Gadolinium chloride pre-treatment reduces the inflammatory response and preserves intestinal barrier function in a rat model of sepsis

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Abstract. The inflammatory response is closely associated with sepsis occurrence and progression. Damage to the function of the intestinal mucosal barrier is considered to be the 'initiation factor' for the development of multiple organ dysfunction syndrome, which is the most severe progression of sepsis. The aim of the present study was to investigate whether gadolinium chloride ($GdCl_3$) could alleviate the systemic inflammatory response and protect the function of the intestinal mucosal barrier in a rat model of sepsis. The mechanism underlying this protective effect was also explored. Sprague-Dawley rats were divided into four groups: Sham, sham + $GdCl_3$, cecal ligation and puncture (CLP; a model of sepsis) and CLP + $GdCl_3$. In each group, blood was collected from the abdominal aorta, and intestinal tissue was collected after 6, 12 and 24 h of successful modeling. Levels of tumor necrosis factor-α, interleukin (IL)-6 and IL-1β were determined using ELISA. Western blot analysis was used to determine levels of occludin, tight junction protein ZO-1 (ZO-1), myosin light chain kinase 3 (MLCK), NF-κB and caspase-3 in intestinal tissues. Hematoxylin-eosin staining was used to observe the degree of damage to intestinal tissue. The results indicated that in CLP sepsis model rats treated with $GdCl_3$, the release of systemic and intestinal pro-inflammatory factors was reduced and tissue damage was alleviated when compared with untreated CLP rats. Additionally, the expression of occludin and ZO-1 was increased, while that of NF-κB, MLCK, and caspase-3 was reduced in the CLP + $GdCl_3$ rats compared with the CLP rats. $GdCl_3$ may alleviate systemic and intestinal inflammatory responses and reduce the expression of MLCK through inhibition of the activation of NF-kB. The results of the present study also indicated that $GdCl_3$ promoted the expression of occludin and ZO-1. $GdCl_3$ was also demonstrated to reduce cell apoptosis through the inhibition of caspase-3 expression.

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Introduction

Sepsis is a clinical syndrome in which the host has an uncontrolled response to infection and develops life-threatening organ dysfunction (1). Sepsis and septic shock are progressive and multifactorial diseases with high morbidity and mortality. Each year, millions of people worldwide suffer from sepsis and >25% of these individuals die from the syndrome, making sepsis a major global health challenge (2).

The early systemic inflammatory response and intestinal barrier dysfunction seen in sepsis are closely associated with progression of the condition and the occurrence of its most severe form, multiple organ dysfunction syndrome (3-5). The release of a large number of pro-inflammatory cytokines in the early stages of inflammation is considered to be an important pathological mechanism in the development of sepsis (6,7). Increasing concentrations of inflammatory cytokines are produced by an excessive inflammatory response, which can cause systemic and intestinal inflammation and the activation of the NF-κB signaling pathway in intestinal epithelial cells (8). Inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, have damaging

effects on the tight junction structure and barrier function of intestinal epithelial cells (9,10). The tight junction is composed of cytoplasmic transmembrane proteins, including occludin and junctional proteins, such as tight junctional protein ZO-1 (ZO-1) (11). Research has indicated that tight junctions are regulated by myosin light chain kinase (MLCK) (12). Studies have also demonstrated that NF-κB activity serves a crucial role in promotion of MLCK expression (13). However, it is not clear whether the impairment of intestinal barrier function due to intestinal inflammatory factors is associated with the regulation of MLCK expression by NF-κB, and the resulting reduction in the expression of tight junctional proteins in intestinal epithelial cells. In the present study, it is hypothesized that the inhibition of the systemic and intestinal inflammatory responses may be an effective means of protecting the intestinal barrier from damage in sepsis.

Gadolinium chloride ($GdCl_3$) is a lanthanide compound that is commonly used to assess the function of Kupffer cells (14,15). As $GdCl_3$ can inhibit the phagocytosis and secretion of Kupffer cells in the liver, it is often used as a tool for studying the functions of monocytes/macrophages and the pathogenesis of disease (15). $GdCl_3$ can induce changes in the phenotype of Kupffer cells and competes to bind to Kuppfer cell calcium receptors, inhibiting the transcription and synthesis of TNF-α (16). $GdCl_3$ has not been indicated to trigger an immune response, so it has been used in animal models of a variety of experimental diseases, including hepatic ischemia-reperfusion injury models, obstructive jaundice models induced by bile duct ligation, lipopolysaccharide (LPS)-induced endotoxemia models and cecal ligation and puncture (CLP)-induced sepsis models (17). Previous studies have revealed that sepsis-induced acute lung injury can be alleviated by the $GdCl_3$-mediated inhibition of inflammatory mediators release, including the release of TNF-α by macrophages (18). TNF-α and IL-6, which is released by Kupffer cells in the early stages of endotoxemia, may serve an important role in the initiation and progression of ileal mucosal damage (19). It has been suggested that $GdCl_3$ inhibits the secretion of pro-inflammatory cytokines from macrophages by inhibiting the activity of the NF-κB signaling pathway, thereby inhibiting colonic mucosal inflammation and alleviating the severity of intestinal inflammation in mice (20). However, there has been little research into the effects of $GdCl_3$ on intestinal function. $GdCl_3$ has been reported to reduce pulmonary apoptosis in acute lung injury, myocardial apoptosis during myocardial reperfusion and hepatocyte apoptosis in acute liver injury, through the inhibition of caspase-3 expression (15,18,21). However, to the best of our knowledge, there has been limited study into whether $GdCl_3$ can inhibit the expression of caspase-3 in intestinal cells and reduce the apoptosis of intestinal tissue cells in sepsis model rats, thereby protecting the function of the intestinal barrier.

The present study aimed to investigate the effects of $GdCl_3$ on the systemic and intestinal release of cytokines (including TNF-α, IL-1β and IL-6) and the protective effects of $GdCl_3$ on intestinal barrier function in a CLP-model of sepsis. Additionally, whether $GdCl_3$ reduced the expression of NF-κB protein in intestinal tissue and whether $GdCl_3$ could promote the expression of tight junction proteins in intestinal cells to protect the function of the intestinal barrier was investigated. The effect of $GdCl_3$ on intestinal cell apoptosis was also explored to determine whether apoptosis is associated with the expression of caspase-3.

Materials and methods

Animal model. A total of 144 male Sprague-Dawley (SD) rats (weight, 200-250 g; age, 8-10 weeks) were purchased from Xinjiang Medical University (experimental animal production license no. XJYK0011, 2011). Animals were housed at a temperature of 20±1˚C, relative humidity of 45%, noise below 85 decibels and ventilated 8 to 12 times/h on a 12 h light/dark cycle and had free access to standard laboratory feed and tap water. All procedures were approved by the Animal Protection and Use Committee of Shihezi University (no. A20187-174) and were implemented in accordance with the Animal Management Regulations of the Ministry of Health of China (22).

