents of Sham, IR, QIR, IR+TAK and IR+PDTC groups. *P<0.05, compared to Sham group; #P<0.05, compared to IR group.
Figure 4

Protection of Qingchang mixture against hypoxia-reoxygenation (HR)-induced apoptosis through TLR4/NF-κB pathway. For HR, after 12 h of hypoxia, the IEC-6 cells returned to normoxic state for 6 h. For the QHR group, before suffering from HR, the cells were incubated in a medium containing Qingchang mixture (200 μL) for 2 hours. For the IR+TAK or IR+PDTC group, the cells were incubated in a medium containing TAK242 (3 μg) or PDTC (60 μg) for 2 h, and then subjected to HR. After the cells were treatment, (A) the expression of TLR4 and NF-κB was measured by western blot; (B) the cell viability was measured by CCK8 assay; (C and D) the apoptosis was measured by Annexin V/PI analysis; (E) the expression of apoptosis-related proteins was detected by western blot. *P<0.05, compared to NC; ##P<0.05, compared to HR.