

Original Article

Sepsis induces variation of intestinal barrier function in different phase through nuclear factor kappa B signaling

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ABSTRACT The intestinal barrier function disrupted in sepsis, while little is known about the variation in different phases of sepsis. In this study, mouse models of sepsis were established by caecal ligation and puncture (CLP). The H&E staining of sections and serum diamine oxidase concentration were evaluated at different timepoint after CLP. TUNEL assay and EdU staining were performed to evaluate the apoptosis and proliferation of intestinal epithelium. Relative protein expression was assessed by Western blotting and serum concentrations of pro-inflammatory cytokines was measured by ELISA. The disruption of intestinal barrier worsened in the first 24 h after the onset of sepsis and gradually recovered over the next 24 h. The percentage of apoptotic cell increased in the first 24 h and dropped at 48 h, accompanied with the proliferative rate of intestinal epithelium inhibited in the first 6 h and regained in the later period. Furthermore, the activity of nuclear factor kappa B (NF- κ B) presented similar trend with the intestinal barrier function, shared positive correction with apoptosis of intestinal epithelium. These findings reveal the conversion process of intestinal barrier function in sepsis and this process is closely correlated with the activity of NF- κ B signaling.

INTRODUCTION

Sepsis, which defined as the life-threatening organ dysfunction caused by a dysregulated host response to infections, remains a major health burden for its high mortality and morbidity [1-3].

The gut is considered to play a significant role in sepsis and has been defined as the motor of sepsis [4]. The intestinal epithelial barrier, as the first line of defense, prevents the bacteria and toxins in the gut lumen translocate to the bloodstream. During sepsis, the intestinal epithelial barrier impaired with increased intestinal permeability partly resulted from epithelial apoptosis and disruption of tight junctions [5-7]. Clinically, the persistent increase of intestinal permeability is a marker of poor prognosis in sepsis [8,9]. Nevertheless, the process of how intestinal epithelial barrier changes in sepsis remains elusive.

The efficiency of intestinal epithelial barrier is maintained on

the basis of the balance of epithelial cell proliferation and cell death are well regulated. The intestinal epithelium is characterized by its rapid self-renewing rate which totally replaced with newly generated cells within 4-5 days [10]. Apoptosis, which defined as the type I programmed cell death, is critical for the structural integrity of gut, and the excessive apoptosis damages gut integrity and weakens cell adherence, eventually leading to loss of barrier function and thus aggravating the development of sepsis [11,12]. How the balance between proliferation and apoptosis varies during sepsis is still unknown.

The nuclear factor (NF)- κ B, as a crucial transcription factor in inflammation, is not only activated by inflammatory cytokines such as tumor necrosis factor- α (TNF- α) or interleukin-6 (IL-6), but also by bacterial cell wall components like lipopolysaccharides, by viruses and even by physical stress conditions such as gamma- or UV-irradiation. The activation of NF- κ B pathway



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plays a critical role in intestinal epithelial barrier during sepsis [13]. The study by Chen *et al.* [14] showed that activated NF- κ B increased intestinal epithelial apoptosis and intestinal permeability while other studies demonstrated in contrast [15-17]. However, whether NF- κ B activation plays a detrimental or beneficial role in the intestinal tract in response to inflammation remains a matter of debate.

Despite the importance of proliferation and apoptosis of intestinal epithelium is explicit in sepsis, little is known about their variations in different phase of sepsis. In this study, we evaluated the proliferation rate and apoptosis of intestinal epithelium in different time during the sepsis and tried to explore the underline mechanism. The findings will help us better understand the whole process of how intestinal epithelial barrier updated in sepsis.

METHODS

Animal experiment and sepsis model

A total of 60 male C57BL6/J mice (weighing 20–25 g) were purchased from Keygen Biotech, Nanjing, China. The mice were kept in cages in a 12 h light/dark cycled room at 22–25°C with free access to water and a standard specific pathogen free (SPF) laboratory diet. All the experimental protocols were performed in accordance with the National Institutes of Health Guidelines for the Use of Laboratory Animals and were approved by the Institutional Animal Care and Ethics Committee of Wannan Medical College (LLSC-2020-074).

