

Original Article

Effects of gadolinium chloride inhibition of liver Kupffer cells on intestinal mechanical barrier function following cecal ligation and puncture-induced sepsis in rats

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Abstract: *Objective:* The classical “gut-liver axis” theory expounds the relationship among intestinal barrier function, liver Kupffer cells, and systemic inflammatory response. The inflammatory response is closely related to the development and occurrence of sepsis. In this study, we investigated whether inhibition of Kupffer cells by gadolinium chloride (GdCl₃) can alleviate the systemic inflammatory response, thereby protecting the intestinal mucosal barrier function. *Methods:* In this study, sepsis was modeled by cecal ligation and puncture (CLP). Sprague-Dawley (SD) rats were randomly divided into four groups: Sham operation group, GdCl₃ pretreatment sham operation group, CLP group, and GdCl₃ pretreatment group. Rats in the two pretreatment groups were administered 5, 10, 20, or 40 mg/kg GdCl₃, respectively. Blood and intestinal tissue samples were taken from each group for biochemical and histopathological analyses. *Results:* The results showed that the pretreatment with 5 mg/kg GdCl₃ could improve the inflammatory response, intestinal tight junction protein expression, and intestinal tissue apoptosis in septic rats, although the effect was not obvious. With increasing dose, a relatively stable dose range appeared, with 10 and 20 mg/kg pretreatments showing no significant difference with regards to the reduction of intestinal damage in septic rats. However, when the dose reached 40 mg/kg, we observed aggravated intestinal damage. *Conclusions:* The inhibition of Kupffer cells by GdCl₃ had a protective effect on intestinal barrier function, which may be related to the reduction of inflammatory factors secreted by Kupffer cells. Moreover, this protective effect was GdCl₃ dose-dependent.

Keywords: Kupffer cells, intestine, mechanical barrier, sepsis, gadolinium chloride

Introduction

Sepsis is a common complication caused by severe infection, severe trauma, severe burns, shock, and surgery, which in severely affected patients can cause multiple organ dysfunction syndrome (MODS) [1]. According to epidemiological data, millions of individuals worldwide suffer from sepsis every year, more than a quarter of whom will die. Although modern medicine has made considerable progress with regards to anti-infective treatment, fluid resuscitation, and organ function support, the overall mortality rate of individuals with sepsis remains high, and it has become one of the most difficult diseases to overcome [2].

The intestine is the largest “bacteria and endotoxin library” in the human body and a prominent factor contributing to sepsis. The intestine is one of the target organs that are readily and most seriously damaged during sepsis. The intestinal barrier isolates most of these pathogenic substances but allows small amounts of endotoxin to enter the portal vein to maintain activation of the liver reticuloendothelial system [3]. However, the classic “gut-liver axis” theory states that when the body suffers severe trauma, burns, infections, and other significant assaults, the intestinal barrier will be destroyed, intestinal mucosal permeability will increase, and intestinal bacteria and endotoxins will be transferred to the liver. Liver Kupffer cells

release large amounts of cytokines and inflammatory factors into the blood when phagocytizing endotoxins. These inflammatory cytokines enter the corresponding target organs through the blood, and thereby cause damage to organ function. When they enter the intestine, they will further damage the intestinal barrier function, which in turn will aggravate bacterial translocation and increase endotoxin production, thereby aggravating the systemic inflammatory response syndrome (SIRS), which eventually leads to the occurrence of MODS, and death [4, 5].

Kupffer cells are liver-specific macrophages, which have the dual function of purifying endotoxins and secreting large numbers of active substances, including cytokines and inflammatory mediators, in response to activation by endotoxins [6-8]. In recent years, studies have found that these cells are over-activated in a variety of severe disease states. Although the inhibition of Kupffer cell function can alleviate liver damage and SIRS, it can also increase organ damage and the systemic inflammatory response [9, 10]. Gadolinium Chloride ($GdCl_3$) is a lanthanide commonly used to evaluate the function of Kupffer cells. Given that $GdCl_3$ can selectively inhibit the phagocytotic and secretory functions of Kupffer cells, it is widely used as a tool for studying pathogenesis of the mononuclear/macrophage system [11]. Previous studies have shown that, in the early stage of endotoxemia, the $TNF-\alpha$ and IL-6 released by Kupffer cells may play an important role in the initiation and progression of ileal mucosal damage [12, 13]. Studies have confirmed that $GdCl_3$ pretreatment can reduce the apoptosis of lung parenchymal cells and lung inflammation, thereby reducing lung injury in lipopolysaccharide-induced sepsis [14-16]. However, the effects of Kupffer cell inhibition on the intestinal mechanical barrier have rarely been reported.

On the basis of the aforementioned studies, we designed the present study with the aim of investigating the effects of $GdCl_3$ inhibition of Kupffer cells on systemic and intestinal cytokines (including $TNF-\alpha$, IL-6, and IL-1 β), and the protective effect on intestinal mechanical barrier function in cecal ligation and puncture (CLP)-induced sepsis. The functions of the intestinal mechanical barrier were assessed with respect to expression levels of the intesti-

nal tight junction proteins occludin and ZO-1, as well as the apoptotic level of intestinal tissue cells.

Materials and methods

Animals and animal model

Male Sprague-Dawley (SD) rats weighing 200-250 g (age 8-10 weeks) were used in this study. All rats were purchased from Xinjiang Medical University, the Xinjiang Uygur Autonomous Region, China (experimental animal production license no. XJYK0011, 2011). Animals were housed under temperature- ($20 \pm 1^\circ C$) and light cycle (12 h)-controlled conditions and given 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 implemented in accordance with the Animal Management Regulations of the Ministry of Health of China.

Sepsis was modeled by cecal ligation and puncture (CLP). Briefly, under intraperitoneal anesthesia induced by 1% pentobarbital (30 mg/kg; Merck KGaA, Darmstadt, Germany), a midline incision of approximately 2 cm was made in the anterior abdomen. The cecum was carefully isolated and approximately two-thirds of the cecum was ligated using a 4-0 silk suture. The cecum was punctured twice in different places using a 21 needle and was squeezed to extrude the fecal material from the wounds. Finally, the cecum was re-positioned and the abdomen was sutured. The 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 but given access to water for 12 h prior to commencing the experiment. They were randomly divided into four groups: Sham operation group (Sham group, $n = 6$), $GdCl_3$ pretreatment sham operation group (Sham + $GdCl_3$, $n = 24$), CLP group (CLP group, $n = 6$), and $GdCl_3$ pretreatment CLP group (CLP + $GdCl_3$ group, $n = 24$). Rats in the two pretreatment groups were administered 5, 10, 20, or 40 mg/kg $GdCl_3$ (no. 203289-1G, Sigma-Aldrich, St. Louis, MO, USA), respectively, via tail vein injection at 1 and 2 days before the operation, whereas the sham and CLP groups

were administered the same amount of normal saline in the same way. Following successful modeling, the animals were sacrificed after 12 h, and blood samples were taken from the abdominal aorta and intestinal tissue samples were preserved for subsequent analysis.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of tumor necrosis factor (TNF)- α , interleukin (IL)-6 and IL-1 β from serum or the supernatant of intestinal tissue homogenates were assessed using ELISA kits (E-EL-R0019c, E-EL-R0015c, E-EL-R0012c Elabscience, Wuhan, China) according to the manufacturer's instructions. Concentrations of serum diamine oxidase (DAO) were measured using a DAO ELISA kit (E-EL-R0331c, Elabscience).

