Original Article
Early enteral nutrition inhibits TLR4 signaling activation in rats with severe acute pancreatitis

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Abstract: This study examined the effect of early enteral nutrition (EEN) on activation of Toll-like receptor 4 (TLR4) signaling in severe acute pancreatitis (SAP), and to evaluate mechanisms underlying the beneficial effect of EEN in SAP. Sprague Dawley rats were randomly divided into three groups: a sham-operated control group (sham group), a SAP+EEN and a SAP+TPN group (n = 20 each group). Rats were injected with sodium taurocholate to induce SAP and were sacrificed 5 days after nutritional support. Induction of SAP resulted in a decreased survival rate, as compared with the sham group (P < 0.05). SAP rats treated with EEN had a significantly higher survival rate than those treated with TPN (P < 0.05). Expression of intestinal TLR4 and nuclear factor (NF)-κB was increased in SAP rats. EEN treatment could significantly reduce expression of TLR4 and NF-κB in the intestine compared with TPN. In addition, EEN resulted in a decrease in serum levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 as well as a reduction in the serum amylase content and pathological damage to the pancreas. EEN inhibits activation of the TLR4/NF-κB signaling pathway induced by SAP, thus leading to a decrease in inflammation and death.

Keywords: Early enteral nutrition, several acute pancreatitis, TLR4, NF-κB, TNF-α, IL-6

Introduction

Acute pancreatitis (AP) is an inflammatory disease that can be classified into three types: mild acute pancreatitis (MAP), moderately severe acute pancreatitis (MSAP), and severe AP (SAP). SAP represents approximately 20-30% of AP patients and is characterized by severe symptoms, rapid progression, and a high incidence of complications [1]. The pathogenesis of SAP is not clearly elucidated, and the treatment for SAP remains a challenge for physicians [2]. In contrast to the low mortality rates of MAP and MSAP, SAP has a mortality rate as high as 36-50% [3]. The high mortality rate of SAP is believed to be the consequence of multiple organ failure as a result of systematic inflammation response syndrome (SIRS) and infection due to pancreatic necrosis [4]. Inflammatory cells and cytokines play an important role in initiation and progression of AP, and complex immune events contribute to the de-

velopment of AP [5, 6]. Therefore, identification of the mechanisms underlying inflammation and immune responses during SAP may lead to the discovery of a novel target for the treatment of SAP.

AP can cause damage to the intestinal mucosa barrier, which can lead to entry of bacteria and endotoxins into the systemic circulation, and subsequently result in SIRS, secondary pancreatic infection, and multiple organ dysfunction syndrome [7]. Rinderknecht et al. first reported that abnormal activation of the immune system and the release of cytokines contribute to the occurrence of local and systemic complications in AP [8]. A variety of cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8 contribute to the pathogenesis of SAP [9]. Toll-like receptor 4 (TLR4) plays a curial role in innate immunity and inflammatory responses and is associated with the production of inflammatory cytokines [10-12]. Several

studies have demonstrated that TLR4 is important for the development of SAP [13-15]. However, the role of intestinal TLR4 expression in SAP has not been elucidated.

A steady nutrition supply is necessary for AP patients. However, the best route for nutritional support in SAP patients remains debatable. Traditionally, total parenteral nutrition (TPN) is recommended for its minimal effect on pancreatic and intestinal stimulation. However, several lines of evidence have shown that TPN is associated with no advantages on overall patient outcomes compared with enteral nutrition (EN) [16-19]. Furthermore, although the timing to start EN is still controversial, several studies have shown that early EN (EEN) results in a reduction in complications and an improvement in clinical outcomes of patients with AP [20, 21]. However, mechanisms by which EEN produces beneficial effects in AP remain unclear.

In this study, the effects of EEN versus TPN on the expression of TLR4 were compared in the intestine in a rat model of taurocholate -induced SAP. This study was performed to investigate the effect of EEN on the TLR4 signaling pathway in SAP, and to explore the possible mechanisms underlying the effect of EEN in SAP.