Polymicrobial sepsis model was induced *via* caecal ligation and puncture (CLP) as previously described [18]. Briefly, mice were intraperitoneal injected with 1% pentobarbital (50 mg/kg) and a 0.5–1 cm midline abdominal incision was made. Then the cecum was ligated below the ileocecal valve in the distal three quarters of the cecum and followed by a single ‘through and through’ perforation with 21-gauge needle. After the puncture, cecum was returned to the abdomen and a sterile 3-0 silk suture was used to close the incision. The control group were subjected to laparotomy without CLP. Saline solution was injected subcutaneously (50 mg/kg) for resuscitation after the surgery.

H&E staining

The mice underwent CLP were randomly divided into three groups which were sacrificed 6, 24, 48 h respectively after CLP. The small-intestinal tissues were dissected and flushed with ice-cold PBS, then fixed in 10% paraformaldehyde overnight, embedded in paraffin, sliced at 5 μ m and stained with H&E as previous described [19]. The severity of mucosal injury was assessed with Chiu’s score system.

Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF- α , IL-6, diamine oxidase (DAO) and inhibitor kappa B kinase beta (IKK β) in mice serum were measured with ELISA kits (Mlbio, Shanghai, China) according to the manufacturer’s instructions. Briefly, the blood samples were taken before mice sacrificed and then centrifuged at 3,000 g for 15 min at 4°C, followed with the supernatant was collected and frozen at –80°C until assay.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

For cell apoptosis analysis in intestinal epithelium, TUNEL assay was performed using the Dead-End™ Fluorometric TUNEL system (Promega, Madison, WI, USA) on deparaffinized and rehydrated tissue sections, according to the manufacturer’s protocol. Apoptotic cells were quantified as the number of TUNEL positive cells.

EdU staining proliferation

Mice were received intraperitoneal injection of thymidine analog 5-ethynyl-2’-deoxyuridine (EdU, 5 mg/kg, diluted in 0.9% saline; Abcam, Cambridge, MA, USA) 6 h before sacrificed and then EdU was detected in intestinal sections *via* immunohistochemistry according to the manufacturer’s protocol. Briefly, Sections were deparaffinized, rehydrated and incubated in Click-iT Plus reaction cocktail for 30 min at room temperature in the dark, then sections were incubated with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride and coverslipped with antifade mounting medium. The proliferation was assessed by quantifying EdU positive cells using a fluorescence microscope.

Intestinal epithelial cells isolation and protein extraction

Small intestinal epithelium was isolated as previously described [20,21]. Briefly, intestine segments were washed in Hanks Balanced Salt Solution (HBSS) and incubated on a shaker platform in HBSS. Cells were separated from the denser intestinal fragments and then centrifuged five times at 120 g for 3 min. The cells were then lysed in a radioimmunoprecipitation assay lysis buffer containing protease inhibitor and phosphatase inhibitor cocktails. For nuclear-cytoplasmic fractionation, a nuclear extraction kit (Keygen Biotech, Nanjing, China) was used following the manufacturer’s instructions. The protein concentrations were measured using the Protein Assay Kit (Bio-Rad, Richmond, CA, USA).

Western blotting analysis

For western blot analysis, equal amounts of protein (30–80 μ g)

was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA) at 100 V for 1 h at 4°C. Blots were blocked with 5% milk in Tris Buffered Saline-Tween 20 and the following primary antibodies were used: anti-cleaved-Caspase3 (dilution 1:1,000; Cell Signaling Technology, Danvers, MA, USA), anti-proliferating cell nuclear antigen (PCNA) (dilution 1:1,000; Cell Signaling Technology), anti-I κ B α (dilution 1:500; Proteintech, Wuhan, China), anti-zona occludens 1 (ZO-1) (dilution 1:500; Proteintech), anti-occludin (dilution 1:500; Proteintech), anti-p-NF- κ B (p-P65) (1:500; Cell Signaling Technology); anti-LaminB1 (1:500; Proteintech) and anti-GAPDH (dilu-

tion 1:5,000; Sigma, St. Louis, MO, USA).The membranes were analysed by the use of super ECL detection reagent (Appligen, Beijing, China).