Sepsis was induced using CLP. Under intraperitoneal anesthesia induced by 1% pentobarbital (30 mg/kg; Merck KGaA), a midline incision of ~2 cm was made on the anterior abdomen. The cecum was carefully isolated, and ~2/3 of the cecum was ligated using a 4-0 silk suture. The cecum was punctured in two different places using 21-G needles and was squeezed to extrude fecal material. The cecum was then replaced, and the abdomen was sutured. Sham group animals were treated in an identical manner, but no cecal ligation or puncture was performed. Each rat received a subcutaneous injection of 1 ml normal saline for fluid resuscitation after surgery.

SD rats were fasted and given free access to water for 12 h prior to the experiment. They were randomly divided into 4 groups: Sham operation (sham group; n=36), $GdCl_3$ pre-treatment with sham operation (sham + $GdCl_3$ group; n=36), CLP (CLP group; n=36) and $GdCl_3$ pre-treatment with CLP (CLP + $GdCl_3$ group; n=36). The sham + $GdCl_3$ and CLP + $GdCl_3$ groups received 20 mg/kg $GdCl_3$ (no. 203289-1G; Sigma-Aldrich; Merck KGaA) via tail vein injection at 1 and 2 days prior to the operation, while the Sham and CLP groups were given the equivalent amount of normal saline in an identical manner. After successful model establishment (after 2-4 h of modeling, the success of the sepsis model was judged by observing whether the rats had curled up, vertical hair, reduced activity, fecal incontinence, increased secretion from the corner of the eyes and decreased body temperature), the animals were sacrificed after 6, 12 or 24 h. In the western blot experiments, the protein expression level at 12 h of the sham group was used and represented that of each time point of the sham group and sham+$GdCl_3$ group. Blood samples were then collected from the abdominal aorta and intestinal tissue (ileum near the cecum) samples were preserved for subsequent experiments.

ELISA. ELISA kits from Elabscience Biotechnology Inc. were used to assess the concentrations of TNF-α (cat. no. E-EL-R0019), IL-6 (cat. no. E-EL-R0015) and IL-1β (cat. no. E-EL-R0012) in rat serum or supernatant from intestinal tissue homogenization. The serum samples were obtained by centrifugation of blood samples at 3,000 x g for 15 min at 4˚C. The tissue homogenate which was obtained by grinding

intestinal tissue, which was then centrifuged at 5,000 x g for 10 min at 4˚C to obtain a tissue supernatant. The serum concentration of diamine oxidase (DAO) was also measured using a DAO ELISA kit (cat. no. E-EL-R0331; Elabscience Biotechnology Inc.). All kits were used in accordance with the manufacturer's protocol.

Western blot analysis. Total protein was extracted from each group of the ileum about 5 cm above the cecum. The protein was extracted using radioimmunoprecipitation assay buffer (cat. no. D1010; Beijing Solarbio Science & Technology, Inc.) at a ratio of 10 mg tissue to 100 μl buffer. The extracted turbid liquid was placed in an ultra-high-speed centrifuge with at 12,000 x g for 20 min at 4˚C and protein content of the resulting solution was determined using the bicinchoninic acid method (cat. no. P0012, Beyotime Institute of Biotechnology). An equal amount of protein (30 μg/lane) from each sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then transferred onto PVDF membranes. After blocking with 5% skim milk for 2 h at room temperature, the membrane was incubated with the primary antibodies of interest or an anti-β-actin antibody (1:1,000; cat. no. TA-09; ZSGB-BIO; OriGene Technologies Inc.) overnight at 4˚C. The primary antibodies were anti-occludin (1:1,000; cat. no. ab216327; Abcam), anti-ZO-1 (1:500; cat. no. sc-33725; Santa Cruz Biotechnology, Inc.), anti-MLCK (1:5,000; cat. no. ab76092; Abcam), anti-NF-κB (1:1,000; cat. no. 8242; Cell Signaling Technology Inc.) and anti-caspase-3 (1:500; cat. no. ab13847; Abcam). After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000; goat anti-rabbit; cat. no. ZF-0311; ZSGB-BIO or goat anti-mouse; cat. no. ZF-0312, OriGene Technologies, Inc.) at 37˚C for 90 min. Proteins were detected using a chemiluminescence system and visualized using a gel imaging system (ChemiDoc™ Touch; Bio-Rad Laboratories, Inc.). The results were analyzed using intensity quantification software (ImageLab 5.2; Bio-Rad Laboratories, Inc.).

Intestinal permeability assay. An intragastric injection of 600 mg/kg (125 mg/ml) 4 kDa fluorescein isothiocyanate-dextran (FD4; Sigma-Aldrich; Merck KGaA) was administered ~6 h prior to sacrifice. Blood samples were centrifuged at 12,000 x g for 4 min at 4˚C, and the resulting plasma was diluted with an equal volume of PBS; pH 7.4). An excitation wavelength of 480 nm and emission wavelength of 520 nm were used to analyze fluorescence with a full wavelength scanning multifunction reader (Varioskan Flash; Thermo Fisher Scientific Inc.). Standard curves of FITC-dextran concentrations were obtained by serial dilution of the FD4 solution with PBS (0-12.5 mg/ml).

Intestinal epithelial apoptosis. Intestinal tissue was fixed in 4% paraformaldehyde for 48 h at room temperature (~20˚C), embedded in paraffin, and cut into 5-μm sections. A TUNEL apoptosis assay kit (Sigma-Aldrich; Merck KGaA) was used according to the manufacturer's protocol. After dewaxing, hydration and cell permeabilization using 0.2% Triton X-100 (ZSGB-BI; cat. no. ZLI-9308), TUNEL reaction solution, converter-peroxidase, and 3,3'-diaminobenzidine (DAB; ZSGB-BIO; cat. no. ZLI-9018) were added dropwise in sequence. At room temperature, 100 μl DAB substrate was added dropwise to the tissue on the glass coverslip for color development. After dropping, the samples were observed under the microscope, and the color development was stopped when the appropriate amount of yellowish-brown appeared. The stained cells appeared as if the chromatin was condensed, marginalized and divided into blocks (apoptotic bodies), and the nuclear membrane was cracked. After sealing with neutral balsam, the samples were mounted under glass coverslip with glycerol and analyzed under light microscope (magnification, x200). Five fields of view were randomly selected from each tissue and analyzed separately by three professional pathology teachers.