Western blotting

Total protein was extracted from the colon of rats in each group. Equal amounts of proteins from each sample were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The electrophoretically separated proteins were subsequently transferred to PVDF membranes. After blocking with 5% skimmed milk, the membranes were incubated with a primary antibody or β -actin (1:1000, no. TA-09, ZSGB-BIO, Beijing, China) overnight at 4°C. After washing, the membranes were incubated with HRP-conjugated secondary antibody (1:2000, goat anti-rabbit ZF-0311, goat anti-mouse ZF-0312, ZSGB-BIO, Beijing, China) at 37°C for 90 min. Proteins were detected using a chemiluminescent system and visualized using a gel imaging system (ChemiDoc Touch, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The results were analyzed using intensity quantification software (Image Lab 5.2, Bio-Rad Laboratories, Inc.). The primary antibodies included anti-occludin (1:1000, no. ab216327, Abcam, Cambridge, UK), anti-ZO-1 (1:500, no. sc-33725, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-NF- κ B (1:1000, no. #8242, Cell Signaling Technology, Danvers, MA, USA), and anti-caspase-3 (1:500, no. ab13847, Abcam).

Intestinal permeability assay

Gavage injection of 600 mg/kg (125 mg/mL) 4-kD fluorescein isothiocyanate (FITC)-dextran (FD4; Sigma-Aldrich) was administered approximately 6 h prior to sacrifice. Blood samples

were centrifuged at 12,000 \times g for 15 min at 4°C, and the supernatant fluid was collected. A full-wavelength scanning multifunction reader (multifunctional enzyme labeling instrument) (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA), operating at an excitation wavelength of 480 nm and emission wavelength of 520 nm, was used to analyze fluorescence. Standard curves of FITC-dextran were obtained using a concentration range of FITC-dextran solutions prepared by sequential dilution with PBS (0-12.5 mg/mL).

Immunohistochemistry

Paraffin sections were placed in fresh xylene and gradient ethanol for dewaxing and hydration. After antigen retrieval using 0.01 M sodium citrate, the peroxidase was quenched with 3% H₂O₂. After blocking the sections with 10% normal goat serum for 30 min at room temperature, a primary antibody was added dropwise and incubated at 37°C for 60 min. Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG polymer (ZSGB-BIO, Beijing, China) formed an immune complex after 20 min of reaction with the primary antibody bound to the tissue sheet at room temperature. Thereafter, 3,3'-diaminobenzidine (DAB) was added dropwise to the sections for several seconds. The sections were rinsed with tap water and stained with hematoxylin for approximately 20 s. Finally, the sections were dehydrated, cleared, mounted, and observed under a microscope at \times 200 magnification. The primary antibodies used were occludin (1:200; Abcam) and ZO-1 (1:500; Santa Cruz).

Intestinal epithelial apoptosis

The intestinal tissue was fixed and paraffin sections were prepared. A TUNEL Apoptosis Assay Kit (Sigma-Aldrich) was used for analysis according to the manufacturer's instructions. After dewaxing, hydration and cell permeabilization, TUNEL reaction solution, converter-POD, and DAB (ZSGB-BIO) were sequentially added dropwise. Finally, tissue preparations were observed under an optical microscope and photographed.

Intestinal histopathology and damage index

Tissues were fixed with 4% paraformaldehyde at 4°C for more than 24 h, and then embedded using paraffin and serially sectioned (5 μ m).

Table 1. Effect of GdCl₃ pretreatment on TNF- α , IL-6, and IL-1 β expression in the serum following CLP-induced sepsis

Group (n = 6)	TNF- α (pg/ml)	IL-6 (pg/ml)	IL-1 β (pg/ml)
Sham	263.78 \pm 85.40	828.91 \pm 31.38	49.68 \pm 11.07
Sham + GdCl ₃ (5 mg/kg)	296.92 \pm 27.21	877.91 \pm 59.37	50.34 \pm 9.77
Sham + GdCl ₃ (10 mg/kg)	287.88 \pm 26.54	841.45 \pm 82.07	50.24 \pm 7.41
Sham + GdCl ₃ (20 mg/kg)	276.56 \pm 61.44	795.75 \pm 31.84	49.46 \pm 8.75
Sham + GdCl ₃ (40 mg/kg)	476.77 \pm 4.96*	1008.75 \pm 33.66*	62.08 \pm 1.74
CLP	622.43 \pm 10.73*	2663.69 \pm 66.86*	146.93 \pm 5.34*
CLP + GdCl ₃ (5 mg/kg)	583.24 \pm 8.04†	2571.7 \pm 55.64	134.72 \pm 8.2
CLP + GdCl ₃ (10 mg/kg)	512.22 \pm 6.24†	2330.68 \pm 66.99†	107.38 \pm 2.59†
CLP + GdCl ₃ (20 mg/kg)	515.16 \pm 7.51†‡	2169.29 \pm 59.56†‡	101.77 \pm 0.59†‡
CLP + GdCl ₃ (40 mg/kg)	810.54 \pm 10.29†	3195.22 \pm 79.96†	146.23 \pm 2.58

The levels of TNF- α , IL-6, and IL-1 β in the serum of rats in each group were determined by ELISA. The data were obtained at 12 h after operation and are presented as the means \pm SD (n = 6). *P<0.05 compared with the sham group. †P<0.05 compared with the CLP group. ‡P>0.05 compared with the CLP + GdCl₃ (10 mg/kg) group. Abbreviations: GdCl₃, gadolinium chloride; CLP, cecal ligation and puncture; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; IL-1 β , interleukin-1 β ; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation.

The slides were stained with hematoxylin and eosin (HE), and the sections were examined under a DP microscope (Olympus, Tokyo, Japan) at \times 200 magnification. Intestinal injuries were assessed using the Chiu scoring system [17].

Statistical analyses

Data analysis was performed using SPSS 21.0 statistical software. The count data was analyzed using the rank sum test. For measurement data, if the data were conformed to a normal distribution, the mean \pm standard deviation were calculated, and subjected to one-way ANOVA for comparison of multiple sets. If the data were not normally distributed, median (interquartile range) values were used for analysis, and the Kruskal-Wallis nonparametric test was used to compare the differences between groups. P<0.05 was considered statistically significant.