Statistical analysis

The GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA) was adopted for data analysis. All the data were expressed as mean \pm SD from 3 independent experiments. Student's t-test or one-way ANOVA test were used to do significant differences between groups. Spearman correlation coefficients were used to evaluate the relationships between the percentage of apoptotic

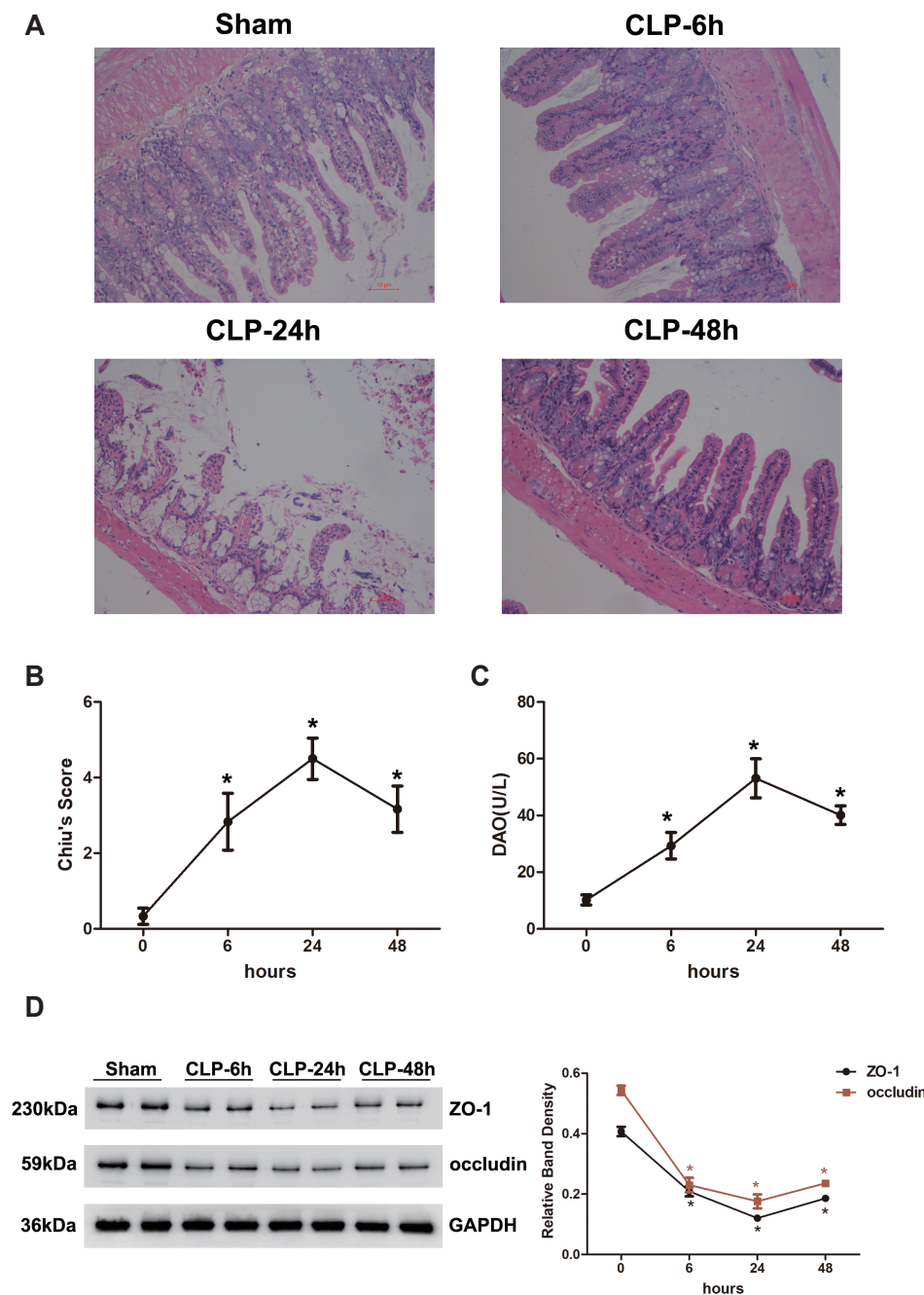


Fig. 1. Changes of intestinal barrier function in sepsis. (A) Typical H&E-stained sections of intestinal tissues at different timepoint after caecal ligation and puncture (CLP) (bar, 10 μ m; magnification, 200 \times). (B) The Chiu's score of H&E-stained sections. (C) The serum concentration of diamine oxidase (DAO) at different timepoint after CLP. (D) The level of zona occludens 1 (ZO-1) and occludin were measured at different timepoint after CLP. The graph represented the relative band densities. * $p < 0.05$ vs. the original timepoint.

cell and IKK β levels. p-values < 0.05 were considered statistically significant.