Intestinal histopathology and damage index. Tissues were fixed with 4% paraformaldehyde at 4˚C for >24 h, embedded in paraffin and serially sectioned (5 μm). Slides were stained with hematoxylin and eosin (H&E, 20% Harris for 10 min and 0.5% eosin for 1 min) at room temperature. The sections were examined under a DP microscope (Olympus Corporation) at x200 magnification. Intestinal injuries were assessed using the Chiu scoring system (23,24). Three senior pathology professors, who were blinded to the study, randomly selected 5 visual fields in each tissue section to score, and finally took the average value.

Statistical analysis. Data analysis was performed using SPSS 21.0 statistical software (IBM Corp.). Normally distributed measurement data are presented as the mean ± standard deviation and were analyzed using a one-way ANOVA. An LSD post-hoc test was used if equal variances were assumed and a Tamhane' T2 post-hoc test was used if equal variances were not assumed. Non-normally distributed data are presented as the median ± interquartile range and were analyzed using Kruskal-Wallis non-parametric test. The Dunn's all-pairwise test was used to analyze differences between two groups following Kruskal-Wallis test. Each analysis was repeated three times. Differences with $P<0.05$ were considered statistically significant.

Results

$GdCl_3$ reduces serum and intestinal inflammatory markers in CLP rats. To verify the effect of $GdCl_3$ on systemic inflammation and the intestinal inflammatory response in sepsis model rats, an ELISA was used to determine TNF-α, IL-6 and IL-1β levels in rat serum and intestinal tissues. The results indicated that serum levels of TNF-α, IL-6 and IL-1β were reduced in the CLP+$GdCl_3$ group compared with those in the CLP group at both 6 and 12 h ($P<0.05$, Fig. 1A, C and E), but that there was no difference between these groups at 24 h (Fig. 1A, C and E). However, TNF-α, IL-6 and IL-1β levels in intestinal tissues were significantly reduced in the CLP+$GdCl_3$ group compared with those in the CLP group at all time points (Fig. 1B, D and F).

$GdCl_3$ reduces intestinal permeability and intestinal injury in CLP rats. ELISA was used to determine levels of DAO, and therefore intestinal barrier integrity, in rat serum. The results

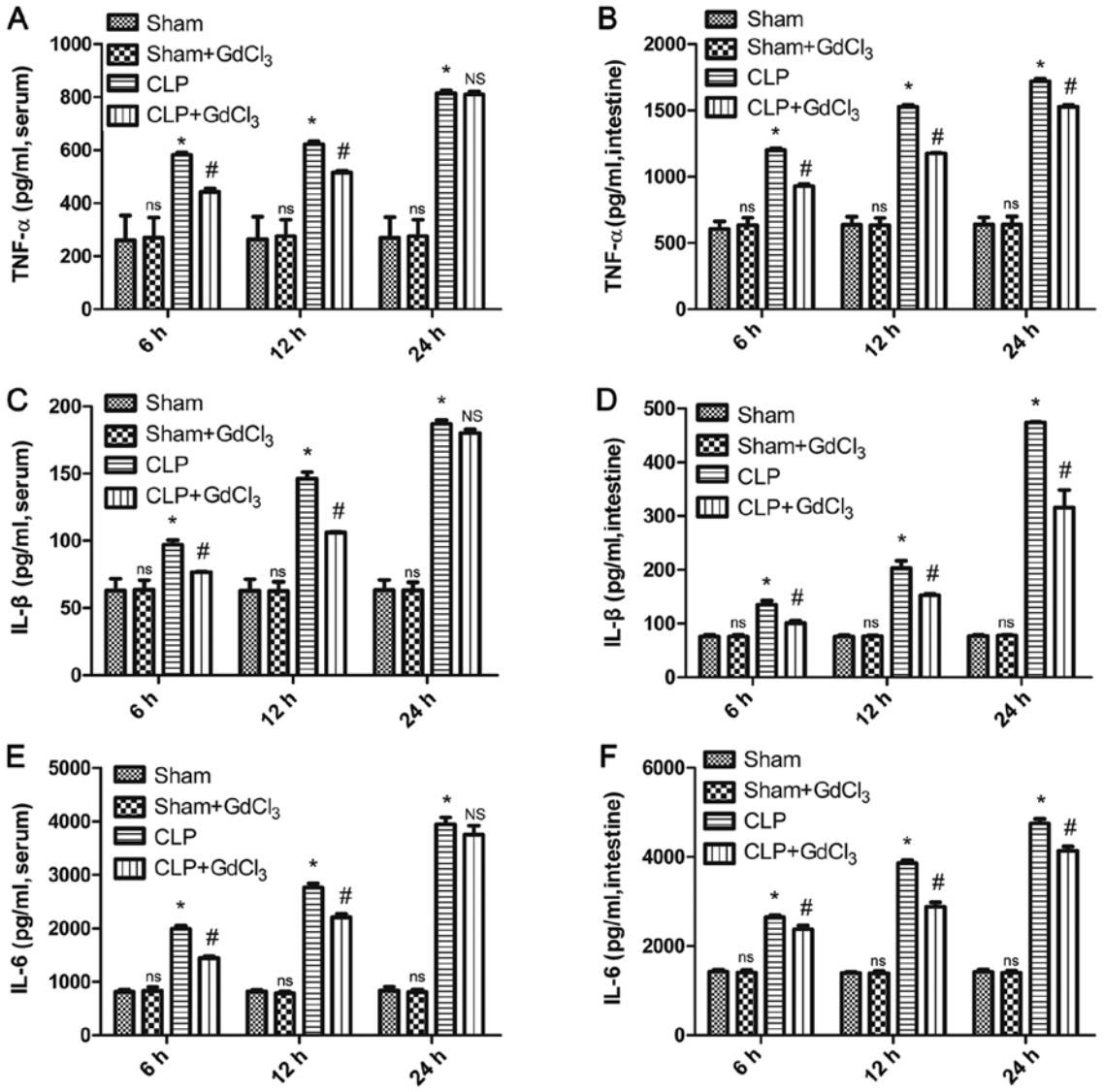

Figure 1. Effect of $GdCl_3$ treatment on systemic and intestinal inflammatory responses following CLP-induced sepsis. Levels of (A) TNF-α in sera, (B) TNF-α in intestinal tissue, (C) IL-1β in sera, (D) IL-1β in intestinal tissue (E) IL-6 in serum and (F) IL-6 in intestinal tissue of CLP-treated rats with or without $GdCl_3$ pretreatment. Data were measured at 6, 12 and 24 h after CLP operation and are presented as the mean ± SD (n=6). *P<0.05 vs. sham group at the same time point; #P<0.05 vs. CLP group at the same time point; ns, not significant vs. sham group at the same time point; NS, not significant vs. CLP group at 24 h. $GdCl_3$, gadolinium chloride; CLP, cecal ligation and puncture; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6.

indicated that the level of DAO was significantly higher in the CLP group compared with the sham group at 6, 12 and 24 h (P<0.05; Fig. 2A). However, the level of DAO in the CLP + $GdCl_3$ group was lower than that in the CLP group at 6, 12 and 24 h (P<0.05; Fig. 2A). To evaluate the degree of intestinal injury more directly, H&E staining of intestinal tissues was performed and the degree of intestinal injury scored according to Chiu's criteria. The results revealed that at 6, 12 and 24 h, the CLP + $GdCl_3$ group exhibited less intestinal tissue damage than the CLP group (Fig. 3A), and the intestinal injury score was lower than that in the CLP group (P<0.05; Fig. 3B). To evaluate the permeability of the intestinal tract, serum levels of FD4 were assessed. The experimental results indicated that the intestinal permeability of the CLP+$GdCl_3$ group was lower compared with the CLP group at each time point (P<0.05; Fig. 2B).