Results

Effects of GdCl₃ inhibition of Kupffer cells on systemic and intestinal inflammatory factors following CLP-induced sepsis in rats

To verify the effect of GdCl₃ pretreatment on systemic inflammation and intestinal inflammatory response in septic rats, we used ELISA to detect TNF- α , IL-6, and IL-1 β in rat serum and intestinal tissues. The results showed that the expression of TNF- α , IL-6, and IL-1 β in the blood

circulation and intestinal tissues of the CLP group was significantly increased compared with that in the sham group (P<0.05, **Tables 1** and **2**). In the pre-treatment sham group, the administration of 5, 10, and 20 mg/kg GdCl₃ had no significant effect on the expression of TNF- α , IL-6, and IL-1 β in blood circulation and intestinal tissues compared with the sham group (P>0.05, **Tables 1** and **2**). However, when

the dose was increased to 40 mg/kg, expression of TNF- α , IL-6, and IL-1 β in the blood circulation and intestinal tissues of rats increased, and levels were significantly higher compared with those in the sham group (P<0.05, **Tables 1** and **2**). After pretreatment with GdCl₃, pro-inflammatory factors in the systemic circulation and intestinal tissues of septic rats gradually decreased with increasing doses. There was no significant difference in the expression of IL-6 and IL-1 β in the blood circulation of rats in the 5 mg/kg GdCl₃ pretreated CLP group and the CLP group (P>0.05, **Table 1**). However, significant differences in the expression of proinflammatory factors in blood circulation and intestinal tissues were observed between rats pretreated with 10 and 20 mg/kg GdCl₃ and those in the CLP group (P<0.05, **Tables 1** and **2**). In contrast, there was no significant difference in the expression of these three inflammatory factors in the blood circulation and intestinal tissues between the 10 mg/kg pretreated CLP group and the 20 mg/kg pretreated CLP group (P>0.05, **Tables 1** and **2**), whereas administration of 40 mg/kg GdCl₃ increased the expression of TNF- α , IL-6, and IL-1 β in septic rats (P<0.05, **Tables 1** and **2**).

Effects of GdCl₃ inhibition of Kupffer cells on intestinal permeability and degree of intestinal damage following CLP-induced sepsis in rats

DAO can reflect the integrity and damage of the intestinal mechanical barrier, and in the pres-

Table 2. Effect of GdCl₃ pretreatment on TNF-α, IL-6, and IL-1β in the intestinal tissues following CLP-induced sepsis

Group (n = 6)	TNF-α (pg/ml)	IL-6 (pg/ml)	IL-1β (pg/ml)
Sham	628.01 ± 23.20	1432.87 ± 21.35	66.04 ± 3.14
Sham + GdCl ₃ (5 mg/kg)	638.26 ± 61.20	1464.2 ± 52.57	66.17 ± 3.43
Sham + GdCl ₃ (10 mg/kg)	642.74 ± 52.65	1434.31 ± 46.65	66.17 ± 3.37
Sham + GdCl ₃ (20 mg/kg)	633.87 ± 54.58	1423.01 ± 51.64	66.91 ± 1.9
Sham + GdCl ₃ (40 mg/kg)	706.09 ± 10.60*	1894.62 ± 48.68*	105 ± 1.46*
CLP	1528.21 ± 14.25*	3811.83 ± 77.16*	208.24 ± 14.47*
CLP + GdCl ₃ (5 mg/kg)	1400.36 ± 13.43†	3185.08 ± 70.46†	180.95 ± 1.24
CLP + GdCl ₃ (10 mg/kg)	1194.44 ± 16.61†	2912.95 ± 47.05†	155.94 ± 2.99†
CLP + GdCl ₃ (20 mg/kg)	1175.23 ± 5.69†‡	2774.03 ± 92.3†‡	154.07 ± 2.9†‡
CLP + GdCl ₃ (40 mg/kg)	1721.64 ± 17.06†	4259.06 ± 171.24†	191.26 ± 2.79

The levels of TNF-α, IL-6, and IL-1β in intestinal tissues of rats in each group were determined by ELISA. The data were obtained at 12 h after operation and are presented as the means ± SD (n = 6). *P<0.05 compared with the sham group. †P<0.05 compared with the CLP group. ‡P>0.05 compared with the CLP + GdCl₃ (10 mg/kg) group. Abbreviations: GdCl₃, gadolinium chloride; CLP, cecal ligation and puncture; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-1β, interleukin-1β; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation.

Table 3. Effect of GdCl₃ pretreatment on DAO and FD4 in the serum following CLP-induced sepsis

Group (n = 6)	DAO (ng/ml)	FD4 (ng/ml)
Sham	30.87 ± 5.05	105.53 ± 8.39
Sham + GdCl ₃ (5 mg/kg)	27.16 ± 1.98	108.62 ± 7.63
Sham + GdCl ₃ (10 mg/kg)	29.82 ± 5.65	106.55 ± 8.44
Sham + GdCl ₃ (20 mg/kg)	25.66 ± 3.37	107.07 ± 8.78
Sham + GdCl ₃ (40 mg/kg)	34.25 ± 5.78	120.22 ± 1.17
CLP	152.25 ± 1.21*	321.35 ± 2.85*
CLP + GdCl ₃ (5 mg/kg)	129.35 ± 0.58†	292.33 ± 2.29†
CLP + GdCl ₃ (10 mg/kg)	129.08 ± 0.93†‡	262.62 ± 2.81†
CLP + GdCl ₃ (20 mg/kg)	128.04 ± 1.14†‡§	257.04 ± 2.78†‡
CLP + GdCl ₃ (40 mg/kg)	166.11 ± 2.96†	335.76 ± 3.46†

The levels of DAO and FD4 in the serum of rats in each group were determined by ELISA and a full-wavelength scanning multifunction reader (multifunctional enzyme labelling instrument), respectively. The data were obtained at 12h after operation and are presented as the means ± SD (n = 6). *P<0.05 compared with the sham group. †P<0.05 compared with the CLP group. ‡P>0.05 compared with the CLP + GdCl₃ (10 mg/kg) group. §P>0.05 compared with the CLP + GdCl₃ (5 mg/kg) group. Abbreviations: GdCl₃, gadolinium chloride; CLP, cecal ligation and puncture; DAO, diamine oxidase; FD4, 4-kD fluorescein isothiocyanate (FITC)-dextran; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation.

ent study, we used ELISA to detect the expression of DAO in rat serum. The results showed that the expression of DAO was significantly higher in the CLP group than in the sham group. The expression of DAO in the CLP group pretreated with 5, 10, and 20 mg/kg GdCl₃ was significantly lower than that in the CLP group (P<0.05, **Table 3**); however, there was no statis-

tically significant differences in DAO expression among the rats receiving these three pretreatments (P>0.05, **Table 3**). The expression level was, nevertheless, significantly increased in the 40 mg/kg pretreatment group. In order to more objectively evaluate the extent of intestinal damage, we also performed HE staining of the intestinal tissue, and scored intestinal tract damage according to the Chiu scoring standard. The results showed that the degree of intestinal tissue damage in the CLP group pretreated with 5, 10, and 20 mg/kg GdCl₃ was less pronounced than that in the CLP group (P<0.05, **Figure 1A**), and the intestinal injury score was lower than that in the CLP group (P<0.05, **Figure 1B**). In order to evaluate the intestinal permeability of rats in each group, we used FD4 as an index. The experimental results showed that the intestinal permeability in CLP group rats pretreated with 5, 10, and 20 mg/kg GdCl₃ was lower than that in CLP group rats (P<0.05, **Table 3**). Interestingly, there was no significant difference between pretreatments with 10 and 20 mg/kg GdCl₃ in the protection of intestinal tissue injury in rats suffering from sepsis (P>0.05, **Table 3**, **Figure 1**). In contrast, however, the degree of intestinal damage and intestinal permeability of rats in the pretreatment CLP group administered