Materials and methods

Animals

Adult male, 3-4 months old Sprague-Dawley rats (weighing between 180-250 g) were housed in a clean cage at room temperature of 25°C, with humidity of 60% and 12-h light/dark cycle. The experimental protocols were approved by the Animal Care Committee at Luzhou Medical College.

All rats were randomly divided into three groups: a sham group (rats with sham operation), a SAP+EEN group and a SAP+TPN group, 20 rats in each group. Rats in the SAP+EEN group received early enteral nutrition. Rats in the SSAP+TPN group received total parental nutrition.

Materials

Antibodies against nuclear factor (NF)-kB p65 were purchased from BioWorld Company, USA.

Polyclonal goat anti-rat TLR4 antibody was purchased from Santa Cruz, USA. ELISA IL-6 kits and ELISA TNF-α kits were obtained from Wuhan Boster Company, China. EEN and TPN fluids were purchased from Huarui Company, China. Sodium taurocholate was obtained from Sigma, USA.

Preparation of SAP rat model and nutritional support

A rat model of taurocholate-induced SAP was created as previously described [22]. Briefly, after anesthesia and antisepsis, a midline incision (2-3 cm) in the upper abdomen was made to expose the pancreas. Sodium taurocholate (3.8%) was injected into the pancreas at the tail via a catheterized needle. SAP was successfully induced as evidenced by edema, exudation, and local hemorrhage in the pancreas.

For the sham group, sham operation was done without injection of taurocholate. For the SAP+EEN and SAP+TPN groups, an epidural catheter (1.2 mm, the enteral nutrient tube) was used to insert at approximately 1.5 cm below the duodenal papilla and into the right jugular vein of rats, respectively. The catheter was tunneled subcutaneously to exteriorize at the back of the neck. After surgery, 2 mL of saline was given subcutaneously to replace the fluid lost. The EEN solution and TPN solution were given at 12 hours after SAP induction. An electronic pump was used to maintain an infusion rate of 2 mL/h. The 50 mL solution was consumed per day, providing the energy of 30 kcal/100 g of body weight/day.

Serum amylase measurement

Blood samples were collected from the heart of sacrificed rats at 5 days after SAP induction. An automated analyzer was used to test the serum amylase contents.

Western blot

Intestinal tissues from rats were homogenized on ice in lysis buffer. After centrifuge (12,000 *g* for 5 minutes at 4°C). Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene fluoride membranes. Membranes were incubated in Tris-buffered saline solution with 0.1% Tween-20 containing 5%

Table 1. The criteria for evaluating pathological scores

	Score	Pathological changes
Edema	0	None
	1	Local interlobular edema
	2	Diffuse interlobular edema
	3	Acinar swelling with enlarged interlobular space
	4	Obvious edema with separated pancreatic lobules
Hemorrhage	0	None
	1	0-25% of pancreatic parenchyma
	2	25-50% of pancreatic parenchyma
	3	50-75% of pancreatic parenchyma
	4	> 75% of pancreatic parenchyma
Necrosis	0	None
	1	Necrosis in the parenchyma around the pancreatic duct
	2	Necrosis in < 20% of pancreatic parenchyma
	3	Necrosis in 20-50% of pancreatic parenchyma
	4	Necrosis in > 50% of pancreatic parenchyma
Inflammation	0	None
	1	Inflammatory cell infiltration around the pancreatic duct
	2	Inflammatory cell infiltration in < 50% of pancreatic parenchyma
	3	Inflammatory cell infiltration in 51-75% of pancreatic parenchyma
	4	Inflammatory cell infiltration in > 75% of pancreatic parenchyma

bovine serum albumin at room temperature for 1 hour. Membranes were then incubated with primary antibodies against TLR4 (polyclonal goat anti-rat TLR4 antibodies, dilution 1:500, Santa Cruz, USA) at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated rabbit anti-goat secondary antibodies (dilution 1:5000, Millipore) at room temperature for 1 hour. Bands were visualized by exposure to Kodak X-ray film. The gray values for each band were analyzed in images acquired after scanning the films.