$GdCl_3$ promotes the expression of tight junction proteins occludin and ZO-1 and reduces MLCK expression in CLP rats. The intestinal occludin and ZO-1 proteins reflect the integrity of the intestinal mechanical barrier. MLCK regulates the permeability of intestinal epithelial cells and the expression of occludin and ZO-1 (25). The results indicated that the expression of occludin and ZO-1 proteins were significantly reduced in the CLP group compared with that in the sham group (P<0.05; Fig. 4A-C). However, the expression levels of occludin and ZO-1 were increased in the CLP + $GdCl_3$ group compared with the CLP group at 6, 12 and 24 h (P<0.05, Fig. 4A-C). The expression of MLCK was reduced in the CLP + $GdCl_3$ group compared with the CLP group (P<0.05, Fig. 4A and D).

$GdCl_3$ reduces expression of NF-κB in the intestines of CLP rats. To verify whether $GdCl_3$ regulates intestinal inflammation in septic rats via the NF-κB pathway, western blot analysis was used to determine the expression of NF-κB p65 protein in rat intestines. The results demonstrated that the expression of

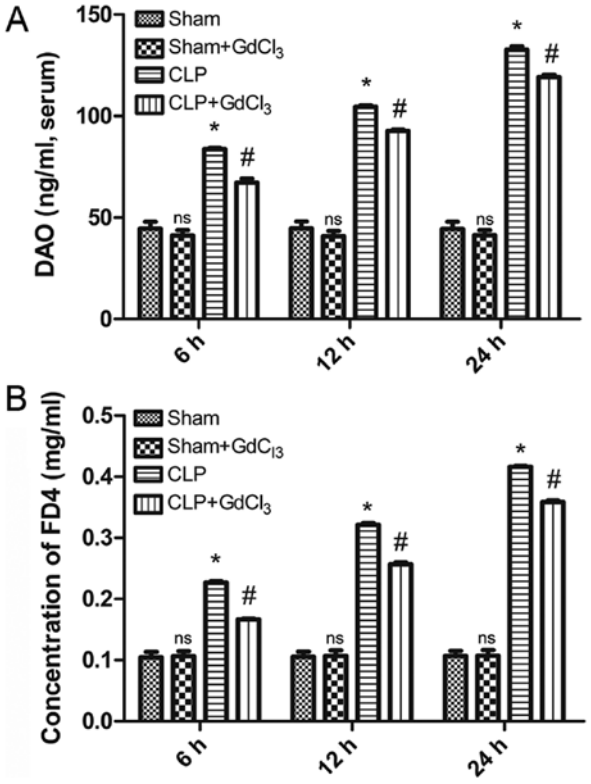

Figure 2. Effect of $GdCl_3$ treatment on intestinal barrier function. Levels of (A) DAO and (B) FD4 in the serum of CLP-treated rats with or without $GdCl_3$ pretreatment. The data were measured at 6, 12 and 24 h after CLP and are presented as the mean ± SD (n=6). *P<0.05 vs. sham group at the same time point; #P<0.05 vs. CLP group at the same time point; ns, not significant vs. sham group at the same time point. $GdCl_3$, gadolinium chloride; CLP, cecal ligation and puncture; DAO, diamine oxidase; FD4, 4-kDa fluorescein isothiocyanate-dextran.

NF-κB was significantly increased in the CLP group compared with the sham group, but was reduced in the CLP + $GdCl_3$ group compared with the CLP group at 6, 12 and 24 h (P<0.05, Fig. 5A and B).

$GdCl_3$ alleviates apoptosis of intestinal tissue cells in CLP rats. Intestinal tissue cell death is also an important indicator of the integrity of the intestinal mechanical barrier (26). Western blot analysis was used to determine the expression of caspase-3 in rat intestinal tissue. The results indicated that the expression of caspase-3 (P<0.05; Fig. 6A and B) were significantly increased in the CLP group compared with the sham group. However, compared with the CLP group, the expression of caspase-3 (P<0.05; Fig. 6A and B) were lower in the CLP + $GdCl_3$ group at 6, 12 and 24 h. TUNEL assays were used to determine the apoptosis level of intestinal cells and apoptotic cells were stained brown and analyzed under a light microscope. A very small amount of brown stained cells was observed in the sham groups and sham $GdCl_3$+ groups (Fig. 7A). In the CLP group, the number of apoptotic cells increased significantly, and the number of expressions gradually increased over time (Fig. 7A). Pretreatment of septic rats with $GdCl_3$ could reduce the number of intestinal apoptotic epithelial cells of three different time points (Fig. 7A). The results indicated that the rate of apoptosis of intestinal cells (P<0.05; Fig. 7B) were significantly increased in the CLP group compared with the sham group. However, compared with the CLP group, the apoptotic rate of intestinal cells (P<0.05; Fig. 7B) were lower in the CLP + $GdCl_3$ group at 6, 12 and 24 h.

$GdCl_3$ has no effect on inflammation, intestinal mechanical barrier, or intestinal injury in non-CLP rats. An ELISA was used to determine the levels of serum and intestinal pro-inflammatory factors in rats. Levels of TNF-α, IL-6 and IL-1β in the serum and intestines of the sham + $GdCl_3$ group were similar to those in the sham group at 6, 12 and 24 h (Fig. 1). Western blot analysis was used to detect the expression of occludin and caspase-3 protein in the intestines. The results demonstrated that there was no difference in the expression of occludin or caspase-3 between the sham and the sham + $GdCl_3$ at any of the three time points (Fig. 8). Intestinal tissue apoptosis levels were determined using a TUNEL assay, and the results indicated no significant difference between the sham and the sham + $GdCl_3$ groups (Fig. 7). The results of a DAO ELISA, an indicator of intestinal damage and use of FD4, and an indicator of intestinal permeability, indicated that there were no differences in intestinal damage or permeability between the sham and the sham + $GdCl_3$ group at any of the three time-points (Fig. 2). H&E staining was used to verify that the sham + $GdCl_3$ treatment did not cause changes in the intestinal tissues of rats compared those in the with sham group (Fig. 3A). Based on Chiu's scoring standard for the degree of intestinal injury, the difference between the sham and the sham + $GdCl_3$ group at any of the three time-points was not statistically significant (Fig. 3B).