RESULTS

Changes of intestinal barrier function in sepsis

Previous studies shown that the intestinal barrier was injured during the sepsis, whereas the process of how intestinal barrier changes is not yet clear [18,19]. In this study, we evaluated the intestinal barrier function at different time point after CLP by examining H&E-stained sections of the intestinal mucosa and measuring DAO concentrations in serum. The intestinal villi

appeared shorten at 6 h after the onset of sepsis and persisted deteriorated in the first 24 h with Chiu's score, a pathological score that assessing the injury of intestinal mucosal, up to 5. As time reached 48 h, the injured intestinal mucosal appeared recovered with a drop of Chiu's score (Fig. 1A, B). The varies of DAO levels in serum presented the similar tendency with Chiu's score (Fig. 1C). Furthermore, we detected the expression of tight junction proteins ZO-1 and occludin in the intestinal epithelium. The expression of ZO-1 and occludin exhibited the same tendency that experienced durative decline in the first 24 h and modest growth 48 h after CLP (Fig. 1D), which suggesting the recovery of tight junction in intestinal epithelium at 48 h.

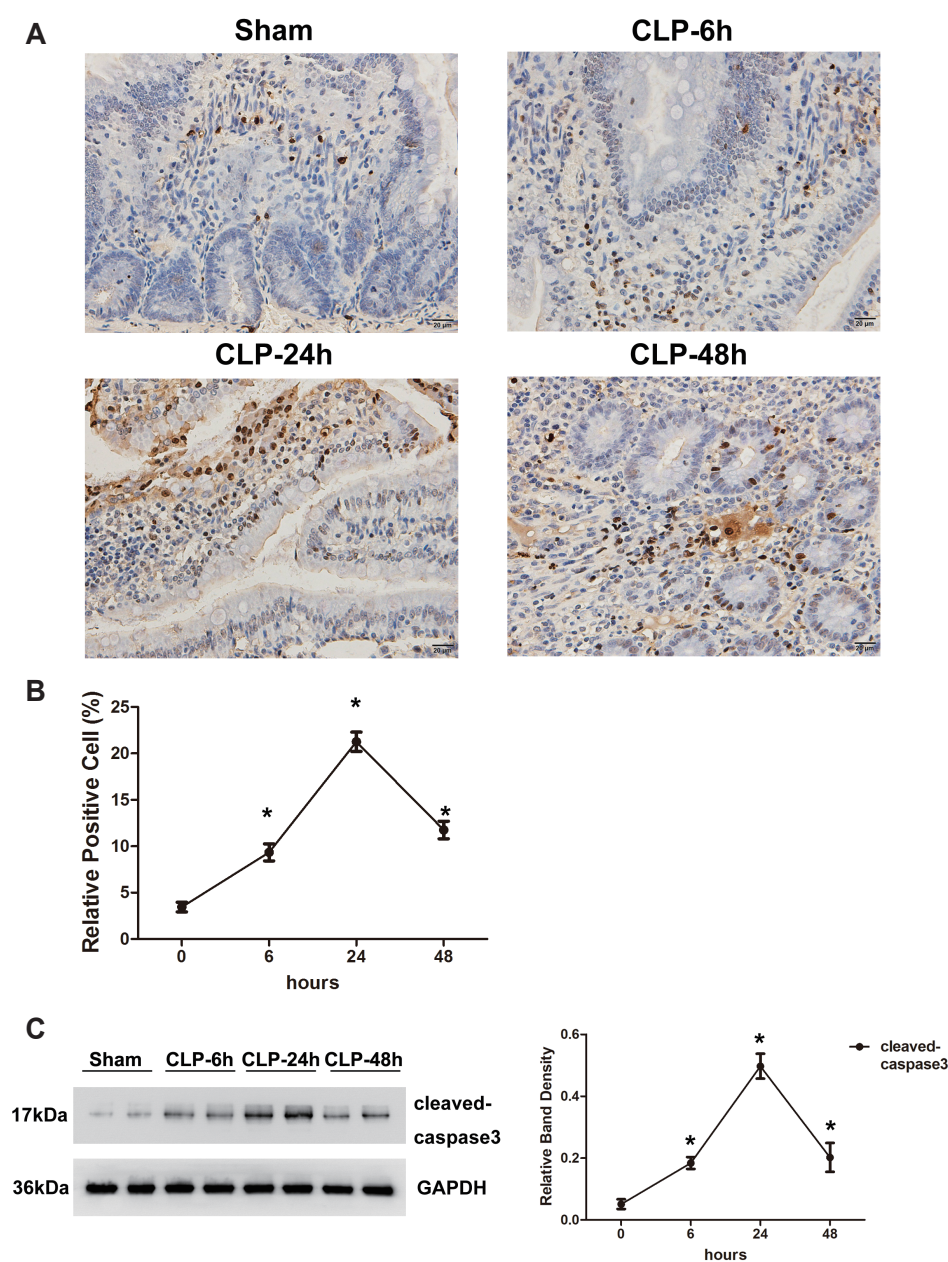


Fig. 2. The variation of apoptosis of intestinal epithelium in sepsis. (A) Representative images of TUNEL staining of the indicated timepoint (bar, 20 μ m; magnification, 400 \times). (B) The percentage of positive TUNEL stained cell at different timepoint after caecal ligation and puncture (CLP). (C) The level of cleaved-caspase3 was measured at different timepoint after CLP. The graph represented the relative band densities. *p < 0.05 vs. the original timepoint.

The variation of apoptosis of intestinal epithelium in sepsis

The integrity of the intestinal barrier depends on the dynamic balance of apoptosis and proliferation in intestinal epithelium. We then quantified the apoptosis of intestinal epithelium using TUNEL staining and cleaved-caspase3 level. The positive cells of TUNEL staining emerged at 6 h and persist increased in the first 24 h, while the number of positive cells underwent a fall in the next 24 h (Fig. 2A, B). The expression of cleaved-caspase3 shared the analogous trend (Fig. 2C), indicating that the apoptosis of intestinal epithelium in sepsis reached a peak at 24 h and then gradually recovered.

The proliferation rate of intestinal epithelium in sepsis

The rapidly renewing rate of intestinal epithelium plays a central role in barrier function [22,23]. The proliferation of intestinal epithelium was assessed with EDU staining and PCNA level. The number of positive EdU staining cell was dramatically decreased in the first 6 h after CLP, then gradually increased during the 48 h, even equal to the initial at 48 h (Fig. 3A, B). The level of PCNA, which a marker protein of proliferation, presented a similar tendency, suggesting the proliferation rate down to bottom at 6 h and then gradually recovered at 48 h (Fig. 3C).