Immunohistochemistry

Intestinal tissue was fixed in formalin and embedded in paraffin. Sample sections (5 μm thick) were prepared for immunohistochemical staining. Sections were incubated with polyclonal primary NF-κB antibody (goat anti-rat, dilution 1:500, BioWorld Company, USA) overnight at 4°C. Then, they were stained using the Envision-plus (DAKO) detection system, counterstained with hematoxylin, and observed under a light microscope by two investigators who were blinded to the experimental conditions. Immunoreactivity was evaluated by a scoring system, including the percentage of stained cells and the intensity. The percentage

of positive stained cells was calculated as follows: 0 point, none; 1 point, ≤ 10%; 2 point, 11-50%; 3 point, 51-80%; and 4 point, 81-100%. The intensity of staining was defined as follows: 0 point, none; 1 point, weak; 2 point, moderate; and 3 point, strong. The final immunoreactivity score was calculated by the sum of the intensity score and the percentage score.

ELISA

The concentrations of TNF-α and IL-6 in the serum were measured using the TNF-α and IL-6 ELISA kits, according to the manufacturer's instruction, respectively. The absorbance of each sample was measured using a plate reader (WellseanMK3, Finland) at a wavelength of 450 nm.

Pancreatic pathology

Tissues in the pancreatic head and tail were removed and cut into small pieces (1 cm × 1 cm × 0.2 cm). The tissues were fixed in 4% paraformaldehyde for 24 hours. Hematoxylin and Eosin (H&E) staining was performed on 5 μm-thick tissue sections. Pathological changes including edema, hemorrhage, necrosis, and inflammation were evaluated using the patho-

Table 2. Survival rate

Group	Survival n (%)
Sham group	20 (100)
SAP+TPN group	10 (50)*
SAP+EEN group	15 (75)#

*P < 0.01 vs. the sham group; #P < 0.05 vs. the SAP+TPN group.

Table 3. Serum amylase contents

Groups	n	Amylase (U/L)
Sham group	20	744.2 ± 40.7
SAP+TPN group	10	3278 ± 219.2*
SAP+EEN group	15	2227 ± 168.9*,#

*P < 0.01 vs. sham group; #P < 0.01 vs. SAP+TNP group.

Figure 1. Expression of TLR4 in the intestine of rats. A. Representative Western blot images showing the expression of TLR4 in the sham-operated (SO), SAP+TPN and SAP+EEN groups, with β-Actin as the control. B. Quantification of the expression of TLR4 normalized to that of β-actin. *P < 0.05 vs. SO group, #P < 0.05 vs. SAP+TPN group.

logical scoring system as reported previously by Rongione *et al*. [23] (**Table 1**). The total pathological score was used for comparison among groups.

Statistical analysis

Quantitative data are expressed as means ± SD. Homogeneity of variance was evaluated using Levene's test. The difference in data with equal variance was compared using one-way analysis of variance. The Kruskal-Wallis H test was used to compare the differences in data with unequal variance, followed by posthoc Q tests. Chi-square test was used to compare differences in categorical data. *P* values < 0.05 were considered statistically significant. Statistical analyses were performed using SPSS 13.60 software.
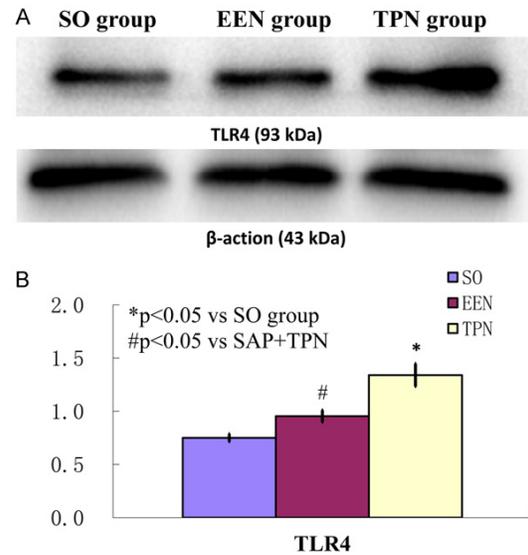
Results

