The effect of early enteral nutrition on intestinal mucosal barrier and remote organs in rats with severe acute pancreatitis

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Abstract

Background

The mechanism of early enteral nutrition on intestinal mucosal barrier is still not fully understood and the direct evidence to demonstrate the protective effects of early enteral nutrition against remote organs injury induced by severe acute pancreatitis is lacking.

Methods

Rats were randomly divided into three groups: 1) only flip pancreas and free diet (n = 8); 2) severe acute pancreatitis and total parenteral nutrition (n = 15); 3) severe acute pancreatitis and early enteral nutrition (n = 15). Severe acute pancreatitis was induced by the retrograde injection of pancreaticobiliary duct with sodium taurocholate. Nutritional supplementation was administered starting 12 hours after induction of pancreatitis. The samples were obtained after the treatment of nutrition 72 hours.

Results

The mortality was not significant difference between the experimental groups. The pathology of ileum and the content of serum diamine oxidase were significantly lower in early enteral nutrition group than total parenteral nutrition group. The expression of tight junction ZO-1, Occludin and Claudin-1 proteins and the intraepithelial CD8⁺ and TCR $\gamma\delta$ ⁺ lymphocytes were significantly higher in early enteral nutrition group than total parenteral nutrition group. The content of the serum amylase and endotoxin were significantly lower in early enteral nutrition group than total parenteral nutrition group. There was a tendency of decreased pancreas and lung pathology scores in early enteral nutrition group, but the difference between total parenteral nutrition and early enteral nutrition groups was not significant.

Conclusion

The early enteral nutrition could significantly improve the expression of the tight junctions and intraepithelial lymphocytes, accompanied by improved intestinal mucosal barrier and endotoxin, but failed to the pancreas and lung injury compared to total parenteral nutrition in the rat model of severe acute pancreatitis.

Background

Severe acute pancreatitis (SAP) is characterized by the presence of persistent single-organ or multiorgan failure (defined by organ failure that is present for ≥ 48 hours). Most patients who have persistent organ failure have pancreatic necrosis and a mortality of at least 30% [1]. When SAP, the loss of large amounts of body fluids results in a sharp decrease in the effective circulating blood volume. The reflex splanchnic

vasoconstriction to preserve the perfusion of vital organs results in intestinal ischemic injury, and resuscitation promotes reperfusion injury [2]. A critical event with the injury to the intestine is the loss of barrier function, increased permeability, and translocation of luminal bacteria and endotoxin, which may promote the development of systemic inflammatory response, multiple organ dysfunction syndrome (MODS) and even death [3]. The necrosis or inflammation of the pancreas and retroperitoneal tissue cause the increase of energy demand and the loss of large amounts of protein. Total parenteral nutrition (TPN) can meet the nutritional requirements by avoiding the stimulation of the pancreas, but it may lead to intestinal barrier dysfunction and then promote the gut derived infection [4]. Many studies including our previous study have demonstrated the importance of early enteral nutrition (EEN) in SAP, which could significantly reduce the rate of respiratory disease, pancreatic-related infections and MODS [5]. The gut has long been characterized as the “motor” of MODS in critical illness. The main theoretical basis of implementing EEN is its function in improving intestinal mucosal barrier and reducing the gut derived infection [3]. However, the mechanism of EEN on intestinal mucosal barrier in SAP is still not fully understood and the direct evidence to demonstrate the protective effects of EEN against remote organs injury induced by SAP is lacking.

Intestinal mucosal epithelium is the most important part of intestinal mucosal barrier, which include intestinal mucosal epithelial cells and intercellular connections. The intercellular connections are composed of tight junctions, adhesion junctions and desmosomes. The tight junctions act as selective osmotic barriers, which typically represent rate-limiting steps in paracellular transport[6]. Yasuda et al [7]. found that the tight junction ZO-1 and Occludin proteins were significantly reduced in the rat model of SAP, accompanied by increased apoptosis of intestinal epithelium and increased intestinal permeability. Lutgendorff et al. [8] found that the tight junction Occludin and Claudin-1 proteins were significantly reduced in the rat model of acute pancreatitis, accompanied by increased intestinal permeability. Shen et al. [9] indicated that implementing EN could improve the expression of Occludin compared with PN in the rat model of abdominal infection. However, the study about the effect of EEN on the tight junctions compared with TPN is shortage in SAP. Meanwhile, the gut-associated lymphoid tissue (GALT) is the important part of intestinal immune barrier. The GALT are complex multilayered immune tissue and include intestinal intraepithelial lymphocytes, lamina propria lymphocytes, peyer's patch and so on. The intraepithelial lymphocytes are closest to enteric cavity among the tissue of immune defense, which is regarded as the sentinel of the mucosal barrier [10]. Peng et al. [11] indicated that the EEN treatment significantly increased the expression of MAdCAM-1, CD4⁺ and CD8⁺ in Peyer's patch compared with TPN, accompanied by a decrease in the serum levels of endotoxin and bacterial translocation in the rat model of SAP. Zou et al. [12] indicated that the EN treatment significantly increased the percentage of CD3⁺ and CD4⁺ lymphocyte and the ratio of CD4⁺ and CD8⁺ lymphocyte subsets compared with PN in plasma of the pig model of SAP. However, the study about the effect of EEN on the intestinal intraepithelial lymphocytes compared with TPN is unclear in SAP.