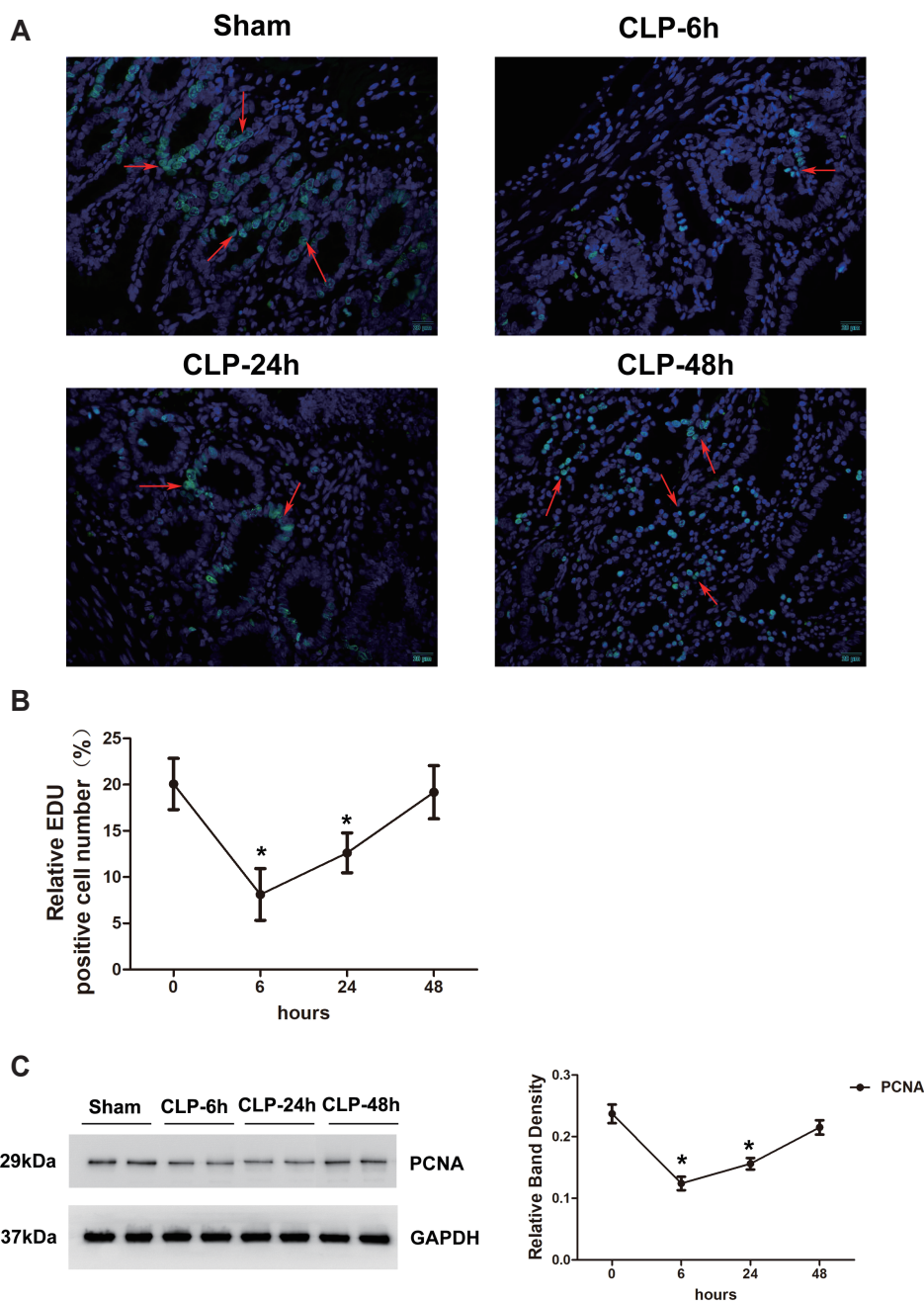


Fig. 3. The proliferation rate of intestinal epithelium in sepsis. (A) The representative images of EdU assays of the indicated timepoint (bar, 20 μ m; magnification, 400 \times). The positive cells were marked with red arrows. (B) The percentage of positive EdU stained cell at different timepoint after caecal ligation and puncture (CLP). (C) The level of proliferating cell nuclear antigen (PCNA) was measured at different timepoint after CLP. The graph represented the relative band densities. * $p < 0.05$ vs. the original timepoint.

The activity of NF- κ B signaling positively corrected with apoptosis during sepsis

Furthermore, the levels of pro-inflammatory cytokines (TNF- α and IL-6) were measured by ELISA and found that the serum concentrations of TNF- α and IL-6 is elevated in the first 6 h and peaked at 24 h, then gradually decreased (Fig. 4A, B). NF- κ B pathway is a classic signalling pathway in inflammatory response. To define whether NF- κ B pathway was involved in the process, the nuclear protein expression of p-NF- κ B (p-P65), the level of IKK β in serum and the protein expression of NF-kappa-B inhibitor alpha (I κ B- α) were measured. The nuclear protein expression of p-P65 gradually increased in the first 24 h and plunged at 48 h

(Fig. 4C). The expression of I κ B- α decreased in the first 24 h, and then slowly increased at 48 h (Fig. 4D). The level of IKK β raised first and then declined (Fig. 4E). These results indicating the NF- κ B signaling activated in the first 24 h and then the activity decreased during the next 24 h. Moreover, a strong correlation between NF- κ B activity and apoptosis was established by analysing the serum IKK β levels and apoptotic cells of intestinal epithelium in sepsis (Fig. 4F).