Survival rate

All rats survived in the sham group. There were 10 rats in the SAP+TPN and 5 rats in the SAP+EEN group died within 5 days after SAP induction. The 5-day survival rates in the SAP+TPN (50%) and SAP+EEN (75%) groups were significantly lower than that of the sham group (100%, P < 0.05, **Table 2**). The SAP+EEN group had a significantly higher survival rate than the SAP+TPN group did (P < 0.05, **Table 2**).

Serum amylase contents

Serum amylase content was 3278 ± 219.2 U/L in the SAP+TPN versus 2227 ± 168.9 U/L

in the SAP+EEN group (P < 0.05, **Table 3**), and both were significantly higher than that in the sham group (744.2 ± 40.7, both P < 0.05, **Table 3**).
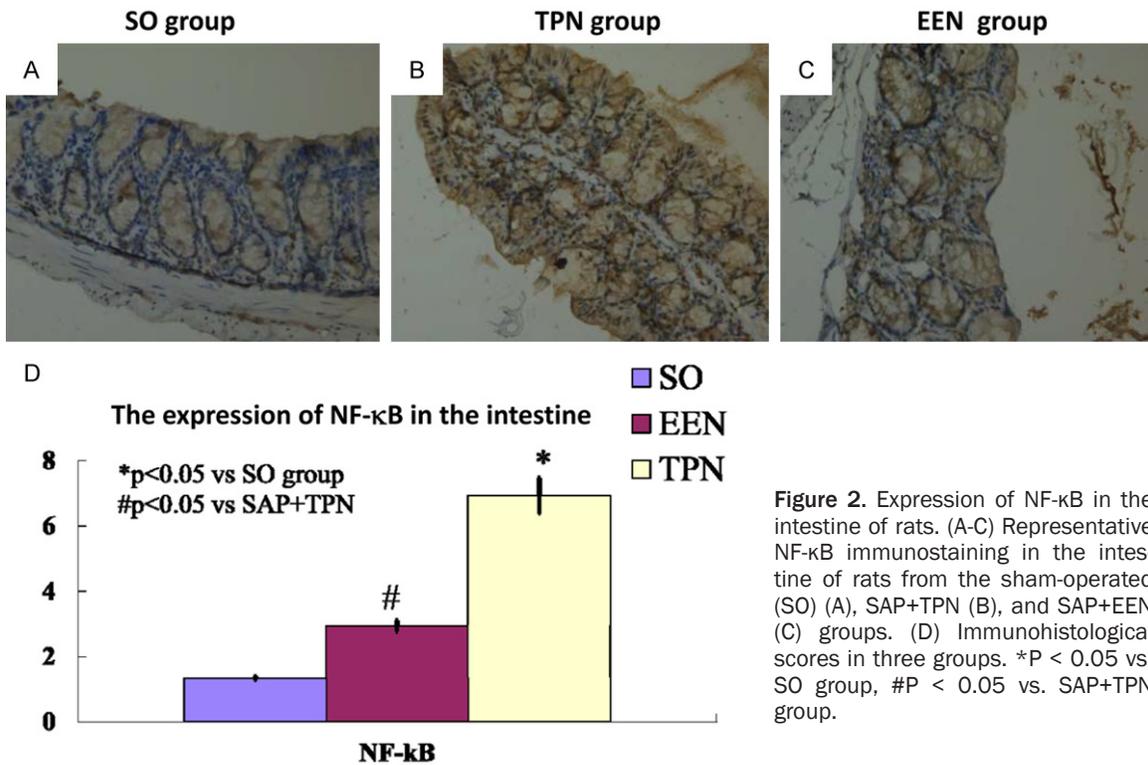
TLR4 expression in the intestine