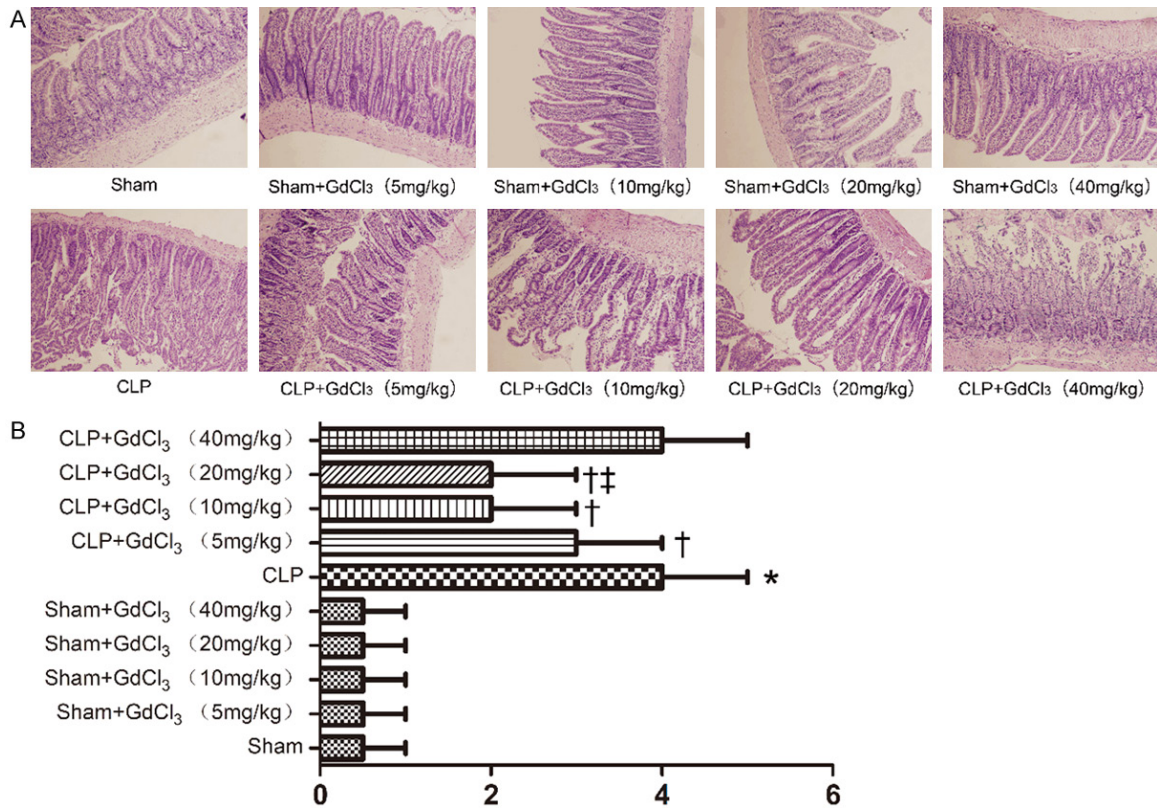


Figure 1. Effect of GdCl₃ pretreatment on intestinal tissue damage in CLP-induced septic rats. HE staining was performed on intestinal histopathology in each group (A), and the degree of intestinal tissue damage was scored using Chiu's scale (B). The magnification of the HE staining image for each group is $\times 200$. * $P < 0.05$ compared with the sham group. † $P < 0.05$ compared to with the CLP group. * $P > 0.05$ compared with the CLP + GdCl₃ (10 mg/kg) group. Abbreviations: GdCl₃, gadolinium chloride; CLP, cecal ligation and puncture; HE, hematoxylin-eosin.

with 40 mg/kg GdCl₃ were aggravated to an even greater extent than those in the CLP group ($P < 0.05$, Table 3, Figure 1).

Effects of GdCl₃ inhibition of Kupffer cells on the expression of occludin and ZO-1 in intestinal tissues following CLP-induced sepsis in rats

The levels of intestinal tissue occludin and ZO-1 proteins can reflect the integrity of the intestinal mechanical barrier, and in the present study, we used western blotting and immunohistochemical analyses to detect their expression. The results showed that the expression levels of occludin and ZO-1 proteins were significantly decreased in the CLP group compared with the sham group ($P < 0.05$, Figure 2A-C). There were significant increases in the expression of occludin and ZO-1 in the CLP group pretreated with 5, 10, and 20 mg/kg GdCl₃ compared with those in the CLP group ($P < 0.05$,

Figure 2A-C), whereas in contrast, the expression of occludin and ZO-1 in the CLP group pretreated with 40 mg/kg GdCl₃ decreased significantly. Moreover, the expression levels of occludin (Figure 3A) and ZO-1 (Figure 3B) detected immunohistochemically were similar to those determined by western blotting.

Effects of GdCl₃ inhibition of Kupffer cells on intestinal tissues apoptosis following CLP-induced sepsis in rats

The growth state of intestinal tissue cells is also an important index that can be used to evaluate the integrity of the intestinal mechanical barrier, and therefore we performed western blotting to detect the expression of caspase-3 in the intestinal tissue of rats, and TUNEL to detect the apoptosis of intestinal tissue cells. The results showed that the expression of caspase-3 ($P < 0.05$, Figure 4A, 4B) and the apoptosis rate of cells ($P < 0.05$, Figure 5A,