DISCUSSION

The present study shown that while intestinal permeability is

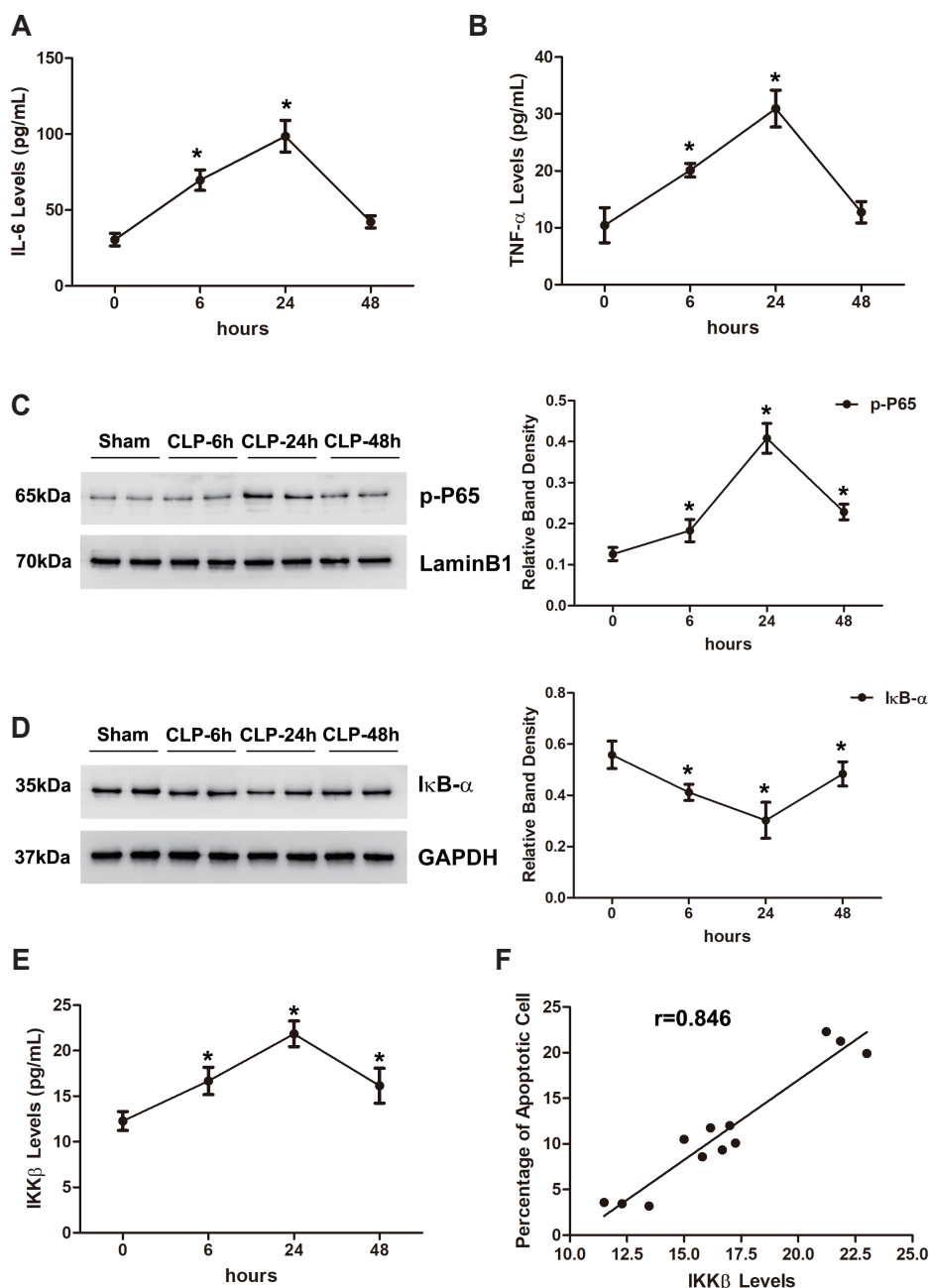


Fig. 4. The activity of nuclear factor kappa B (NF- κ B) signaling positively corrected with apoptosis during sepsis. (A, B) The serum concentrations of tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6) were determined by ELISA at different timepoint after caecal ligation and puncture (CLP). (C) The level of nuclear p-NF- κ B (p-P65) in intestinal epithelium was measured at different timepoint after CLP. The graph represented the relative band densities. (D) The level of I κ B- α in intestinal epithelium was measured at different timepoint after CLP. The graph represented the relative band densities. (E) The serum level of inhibitor kappa B kinase beta (IKK β) at different timepoint after CLP. (F) Correlation analysis for the percentage of apoptotic cells and IKK β levels. * $p < 0.05$ vs. the original timepoint.

elevated 6 to 48 h after the onset of sepsis it reaches a peak at 24 h following induction of sepsis, which is similar to the previous study [24]. Moreover, the apoptosis of intestinal epithelium shared the similar trend, indicating the vital role in intestinal epithelial barrier during sepsis.