Using Western blot, expression of TLR4 in the intestine of rats from each group was examined. Compared with the sham group, the SAP+TNP and SAP+EEN groups had significantly increased TLR4 expressions (P < 0.05, **Figure 1**). In addition, the SAP+EEN group exhibited a significantly lower TLR4 expression than the SAP+TPN group did (P < 0.05, **Figure 1**).

NF-κB expression in the intestine

Using immunohistochemistry, NF-κB expression was investigated in the intestine of rats from each group. In the sham group, NF-κB was weakly expressed in the cytoplasm and rarely in the nuclei (**Figure 2A**). In contrast, in the SAP+TPN group, NF-κB was strongly expressed in the cytoplasm and the nuclei (**Figure 2B**). In the SAP+EEN group, NF-κB was expressed in the cytoplasm and the nuclei, but the expression level was weaker than that in the SAP+TPN group (**Figure 2C**). Compared with the sham group, the SAP+TPN and SAP+EEN groups

Figure 2. Expression of NF-κB in the intestine of rats. (A-C) Representative NF-κB immunostaining in the intestine of rats from the sham-operated (SO) (A), SAP+TPN (B), and SAP+EEN (C) groups. (D) Immunohistological scores in three groups. *P < 0.05 vs. SO group, #P < 0.05 vs. SAP+TPN group.

Table 4. Serum levels of TNF-α and IL-6

Groups	TNF-α (ng/L)	IL-6 (ng/L)
Sham group (n = 20)	81.57 ± 18.25	362.83 ± 41.32
SAP+EEN group (n = 15)	223.21 ± 29.94*,#	628.62 ± 142.24*,#
SAP+TPN group (n = 10)	465.72 ± 42.47*	932.46 ± 57.21*

*P < 0.05 vs. sham group, #P < 0.05 vs. SAP+TNP group.

exhibited significantly higher levels of NF-κB expression (P < 0.05, **Figure 2D**). Moreover, the SAP+EEN group had a significantly lower NF-κB expression level as compared with the SAP+TPN group (P < 0.05, **Figure 2D**).

Serum levels of TNF-α and IL-6

The serum levels of TNF-α and IL-6 in both SAP+TPN and SAP+EEN groups were significantly higher than that of the sham group (all P < 0.05, **Table 4**). These two cytokines in the SAP+EEN group were significantly lower than that in the SAP+TPN group (P < 0.05, **Table 4**).

Pathological damage to pancreatic tissues

The sham group exhibited a clear and regular arrangement of pancreatic structure, without evidence of hemorrhage and necrosis. Mild congestion and edema was occasionally observed (**Figure 3A**). In the SAP+TNP group, diffuse lobular necrosis was observed with infiltration of numerous inflammatory cells. Hemorrhage and damaged blood vessels were observed at the locus of necrosis. The structures of the pancreatic acini and pancreatic lobules were unclear (**Figure 3B**). In the SAP+EEN group, congestion, necrosis, and inflammatory cell infiltration were reduced compared with the SAP+TPN group. Necrosis and shedding of the pancreatic ductal epithelium were seen (**Figure 3C**). The pathological scores of pancreas in the SAP+EEN and SAP+TPN groups were significantly higher than that in the sham group (P < 0.05, **Figure 3D**). However, the SAP+EEN group had a significant lower pathological score than that in the SAP+TPN group (P < 0.05, **Figure 3D**).