A

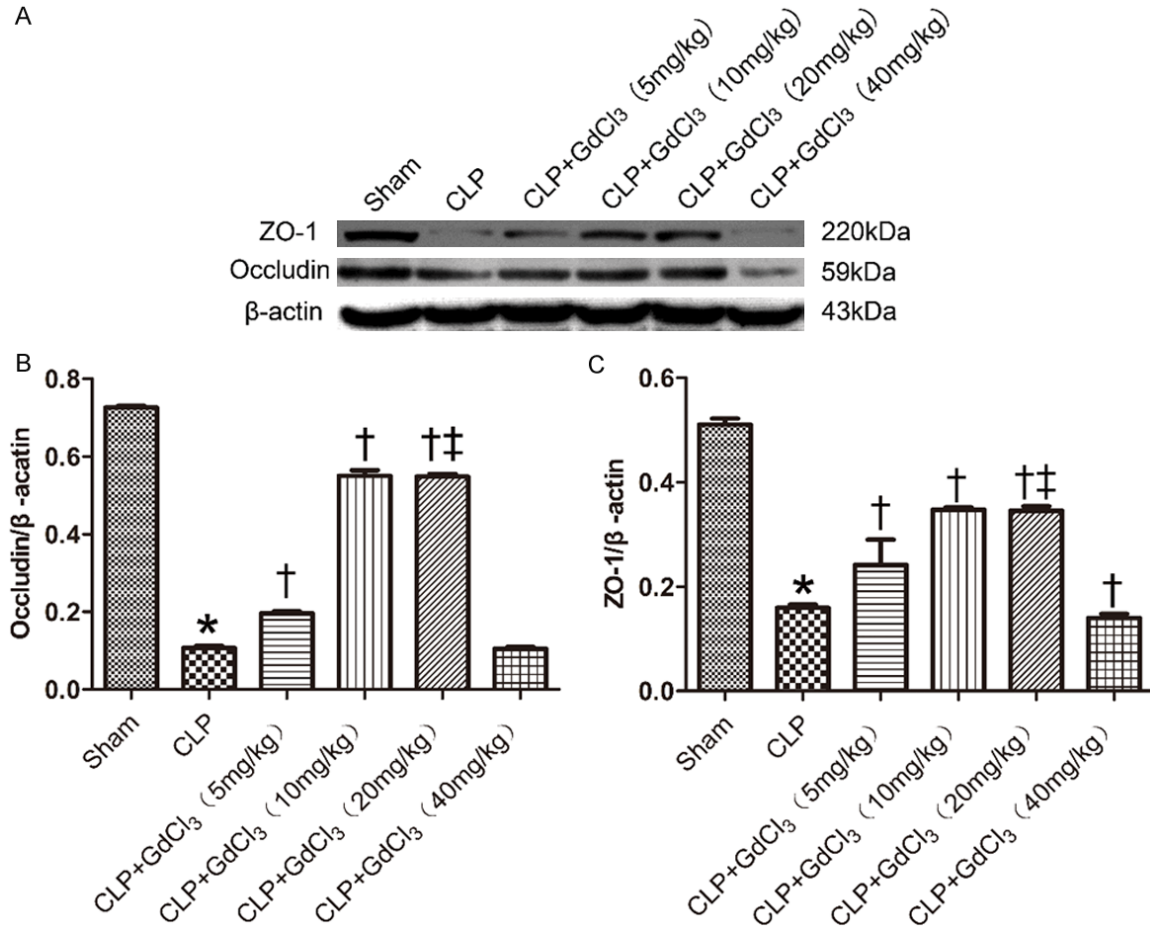


Figure 2. Effect of GdCl₃ pretreatment on occludin and ZO-1 protein expression in intestinal tissues during CLP-induced sepsis. The expression of these proteins was examined by western blotting. A. Representative western blot images for these proteins obtained 12 h after operation. B. A histogram of occludin protein. C. A histogram of ZO-1 protein. *P<0.05 compared with the sham group. †P<0.05 compared with the CLP group. ††P>0.05 compared with the CLP + GdCl₃ (10 mg/kg) group. Abbreviations: GdCl₃, gadolinium chloride; CLP, cecal ligation and puncture; ZO-1, zonula occludens 1.

5B) were significantly higher in the CLP group than in the sham group. However, the expression of caspase-3 and the apoptotic rate of cells were decreased in the CLP group pretreated with 5, 10, and 20 mg/kg GdCl₃ compared with the CLP group. In rats pretreated with 40 mg/kg, there were increases in the expression level of caspase-3 and the apoptotic rate of intestinal tissue cells. Interestingly, there was no significant difference in intestinal tissue apoptosis between the CLP group pretreated with 5 mg/kg GdCl₃ and the CLP group (P>0.05, **Figure 5A, 5B**). Similarly, no significant difference was detected in the expression of caspase-3 and apoptotic rate between the 10 and 20 mg/kg GdCl₃ pretreated CLP groups (P>0.05, **Figures 4, 5**).

Effects of GdCl₃ inhibition of Kupffer cells on the expression of NF-κB protein in intestinal tissues following CLP-induced sepsis in rats

To verify whether regulation of the intestinal inflammatory response is related to the NF-κB pathway, we performed western blotting to determine the expression of NF-κB protein in rat intestines. The results showed that the expression of NF-κB protein was significantly increased in the CLP group compared with the sham group (P<0.05, **Figure 6A, 6B**). Furthermore, compared with the CLP group, there was a decrease in the expression of NF-κB protein in the CLP group treated with 5, 10, and 20 mg/kg GdCl₃ (P<0.05, **Figure 6**). Interestingly, the expression level NF-κB protein increased again in response to pretreatment with 40 mg/