The intestinal epithelium, which is consisted of monolayer cells linked close to the apical surface by the tight junctions, act as an important role in determining intestinal permeability. The homeostasis of intestinal epithelium, which is determined by the balance between cell proliferation and apoptosis that regulate intestinal cell turnover and mucosal cellularity, is critical in the determination of intestinal barrier function [25]. In this study, the proliferation rate of intestinal epithelium significantly decreased in the first 6 h and gradually recovered in 48 h, which also contributing to the recovery of intestinal barrier function in the later stage of sepsis.

The disruption of intestinal barrier function in early stage is widely studied [26-28], while little attention is paid to the late sepsis. The CLP model of sepsis has previously been described to have at least two phases based on the adrenomedullin or blood glucose level and defined the first phase of sepsis occurred within 6 h, while the second phase was present at 18 h [29-31]. In our study, we defined the first 24 h after CLP as acute phase on the basis of the plasma cytokines. The plasma levels of IL-6, which is reported to predict the early deaths of sepsis [32,33], is elevated in the first 6 h and peaked at 24 h, then gradually decreased. The level of TNF- α shared the similar tendency with IL-6 in this study, which provide support for the definition of early and late sepsis. Indeed, the peak time of pro-inflammatory cytokines (IL-6 and TNF- α) after CLP were various in different studies partly due to the different ligation position and cecal-puncture diameter [34,35].

During sepsis, the activated NF- κ B increase the pro-inflammatory cytokines (IL-6 and TNF- α) levels, in turn, the elevated pro-inflammatory cytokines directly or indirectly activate the NF- κ B pathway, thus building a positive feedback loop which amplify the inflammation response [36,37]. On this basis, inhibiting the NF- κ B activity seems an effective therapy of sepsis theoretically. However, the fact is not optimistic in clinical [38,39]. Other studies also demonstrated that NF- κ B acts as an anti-inflammatory factor and promotes the production of IL-10, indicating the NF- κ B as a double-edged sword in sepsis [40]. In this study, the activity of NF- κ B pathway was reflected by the level of nuclear p-P65, the expression of I κ B- α and the serum IKK β concentration. In canonical NF- κ B pathway, the inhibitor kappa B kinase (IKK) complex is triggered by microbial products and proinflammatory cytokines, leading to activation of p65/p50/I κ B trimer, degrading the I κ B and finally promoting the nuclear translocation of p65/p50 dimer [41]. The changes of nuclear p-P65, I κ B- α and IKK β in this study revealed that the activity of NF- κ B pathway rocketed in first 24 h and then gradually recovered in the next 24 h after CLP.

The NF- κ B pathway act as a critical factor in regulating the apoptotic program. It seems that the NF- κ B has dual function in

the apoptotic process. On one hand, NF- κ B was considered as a pro-apoptotic factor for its rapid activation in response to apoptotic signals and involvement in the expression of some apoptotic genes, such as TNF- α , c-myc, and fas ligand (FasL) [42-45]. On the other hand, several studies revealed an anti-apoptotic effect of NF- κ B in response to various of apoptotic stimuli [46,47]. In this study, the activity of NF- κ B shared positive correction with apoptosis, suggesting NF- κ B act as a promoting role in sepsis-induced apoptosis of intestinal epithelium.

Limitations of the present study include that we have only assessed the changes of intestinal permeability, proliferation and apoptosis, the potential mechanism that caused these changes needs to be further explored. Moreover, it would be beneficial to use selective NF- κ B knockout mice to verify the relationship NF- κ B and intestinal epithelium apoptosis in sepsis induced intestinal barrier dysfunction.

In conclusion, the present study highlights that the intestinal permeability reached a peak at 24 h following induction of sepsis, and this is highly correlated with the apoptosis of intestinal epithelium induced by the activation of NF- κ B pathway. The variation of intestinal permeability also implies a novel target for the treatment of sepsis-induced intestinal barrier dysfunction.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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