Discussion

In the present study, expression of TLR4 in the intestine from SAP rats was investigated after receiving TPN or EEN. Intestinal expression of TLR4 was found to be increased in SAP rats. EEN treatment led to a reduction in TLR4
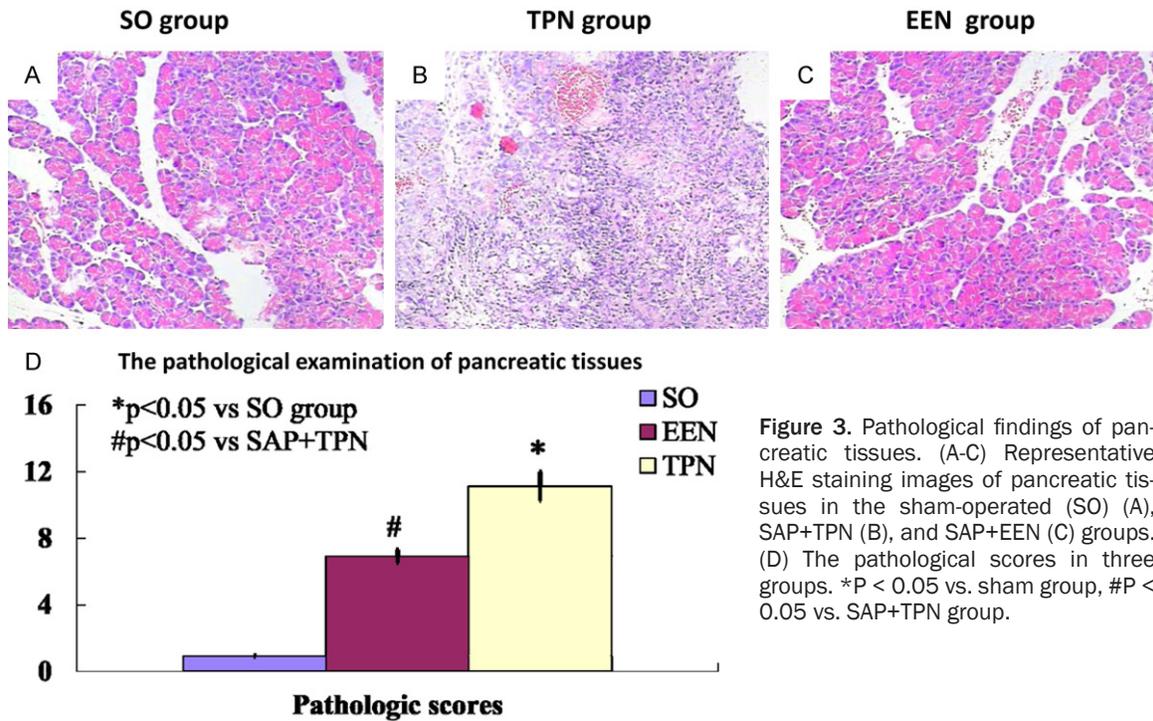
Figure 3. Pathological findings of pancreatic tissues. (A-C) Representative H&E staining images of pancreatic tissues in the sham-operated (SO) (A), SAP+TPN (B), and SAP+EEN (C) groups. (D) The pathological scores in three groups. *P < 0.05 vs. sham group, #P < 0.05 vs. SAP+TPN group.

expression in the intestine as compared with TPN, as well as a decrease in the intestinal NF-κB expression and the serum levels of TNF-α and IL-6. EEN treatment also alleviated pathological pancreatic damage to SAP. Our study suggests that EEN may inhibit the TLR4/NF-κB signaling pathway in the intestine, thus reducing release of inflammatory cytokines into the systemic circulation and causing damage to the pancreas in SAP. This study provides the evidence that EEN, not TPN, should be used for the treatment of SAP.