Discussion

The release of a large number of pro-inflammatory cytokines at an early stage of the inflammatory response is considered to be an important pathological mechanism for the development of sepsis, and the resulting intestinal tissue inflammation can cause destruction of intestinal barrier function (27). Inflammatory cytokines, including TNF-α and IL-1β, have been demonstrated to serve a role in this dysfunction (28). Intestinal barrier function damage in sepsis leads to an increase in intestinal permeability (29). This allows multiple antigens, bacteria and other toxic metabolites in the intestinal lumen to invade the intestinal tissue, causing further damage to the intestinal tract, aggravating the inflammatory response of the intestinal tissue and destroying the integrity of the intestinal epithelial barrier. This may progress to invasion of the lymphatic tissue and circulating blood, resulting in systemic inflammation (30). This creates a cycle that causes the eventual outcome of increased distal organ damage and risk of death (31,32). It is therefore hypothesized that the inhibition of intestinal inflammation may be an effective method for preventing intestinal barrier dysfunction in sepsis.

$GdCl_3$ acts to inhibit the phagocytosis and secretion of Kupffer cells, thereby alleviating the inflammatory response (33). Studies have also demonstrated that endotoxemia and excessive activation of Kupffer cells in numerous severe disease states (34). Inhibition of Kupffer cell function can ameliorate systemic inflammatory response syndrome (SIRS), while activation of Kupffer function can aggravate

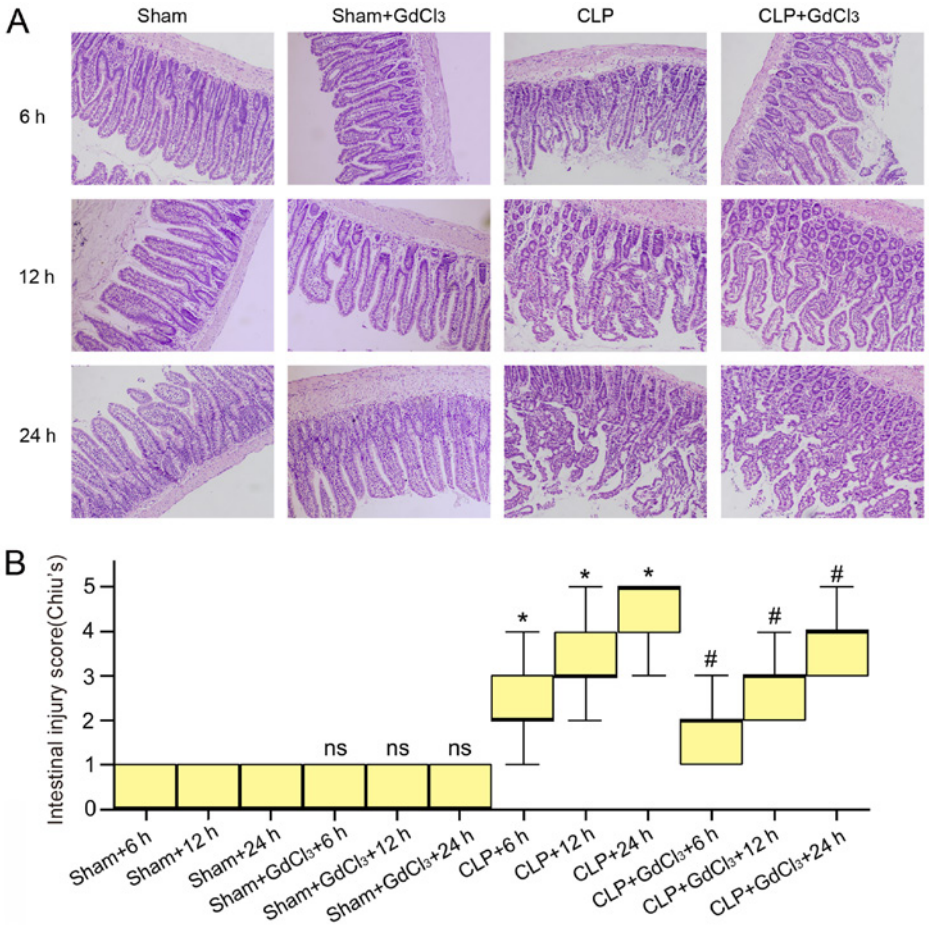

Figure 3. Effect of GdCl3 treatment on intestinal tissue damage in CLP-induced septic rats. (A) H&E staining was performed to evaluate intestinal histopathology. Magnification x200. (B) The degree of intestinal tissue damage was scored by Chiu's criteria. *P<0.05 vs. sham group at the same time point; #P<0.05 vs. CLP group at the same time point; ns, not significant vs. sham group at the same time point. $GdCl_3$, gadolinium chloride; CLP, cecal ligation and puncture; H&E, hematoxylin-eosin.