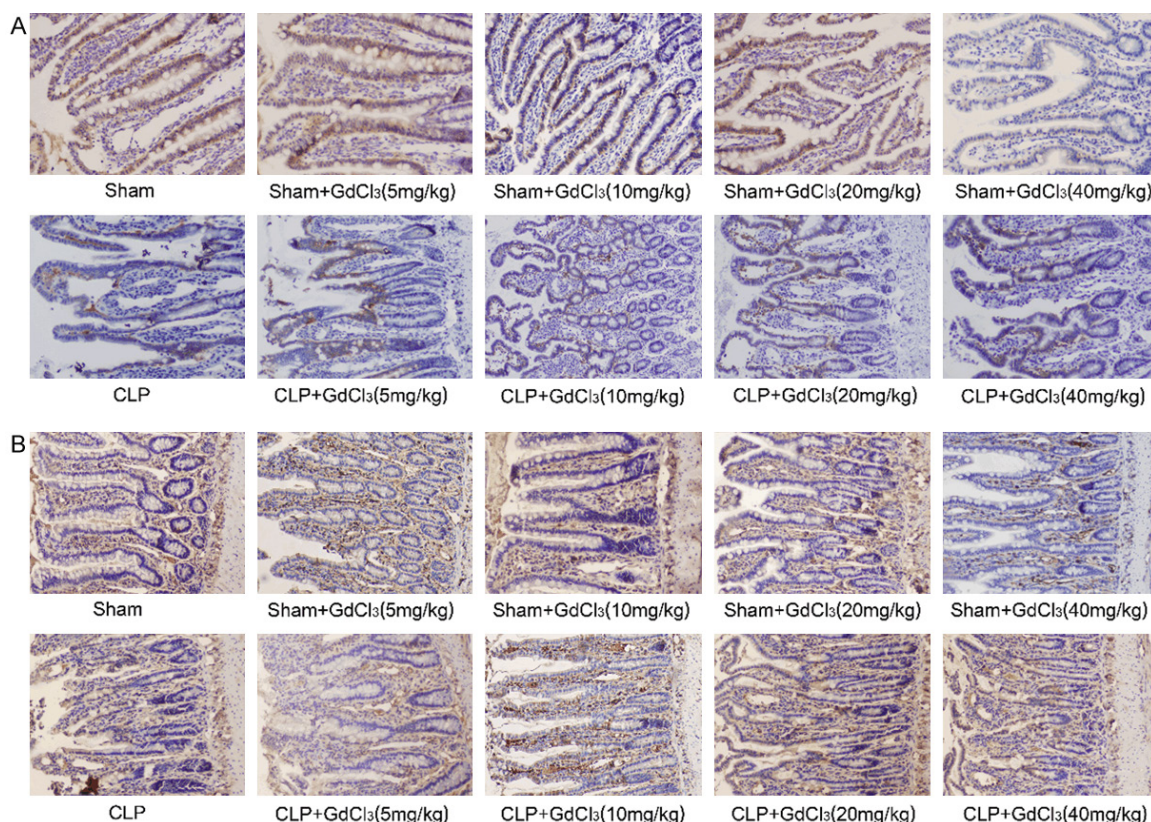


Figure 3. Effect of $GdCl_3$ pretreatment on the expression levels of intestinal occludin and ZO-1 proteins detected immunohistochemically during CLP-induced sepsis. The levels of (A) Occludin and (B) ZO-1 in the intestine at 12 h after operation. Magnification $\times 200$. Abbreviations: $GdCl_3$, gadolinium chloride; CLP, cecal ligation and puncture; ZO-1, zonula occludens 1.

kg $GdCl_3$. There was, however, no significant difference in the expression of NF- κ B between the 10 and 20 mg/kg groups ($P>0.05$, **Figure 6**).

Discussion

With the accumulation of in-depth studies of critical illnesses, the importance of gastrointestinal lesions in critical care has become increasingly recognized, and such studies indicate that the gastrointestinal tract is the trigger and initiator of SIRS and the central organ of MODS [18, 19].

In 1998, Marshall creatively proposed the concept of the gut-liver axis, which provided an important theoretical basis for revealing the relationship between these two organs [20, 21]. When the intestinal barrier function is impaired due to external factors, there follows a translocation of intestinal bacteria, and these bacteria, along with other harmful agents such as endotoxins, will invade the portal system

and activate liver Kupffer cells. If the inactivation of bacteria and endotoxins by the Kupffer cells is insufficient, systemic infections are induced, causing a systemic inflammatory response, whereas if the Kupffer cells are over-activated, a large number of inflammatory factors are released in the body, including TNF- α , IL-6, and IL-1 β . In either case, damage may occur to the intestinal mucosa and distal organ tissues [22-24]. This pattern of activation suggests the hypothesis that an appropriate inhibition of Kupffer cell function could reduce intestinal damage during sepsis.

In the early stage of sepsis, the release of a diverse range of pro-inflammatory cytokines is generally considered to be an important pathological mechanism for the development of sepsis. Intestinal tissue inflammation can cause damage to the intestinal epithelial barrier function, and the relationship between the damaging effects of inflammatory cytokines such as TNF- α and IL-1 β on the tight junctions between

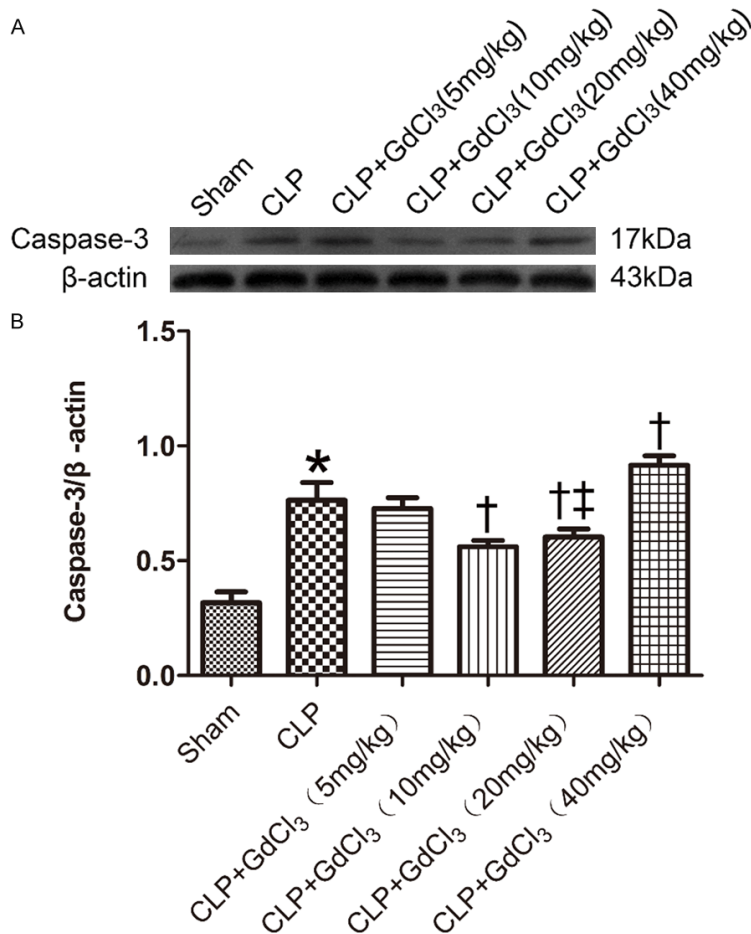


Figure 4. Effect of GdCl₃ pretreatment on the apoptosis-related protein caspase-3 in intestinal tissue of CLP-induced septic rats. The expression of caspase-3 in intestinal tissues was examined by western blotting. (A) Representative western blot images for caspase-3 obtained 12 h after operation. (B). A histogram of caspase-3 protein expression level. * $P < 0.05$ compared with the sham group. † $P < 0.05$ compared with the CLP group. ‡ $P > 0.05$ compared with the CLP + GdCl₃ (10 mg/kg) group. Abbreviations: GdCl₃, gadolinium chloride; CLP, cecal ligation and puncture.

intestinal epithelial cells and intestinal barrier function have been confirmed [25, 26]. Therefore, inhibiting intestinal inflammatory responses may be an effective method for preventing intestinal barrier dysfunction in sepsis. Studies have confirmed that GdCl₃ can inhibit the phagocytotic and secretory activity of Kupffer cells, thereby reducing the inflammatory response. Therefore, GdCl₃ has been used in various animal models of experimental diseases, including lipopolysaccharide (LPS)-induced endotoxemia and CLP-induced sepsis. Studies have confirmed that GdCl₃ pretreatment can reduce the apoptosis of lung parenchymal cells and lung inflammation, reducing lung injury in LPS-induced sepsis [16]. In the present study,

rats were pretreated with different doses of GdCl₃, and we found that 5, 10, and 20 mg/kg GdCl₃ reduced the release of TNF- α , IL-6, and IL-1 β from systemic and intestinal sources. Interestingly, when the dose was increased to 40 mg/kg, the release of pro-inflammatory factors increased. This may be related to the necrosis of Kupffer cells caused by a high dosage of GdCl₃, and thus these cells are unable to phagocytize bacteria and endotoxins, thereby resulting in infection and inflammation. The results showed that inhibition of Kupffer cells with GdCl₃ attenuated the systemic inflammatory response and intestinal inflammation; however, the degree of inhibition was GdCl₃ dose-dependent.