EEN has been demonstrated to better improve clinical outcomes than TPN does [16-19]. EEN treatment was superior to TPN in increasing survival rate of SAP rats. In addition, as compared with TPN, EEN could more effectively reduce the serum amylase content and alleviate pathological damage to the pancreas after SAP. It has been reported that intestinal barrier dysfunction is a main cause leading to SIRS, secondary pancreatic infection, multiple organ dysfunction syndrome as well as increased risk of death in patients with SAP [7]. Evidence has shown that enteral nutrition deficiency leads to intestinal barrier dysfunction in a mice model [24], while supplementation with EEN helps to maintain the intestinal epithelial barrier function in SAP patients [25]. Thus, our results that EEN reduced pancreatic damage and

improved the survival rate suggest that the beneficial effect of EEN may be because EEN can promote the maintenance of the integrity of the intestinal barrier.

In the present study, expression of TLR4 was further investigated in the intestine in SAP rats and found to be significantly increased as compared with those sham-operated rats, suggesting that SAP induced upregulation of TLR4 in the intestine. The mechanisms underlying upregulation of TLR4 in SAP remains unclear. It is well known that TLR4 recognizes lipopolysaccharide (LPS), a unique glycolipid on the outer wall of Gram-negative bacteria [10]. In addition, LPS stimulation upregulates TLR4 expression [26]. Therefore, it is likely that SAP impaired the intestinal epithelial barrier, leading to an increase in permeability of the intestine to Gram-negative bacteria. Excessive exposure to LPS may subsequently lead to upregulation of TLR4 in the intestine. Furthermore, increase of TLR4 expression in the SAP+EEN group was significantly less than that of the SAP+TPN group, suggesting that EEN was superior to TPN in inhibiting SAP-induced upregulation of TLR4 expression. It is unclear how EEN support inhibits SAP-induced upregulation of TLR4 expression. EEN support promotes the maintenance of the intestinal barrier function in SAP patients [25]. Thus, it is likely that EEN support may

inhibit upregulation of TLR4 in the intestine by protecting the integrity of the intestinal epithelial barrier. In addition, it has been reported that TLR4 expression on peripheral blood monocytes is altered during the early stage of AP [27], suggesting that TLR4 expression levels may be used to monitor the progression of AP.

Expression of NF-κB, the downstream transcription factor of TLR4, was further examined in the intestine of SAP rats receiving EEN or TPN and was significantly up-regulated in SAP rats, suggesting that TLR4 activated NF-κB in the intestine in SAP. Consistent with this, several studies have shown that the TLR4/NF-κB signaling pathway is activated in the pancreas, liver, kidney, and small intestine of SAP rats [13, 28]. It is well known that activation NF-κB by TLR4 results in the release of various inflammatory cytokines, such as TNF-α, IL-6, and IL-8 [10], and that inflammatory cytokines play an important role in SIRS and multiple organ dysfunction syndrome, thus contributing to the pathogenesis of SAP [5, 6, 9]. Consistent with these studies, we found that the serum levels of TNF-α and IL-6 were significantly higher in SAP rats compared with controls and suggest that activation of the TLR4/NF-κB signaling pathway in the intestine may promote the release of inflammatory cytokines into the circulation, thus leading to the development of SAP. In addition, expression of TLR4 and NF-κB was significantly reduced and serum levels of TNF-α and IL-6 were significantly lower in the SAP+EEN group than in the SAP+TPN group, suggesting that EEN treatment may inhibit activation of the TLR4/NF-κB signaling pathway induced by SAP and reduce the release of inflammatory cytokines.

In summary, for the first time, we found that EEN was better than TPN in reducing death and pancreatic damage in a rat model of taurocholate-induced SAP. EEN was more potent than TPN in inhibiting the expression of intestinal TLR4 and NF-κB and reducing serum levels of TNF-α and IL-6. Our findings suggest on one hand that activation of the TLR4/NF-κB signaling pathway might contribute significantly to the pathogenesis of SAP, and on the other hand that EEN might exert its beneficial effect against SAP by inhibiting the TLR4/NF-κB signaling pathway. However, our findings from this animal study must be confirmed by future clinical studies.

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