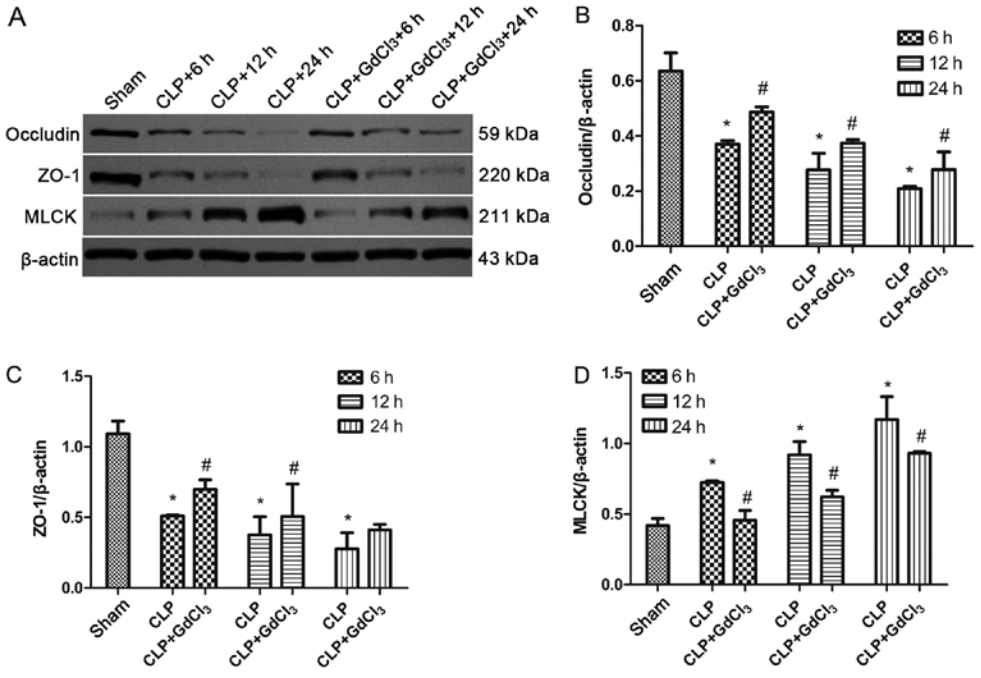

Figure 4. Effects of $GdCl_3$ treatment on tight junction proteins occludin and ZO-1 and on MLCK expression in intestinal tissues during CLP-induced sepsis. Protein expression in intestinal tissue was examined by western blotting. (A) Representative western blot images obtained at 6, 12 and 24 h after CLP operation. (B-D) Histograms of occludin, ZO-1, and MLCK protein expression levels. *P<0.05 vs. sham group at the same time point; #P<0.05 vs. CLP group at the same time point. $GdCl_3$, gadolinium chloride; CLP, cecal ligation and puncture; ZO-1, tight junction protein ZO-1; MLCK, myosin light chain kinase.

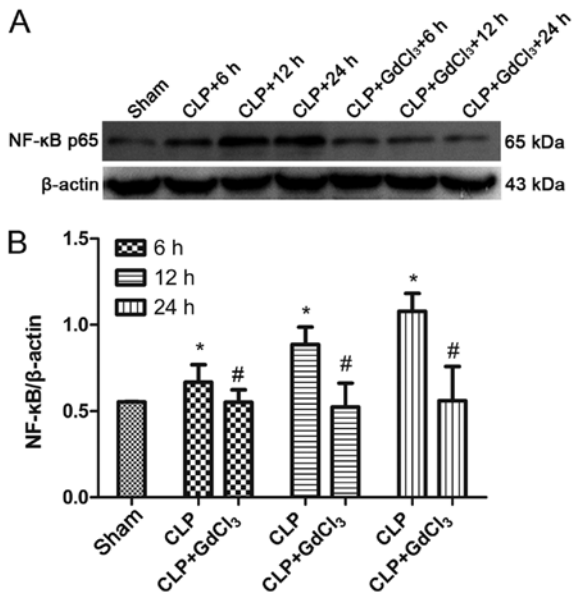

Figure 5. Effect of GdCl3 treatment on NF-κB expression in intestinal tissues of CLP-induced septic rats. Protein expression in intestinal tissues was examined by western blotting. (A) Representative western blot images for NF-κB obtained at 6, 12 and 24 h after CLP operation. (B) Histogram of NF-κB protein expression level. *P<0.05 vs. sham group at the same time point; #P<0.05 vs. CLP group at the same time point. $GdCl_3$, gadolinium chloride; CLP, cecal ligation and puncture.

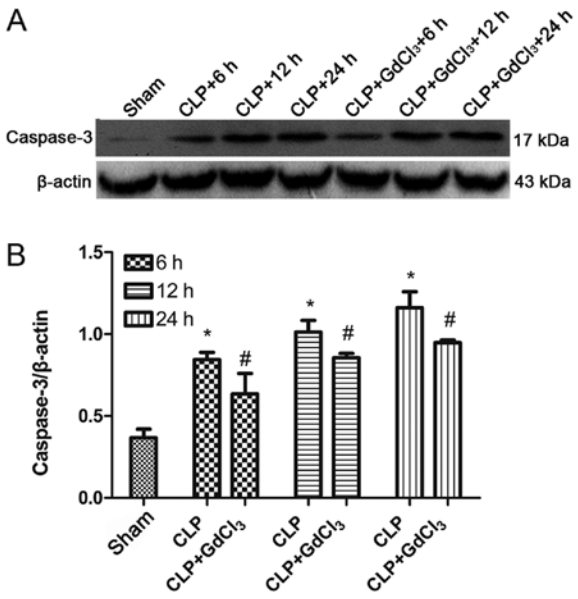

Figure 6. Effect of GdCl3 treatment on apoptosis protein caspase-3 in intestinal tissues of CLP-induced septic rats. Protein expression in intestinal tissues was examined by western blotting. (A) Representative western blot images for caspase-3 obtained at 6, 12 and 24 h after CLP operation. (B) Histogram of caspase-3 protein expression level. *P<0.05 vs. sham group at same time point; #P<0.05 vs. CLP group at the same time point. $GdCl_3$, gadolinium chloride; CLP, cecal ligation and puncture.

SIRS, thereby increasing the likelihood of multiple organ damage, including intestinal damage (35). Studies have confirmed that $GdCl_3$ pretreatment can reduce the apoptosis of lung parenchymal cells and lung inflammation, thereby reducing lung injury in LPS-induced sepsis (18). However, the effects of $GdCl_3$ pretreatment on the intestinal tract have rarely been reported.

The results of the present study indicated that in healthy rats, $GdCl_3$ had no effect on the inflammatory response, intestinal tight junction protein expression or intestinal cell apoptosis. In contrast, in the CLP-induced septic rats, expression of intestinal pro-inflammatory cytokines was reduced at 6 and 12 h by treatment with $GdCl_3$. At 24 h, the expression of TNF-α, IL-6 and IL-1β in the circulating blood of rats was not significantly different in CLP + GdCl3 rats compared to CLP rats, but levels in the intestinal tract were reduced in CLP + GdCl3 rats compared with the CLP group at 24 h. This finding indicated that localized inflammation is likely to have progressed into a systemic inflammatory response as the duration of sepsis was prolonged, at which point it could not be suppressed by the inhibition of Kupffer cells alone. These findings have some similarities with previous research (14). This study suggests that inactivation of Kupffer cells by $GdCl_3$ had no effect on inflammation and systemic inflammatory response following CLP-induced sepsis. However, there were some differences compared with the previous research. The previous experimental research was based on the experimental data obtained from blood sample of mice collected 8 h after the successful establishment of the CLP model, but we obtained the data from blood sample of rats collected at the 24 h time point (14). These differences may be associated with the rat species used. In sepsis, a large number of inflammatory cytokines, including TNF-α and IL-1β, can cause systemic and intestinal inflammatory reactions and activate NF-κB signaling pathways in intestinal tissues (36). Following the activation of NF-κB in the intestinal mucosa, and NF-κB can bind to inflammatory cytokine gene promoter sequences in immune cells to promote their expression (10). Western blot analysis was used to determine the expression of NF-κB p65. The results indicated that, at 6, 12 and 24 h, $GdCl_3$ treatment could inhibit the expression of NF-κB in intestinal tissues of septic rats. Taken together, the results of ELISAs and western blot analysis indicated that $GdCl_3$ could alleviate intestinal tissue inflammation in sepsis model rats and that this may be due to inhibition of NF-κB pathway activation.