Hence, our study focused on investigating the mechanism of EEN on intestinal mucosal barrier and the protective effect of EEN against remote organs injury induced by SAP.

Materials And Methods

Animals

Male Sprague-Dawley rats (6-8 weeks old), weighing 250–300 g, were used. Rats were acclimated for 1 week to a temperature-controlled room on a 12-h light/dark cycle and the ad libitum chow and water. Thirty-eight rats were randomly assigned to 3 groups: sham-operated group (SO group, n=8), SAP group receiving total parental nutrition (TPN group, n=15), and SAP group receiving early enteral nutrition (EEN group, n=15).

Animal model of SAP and nutrition support

SAP was induced in rats by infusion of 4% of sodium taurocholate as our previous study described by Kang et al. [13] Briefly, the rats were fasted overnight but allowed free access to water until 4 h before laparotomy. Animals were anesthetized by 4% isoflurane gas in table top animal anesthesia machine (RWD Life Science, Shenzhen, China). After the pancreatic duct was catheterized with a needle, the 4% sodium taurocholate was slowly injected from the tail of the pancreas. Approximately 2 min, the pancreas exhibited edema, exudation, and local hemorrhage, which indicated that the SAP was successfully induced. Rats in the SO group received the same surgical procedure without injection of sodium taurocholate. In all rats, one tube (1 mm epidural catheter) was placed at jejunum. Then this tube was tunneled subcutaneously and exited at the midpoint of back. The other tube (1 mm epidural catheter) was inserted into the right jugular vein. Then this tube was tunneled subcutaneously and exited at the midpoint of neck. After surgery, all rats were administered 4ml 0.9% saline subcutaneously to replace the fluid loss during surgery.

In EEN group, the rats received the EEN solution at 12h after surgery. The EEN solution contains 3.8% protein, 13.8% carbohydrates, 3.4% lipids, electrolytes, and multivitamins, with the calorie/volum ratio of 1:1 (1 kcal/L). In TPN group, the rats received the TPN solution at 12h after surgery. The TPN solution contains 4.1% protein, 14.0% carbohydrates, 3.0% lipids, electrolytes, and multivitamins, with the calorie/volum ratio of 1:1 (1 kcal/L). The EEN and TPN solutions were almost isocaloric and isonitrogenous to ensure the rats had the same nutritional intake. Each rat in both groups received the energy of 125 kcal/kg in the first day and 250 kcal/kg/d later. The samples were obtained after the treatment of nutrition 72 hours.

Enzyme-linked immunosorbent assay (ELISA)

We utilized the commercial ELISA kits (Jianglai biotechnology, Shanghai, China) to determine the serum amylase, diamine oxidase and endotoxin contents of abdominal aorta. The procedure was in accordance with the manufacturer's instructions.

Hematoxylin eosin (HE) Staining

The tissues (2 cm) specimens obtained during the rat experiment were immediately fixed in 10% paraformaldehyde and incubated overnight at room temperature. Next, tissue samples were embedded in paraffin and 5µm sections were cut. Sections were deparaffinized in xylene and rehydrated in graded ethanol to distilled water and stained with hematoxylin and eosin for histological analysis. The pathology of pancreas was evaluated by improved Schmidt score method [14]. The pathology of lung was evaluated by Smith score method [15]. The pathology of ileum was evaluated by Chiu score method [16].

Immunohistochemistry assay

For immunohistochemistry, sections were deparaffinized in xylene and rehydrated in graded ethanol to distilled water. Subsequently, the sections were processed using the DAB Detection Kit (ZSJQ, Beijing, China) according to the manufacturer's protocol. The IHC features shown in the figures are representative of all tissue samples studied. The primary antibodies are as following: Occludin Polyclonal Antibody (Invitrogen; 1:100 dilution); ZO-1 Polyclonal Antibody (Invitrogen; 1:100 dilution); Claudin-1 Polyclonal Antibody (MH25) (Invitrogen; 1:100 dilution).