In animal studies, it has been shown that when the intestinal epithelial barrier is impaired, Fitc-Dextran more readily enters interstitial spaces and blood circulation through the gaps between epithelial cells, resulting in a significant increase in plasma Fitc-Dextran content [27]. Studies have confirmed that DAO in plasma is mainly derived from intestinal mucosal epithelial cells, and that the activity of DAO in peri-

pheral blood is relatively stable. 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 [28]. The results of the present study indicated that DAO and FD4 values in pretreated CLP rats were decreased in responses to the administration of different doses of GdCl₃ (5, 10, and 20 mg/kg). We also obtained comparable results based on HE staining of intestinal tissue and Chiu's score of intestinal tissue damage. These findings indicate that treatment with GdCl₃ can enhance intestinal barrier function in septic rats.

In order to observe more specifically the effects of GdCl₃ inhibition of Kupffer cells on the intes-

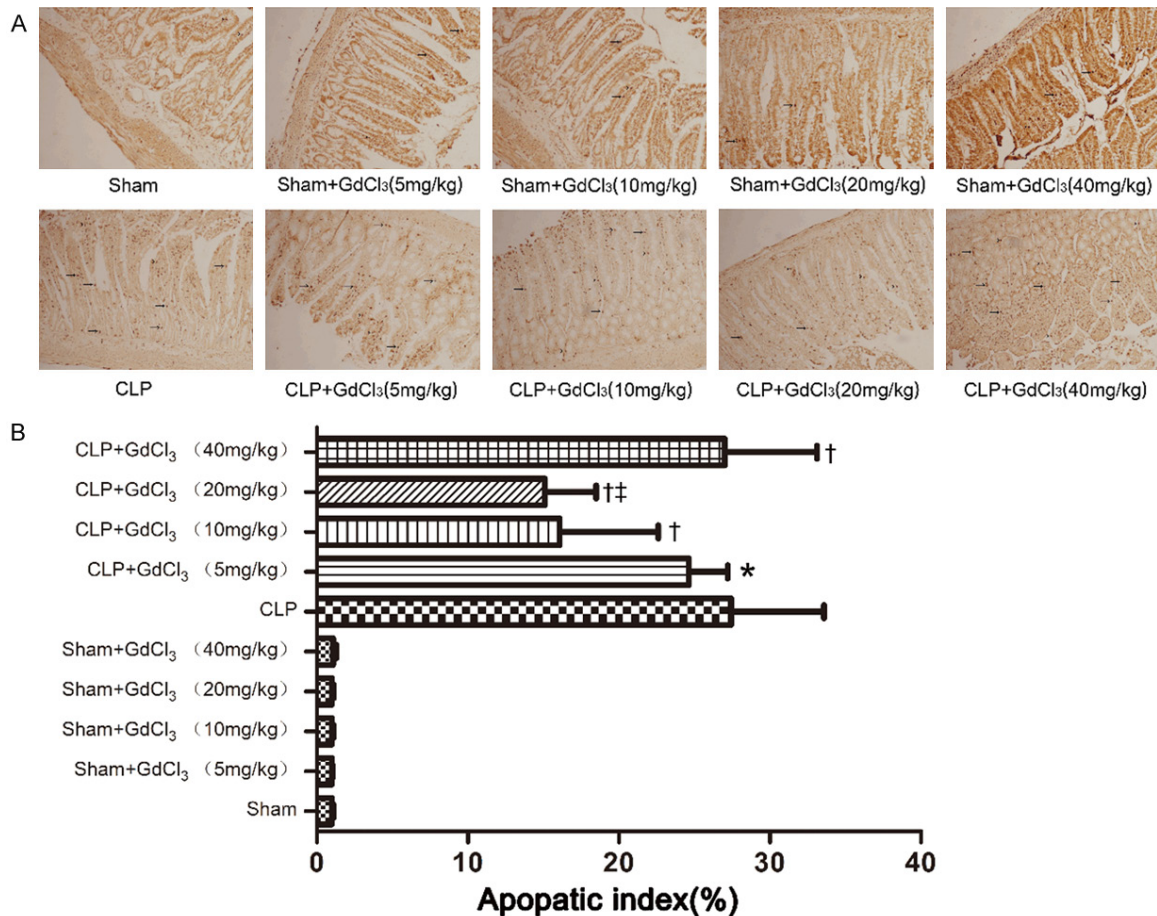


Figure 5. Effect of GdCl₃ pretreatment on the apoptosis of intestinal tissue in CLP-induced septic rats. Intestinal tissue apoptosis was detected by TUNEL analysis and the apoptotic rate was determined based on count data (B). Cells in which the nucleus stained brown are apoptotic cells, which are indicated by arrows (A). Magnification × 200. *P<0.05 compared with the sham group. †P<0.05 compared with the CLP group. ††P>0.05 compared with the CLP + GdCl₃ (10 mg/kg) group. Abbreviations: GdCl₃, gadolinium chloride; CLP, cecal ligation and puncture; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

tinal mechanical barrier, we also examined the expression of intestinal tight junction proteins (occludin and ZO-1) and the apoptosis of intestinal tissues. The intestinal barrier is a selective barrier, and there are two pathways whereby substances in the intestinal cavity can pass through the intestinal mucosa, namely the transcellular and paracellular pathways. The intestinal paracellular pathway is primarily regulated by tight junction proteins, which mainly comprise occludin, claudin, and zonula occludens (ZO) proteins. Among these, occludin and ZO-1 play the most important roles in tight junction function. Studies have shown that the expression of ZO-1 and occludin in intestinal tissues is reduced in sepsis [29]. In the present study, we performed western blotting and immunohistochemical analyses to determine the expression

of ZO-1 and occludin, and found that pretreatment of septic rats with 5, 10, and 20 mg/kg GdCl₃ increased the expression of ZO-1 and occludin in their intestinal tissues. However, when septic rats were treated with 40 mg/kg GdCl₃, the expression of ZO-1 and occludin was found to be decreased to levels even lower than those in the CLP group.