FD4 is an indicator that is used to evaluate the function of the intestinal epithelial barrier. It cannot be absorbed in bowel lumen or degraded in the blood (4). In healthy animals, it is rarely able to enter the circulation through gaps between intestinal epithelial cells (37). Studies have confirmed that DAO in plasma is mainly derived from intestinal mucosal epithelial cells (38). DAO is released into the blood after intestinal mucosal cells are damaged or necrotic, which leads to an increase of DAO concentration in the circulation. DAO activity in peripheral blood is relatively stable (39). Accordingly, the degree of damage and integrity of the intestinal mucosal mechanical barrier can be indirectly determined by assessing the changes in DAO in peripheral blood (40). The results of the present study indicated that the levels of DAO and FD4 in CLP + $GdCl_3$ rats were reduced at each time point (6, 12, and 24 h) when compared with CLP model rats. This indicated an improvement in the intestinal barrier function of sepsis model rats treated with $GdCl_3$. Similar results were obtained using H&E staining of intestinal tissue and Chiu's score to evaluate the severity of intestinal injury.

The intestinal barrier is a selective barrier. The material in the intestinal lumen has two potential pathways through

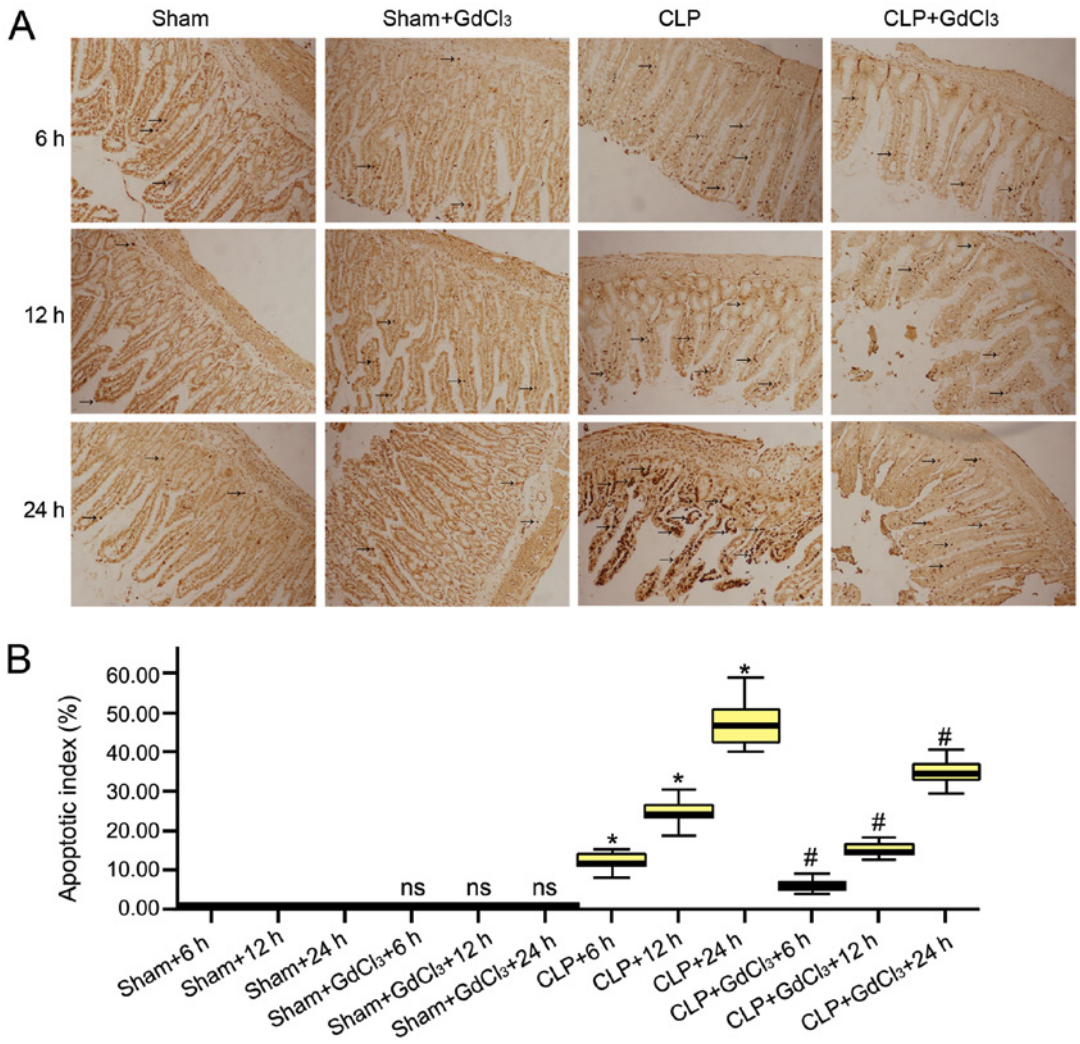

Figure 7. Effect of GdCl3 treatment on apoptosis of intestinal cells in CLP-induced septic rats. Intestinal tissue apoptosis was detected by TUNEL. (A) Cells in the nucleus stained brown were apoptotic cells (arrows). Magnification x200. (B) Apoptotic rate was calculated. *P<0.05 vs. sham group at the same time point; #P<0.05 vs. CLP group at the same time point; ns, not significant vs. sham group at the same time point. $GdCl_3$, gadolinium chloride; CLP, cecal ligation and puncture.

the intestinal mucosa: The transcellular pathway and the paracellular pathway (41,42). The intestinal paracellular pathway is largely regulated by tight junction proteins (43). Tight junctions are composed of occludin, claudins, ZO proteins and linked mature molecules. Among them, occludin and ZO-1 proteins are the most important. Studies have shown that sepsis can reduce the expression of ZO-1 and occludin in the intestinal epithelium (44). MLCK is a Ca^{2+}/calmodulin-dependent protein kinase that is part of an important signaling pathway in regulation of the function of tight junction proteins (42). Experiments have demonstrated that MLCK can also regulate the structure of tight junction proteins and affect the permeability of the intestinal mucosa by regulating the expression of occludin, claudins and Zos (42). The expression of MLCK is associated with the activation of NF-κB. After activation of NF-κB in the intestinal mucosa, it can bind to the MLCK gene promoter sequence in intestinal epithelial cells to promote the expression of MLCK (45). Previous studies have also indicated that inflammatory cytokines can disrupt tight junctions between epithelial cells by activating the NF-κB and MLCK pathways (46,47). The results of the present study suggested that the expression of ZO-1 and occludin was significantly upregulated in the intestinal tissues of septic rats treated with $GdCl_3$, while expression of MLCK was significantly downregulated. Taken together, with the result that expression of NF-κB in intestinal tissue is reduced by $GdCl_3$, the results indicated that $GdCl_3$ reduced the expression of MLCK through inhibition of the activation of NF-kB, which increased the expression of occludin and ZO-1, which served a role in protecting intestinal barrier function.

Intestinal mucosal barrier dysfunction is thought to be associated with excessive intestinal epithelial cell apoptosis, and apoptosis serves an important role in maintaining intestinal mucosal epithelial homeostasis (48). Apoptosis is a process of active cell death under the control of genes, which plays an important role in regulating the development of the body, maintaining the stability of the internal environment and ensuring normal physiological functions (49). If apoptosis is abnormal, that is, and the normal order of apoptosis is disrupted, it can cause a series of diseases. In recent years, it has been demonstrated that intestinal cell apoptosis serves an important role in diseases with impaired intestinal mucosal barrier (50). If cell apoptosis is dysregulated, it can cause intestinal mucosal atrophy, which

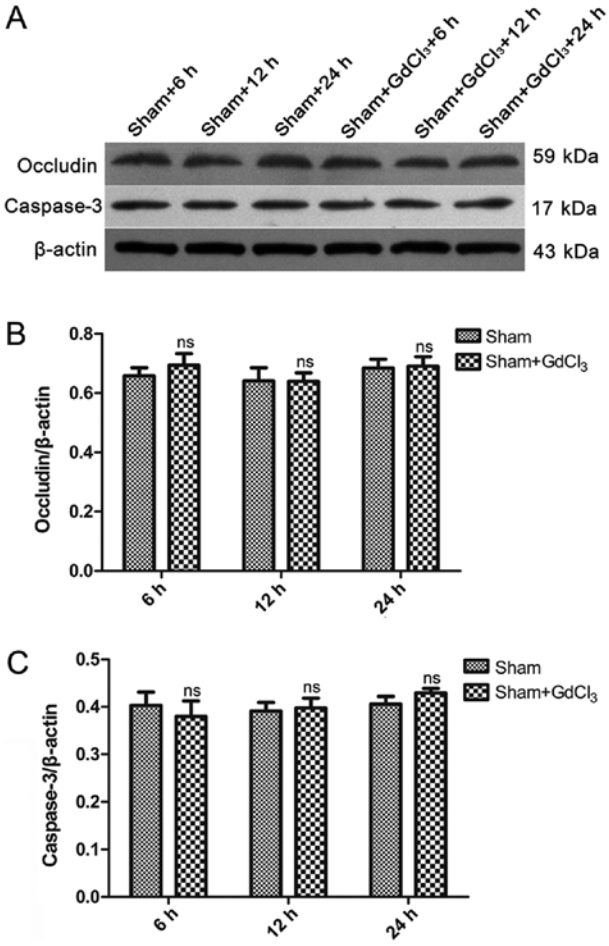

Figure 8. Effects of $GdCl_3$ on expression of intestinal occludin and caspase-3 in non-septic rats. Expression in intestinal tissues was examined by western blotting. (A) Representative western blot membrane images for occludin and caspase-3 obtained at 6, 12 and 24 h after sham operation. (B) Histogram of occludin expression levels. (C) Histogram of caspase-3 expression levels. ns, not significant compared to sham group at the same time point. $GdCl_3$, gadolinium chloride; CLP, cecal ligation and puncture.

leads to intestinal dysfunction (51). In animal models of sepsis, intestinal epithelial cell apoptosis is significantly elevated, and inhibition of this intestinal epithelial cell apoptosis can improve the survival rate of septic mice (52). Studies have demonstrated that the key to a series of cellular apoptosis-related reactions is the activation of caspase protease (53). Caspase-3 is the key to regulate apoptosis and serves a decisive role in the final stage of apoptosis, if caspase-3, which is also known as the 'death protease' is activated, apoptosis is inevitable (54,55). In the present study, apoptosis of intestinal cells was evaluated using a TUNEL assay and western blot analysis of caspase-3. The results indicated that the apoptotic rate of intestinal cells and expression of caspase-3 was decreased in CLP + $GdCl_3$ rats compared with CLP rats suggesting that $GdCl_3$ treatment reduces the apoptosis of intestinal tissue cells in septic rats, and that this effect may be associated with the inhibition of the caspase-3 expression.

In conclusion, the results of the present study suggested that $GdCl_3$ may alleviate the systemic and intestinal inflammatory response. However, there were no differences in cytokine or chemokine levels between $GdCl_3$-treated and $GdCl_3$-untreated septic rats at 24 h, suggesting that levels of pro-inflammatory factors in the circulation may not reflect the cytokine secretion levels of Kupffer cells. Studies have indicated that a protective effect of $GdCl_3$ on intestinal inflammatory injury may be achieved by inhibiting the production of pro-inflammatory cytokines in Kupffer cells or by inhibiting intestinal macrophages (20). The results of the present study indicated that $GdCl_3$ injection into the tail vein can ameliorate intestinal inflammation in rats. However, it is necessary to further clarify whether $GdCl_3$ functions by downregulating the release of pro-inflammatory cytokines from intestinal mucosal macrophages or from liver Kupffer cells. The results of the present study demonstrated that, the expression of tight junction proteins in the intestines was increased in CLP + $GdCl_3$ rats compared with CLP rats, and the apoptosis of intestinal cells was also decreased, thereby reducing the degree of intestinal damage. It is therefore hypothesized that the protective effect of $GdCl_3$ on intestinal barrier function in sepsis model rats may be due to a reduced intestinal inflammatory response and reduced expression of NF-κB. This may induce reduced expression of MLCK, which increases the expression of occludin and ZO-1 in the intestine. It is also hypothesized that the protective effect of $GdCl_3$ on intestinal barrier function in septic rats may be associated with the inhibition of caspase-3 overexpression.

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Availability of data and materials

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

Authors' contributions

YHZ performed all animal experiments and revised the manuscript. SWZ and YHZ were major contributors in writing the manuscript and performed the statistical analysis. WJZ, JTD and YHZ jointly designed the study. SWZ, HJZ, HYQ, YQZ, XLL, SL, HZ, JDW, ZYZ, HZW, MS and JL participated in and completed animal experiments. JZ and FW participated in and guided the statistical analysis. SWZ, FW and YHZ confirmed the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Shihezi University (Shihezi, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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