A further mechanism contributing to intestinal mucosal barrier dysfunction may be associated with the excessive apoptosis of intestinal epithelial cells. Animal models and human autopsy studies have found that sepsis can cause massive apoptosis of intestinal epithelial cells. In an animal model of sepsis, apoptosis of intestinal epithelial cells was found to be significantly increased in mice after CLP, and inhibition of

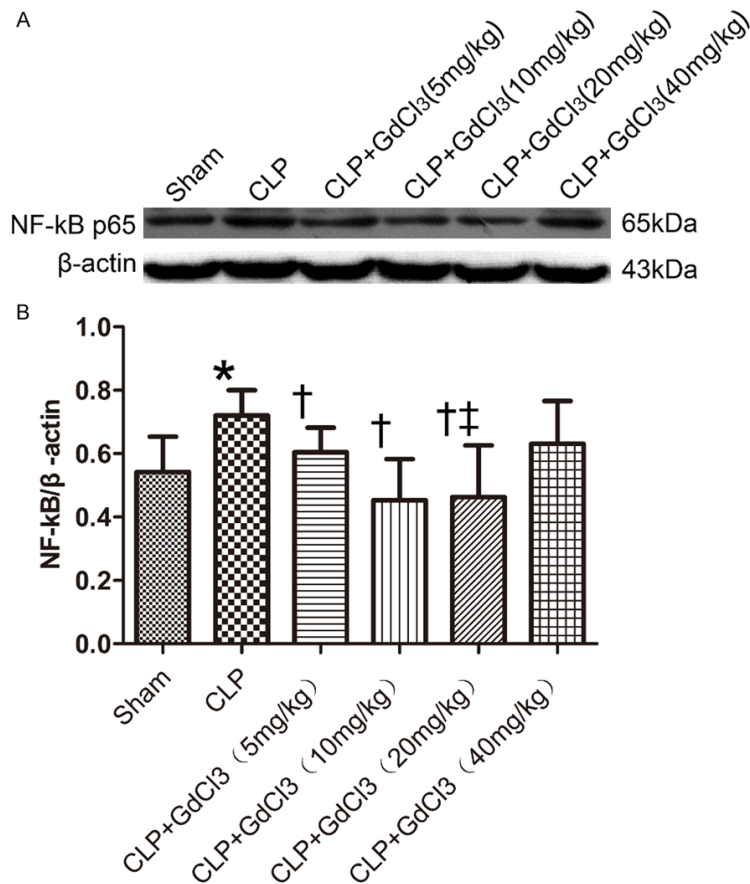


Figure 6. Effect of $GdCl_3$ pretreatment on NF- κ B expression in the intestinal tissue of CLP-induced septic rats. The expression of NF- κ B protein in intestinal tissues was examined by western blotting. (A). Representative western blot images for NF- κ B obtained 12h after operation. (B). A histogram of NF- κ B protein expression level. * $P < 0.05$ compared with the sham group. † $P < 0.05$ compared with the CLP group. ‡ $P > 0.05$ compared with the CLP + $GdCl_3$ (10 mg/kg) group. Abbreviations: $GdCl_3$, gadolinium chloride; CLP, cecal ligation and puncture; NF- κ B, nuclear factor-kappa B.

this apoptosis enhanced the survival rate of septic mice. More recent studies have indicated that activation of caspase protease is a key event in the regulation of cell apoptosis. If caspase-3 is activated, cell apoptosis is inevitable [30, 31]. In the present study, we examined intestinal cell apoptosis in each group based on TUNEL analysis and the expression of caspase-3 in the intestine detected by western blotting. The results showed that treatment of septic rats with 5, 10, and 20 mg/kg $GdCl_3$ decreased the expression of caspase-3 and the rate of intestinal tissue cell apoptosis. However, in septic rats treated with 40 mg/kg $GdCl_3$, the reductions in caspase-3 expression and intestinal tissue cell apoptosis were not significantly different from those in the CLP

group. These findings thus indicate that treatment with $GdCl_3$ can reduce the apoptosis of intestinal tissue cells in septic rats, and that this may be related to activation of the caspase pathway. On the basis of the aforementioned results, we speculate that inhibition of Kupffer cell activity by $GdCl_3$ can improve the intestinal mucosal barrier function in septic rats, and that this protective effect of $GdCl_3$ is dose-dependent.

In animals suffering from sepsis, a range of different inflammatory cytokines, including TNF- α , IL-1 β , and IL-6, are produced due to excessive inflammatory reactions. Systemic and intestinal inflammatory responses activate the NF- κ B signaling pathway and the expression of myosin light chain kinase (MLCK) in intestinal tissue [32]. MLCK is a protein kinase that regulates tight junctions, and studies have shown that this regulation can affect permeability of the intestinal mucosa by regulating the expression of occludin, claudins, and ZO-1. Previous studies have also indicated that NF- κ B activated in the intestinal

mucosa by inflammatory cytokines can bind to the MLCK gene promoter sequence in intestinal epithelial cells to promote MLCK expression, resulting in the disruption of tight junctions between intestinal epithelial cells [33]. NF- κ B is a nuclear protein factor found in almost all cells, which is associated with the regulation of cell differentiation, apoptosis, and tissue damage. NF- κ Bp65, a subunit of NF- κ B, directly and indirectly inhibits the activation and synthesis of caspase-3 [34]. In the present study, we examined the expression of caspase-3, and also performed western blotting to determine the expression level of NF- κ Bp65. The results showed that, in septic rats, expression levels of NF- κ Bp65 were related to changes in the expression of intestinal proinflamma-

tory cytokines, intestinal tight junction proteins, and caspase-3 expression. These results accordingly indicate that the protective effect of GdCl_3 pretreatment on the intestinal barrier in septic rats may be due to the inhibition of NF- κ B pathway activation.

The use of GdCl_3 to inhibit liver Kupffer cells was found to reduce the systemic and intestinal expression of TNF- α , IL-1 β , and IL-6, and also altered the expression levels of intestinal mechanical barrier-related indicators, such as occludin, ZO-1, and caspase-3. Moreover, we detected certain correlations between the change trends of inflammatory factors and those of intestinal mechanical barrier-related indicators. We therefore justifiably believe that the inhibition of Kupffer cells by GdCl_3 can protect intestinal barrier function by reducing the release of inflammatory factors in intestinal tissue. Although this assumption is consistent with the previously described mechanism of hepatointestinal circulation, it has also been proposed that the protective effect of GdCl_3 on intestinal inflammation injury is due to the inhibition of intestinal macrophages by GdCl_3 itself [35]. Therefore, it will be necessary to further investigate its mechanism of action. We also discovered that the protective effect of GdCl_3 on the intestine was dose-dependent, with a low dose of GdCl_3 producing no significant reduction in intestinal inflammatory damage or improvement of intestinal barrier function. However, with an increase in dose, we observed a relatively stable dose range, beyond which the reduction of damage in the intestinal tract did not increase. This may be related to the fact that GdCl_3 not only inhibits Kupffer cell secretion, but also inhibits the phagocytotic activity of these cells. However, at a higher concentration, GdCl_3 was found to increase intestinal damage. This may be associated with the excessive inhibition or even necrosis of Kupffer cells in response to treatment with GdCl_3 .

In conclusion, GdCl_3 has a protective effect on intestinal barrier function, as a consequence of the inhibition of liver Kupffer cells, which may be related to a decrease in the secretion of inflammatory factors by these cells. Moreover, this protective effect of GdCl_3 is dose-dependent, with high doses not affording protection against intestinal injury.

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Disclosure of conflict of interest

None.

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