Immunofluorescent assay

Frozen sections were cut at 15µm, and mounted on the slides. The nonspecific background was blocked by incubation with 3% bovine serum albumin in PBS for 1h at room temperature. The sections were incubated with Anti-ZO-1 (clone R40.76) (Merck&Millipore, 1:200 dilution), Occludin Rabbit Polyclonal Antibody (Proteintech, 1:50 dilution), Anti-Claudin 1 antibody (Abcam, 1:100 dilution), the mixture of Anti-mouse Monoclonal Anti-TCRγδ antibody (Abcam, 1:50 dilution) and Anti-LAMININ antibody (BOSTER, 1:50 dilution), and the mixture of Anti-CD8 antibody [OX-8] (Abcam, 1:50 dilution) and Anti-LAMININ antibody (BOSTER, 1:50 dilution) at 4°C overnight. The sections were probed with Anti-rabbit IgG (H+L) Alexa Fluor 488 (Cell Signaling Technology, 1:1000 dilution) and Goat Anti-Rat IgG H&L Alexa Fluor 594 (Abcam, 1:100 dilution), the mixture of Anti-rabbit IgG (H+L) Alexa Fluor 488 (Cell Signaling Technology, 1:1000 dilution) and Anti-mouse IgG (H+L) Alexa Fluor 594 (Cell Signaling Technology, 1:1000 dilution), and the mixture of Anti-rabbit IgG (H+L) Alexa Fluor 488 (Cell Signaling Technology, 1:1000 dilution) and Goat Anti-Rat IgG H&L Alexa Fluor 594 (Abcam, 1:100 dilution). The nuclei were counterstained with proLong Gold Antifade Reagent with DAPI (4, 6-diamidino-2-phenylindole, Cell Signaling Technology, Beverly, MA, USA). Slides incubated without any primary antibody were used as negative controls.

Western blot

Proteins were separated by SDS gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked with 3% bovine serum albumin prepared in TBST (Tris-buffered saline, pH 7.5 containing 0.05% Tween-20) for 1 h and then incubated with antibodies overnight at 4°C: Occludin Polyclonal Antibody (Invitrogen; 1:125 dilution); ZO-1 Polyclonal Antibody (Invitrogen; 1:125 dilution); Claudin-1 Polyclonal Antibody (MH25) (Invitrogen; 1:125 dilution) and Anti-beta Actin Antibody (Abcam; 1:1000 dilution). The membranes were then washed four times in TBST and incubated with secondary antibody at room temperature for 1 h. An enhanced chemiluminescence reagent,

WesternBright ECL HRP substrate (Advansta), was used to make the labeled protein bands detectable with Image System Minichemi (Tanon, Shanghai, China).

Statistical analysis

Statistical analyses were performed using SPSS 20.0 software. Quantitative data are expressed as means $\pm$ standard deviation. Levene's tests were used to test homogeneity of variance. For data with equal variance, one-way analysis of variance (ANOVA) was used to compare the difference among groups. Categorical data were analyzed using chi square tests. Probability values less than 0.05 were considered statistically significant.

Results

Mortality

In the SO group, no rats died. In contrast, 8 and 7 rats died in the TPN and EEN groups 72 hours after treatment of nutrition, respectively. The mortality was zero in the SO group, 53.3% in the TPN group, and 46.7% in the EEN group. The mortality was significantly higher in the TPN and EEN groups than in the SO group. The mortality was not significant difference between the EEN group and TPN group.

The contents of serum amylase, diamine oxidase and endotoxin

The serum amylase content was significantly higher in the TPN and EEN groups than in the SO group. The serum amylase content was significantly lower in the EEN group than in the TPN group (Fig. 1A). The serum diamine oxidase and endotoxin contents were significantly higher in the TPN group than in the SO group. The serum diamine oxidase and endotoxin contents were significantly lower in the EEN group than in the TPN group (Fig. 1B and 1C).

The pathology of pancreas, lung and ileum tissues

The pancreas and lung pathology scores were significantly higher in the TPN and EEN groups than in the SO group. Ileum pathology score was significantly higher in the TPN group than in the SO group. The ileum pathology score was significantly lower in the EEN group than in the TPN group. There was a tendency of decreased pancreas and lung pathology scores in EEN group, but the difference between TPN and EEN groups was not significant (Fig. 2A and 2B).

The expression of the tight junction ZO-1, Occludin and Claudin-1 proteins

In immunohistochemistry, immunofluorescence and western blot, the expression of the tight junction ZO-1, Occludin and Claudin-1 proteins were significantly lower in the TPN group than in the SO group. The expression of the tight junction ZO-1, Occludin and Claudin-1 proteins were significantly higher in the EEN group than in the TPN group (Fig. 3, 4 and 5).

The expression of intraepithelial CD8⁺ and TCR γ δ ⁺ lymphocytes

The expression of intraepithelial CD8⁺ and TCR γ δ ⁺ lymphocytes were significantly lower in the TPN group than in the SO group. The expression of intraepithelial CD8⁺ and TCR γ δ ⁺ lymphocytes were significantly higher in the EEN group than in the TPN group (Fig. 6).

Discussion

In this study, we found that the EEN could significantly improve the intestinal mucosal barrier and endotoxin, but failed to the pancreas and lung injury in the rat model of SAP. The main findings were summarized as following: (1) the results of survival rate, pathology of pancreas and lung and the serum amylase indicated that the rat model of SAP was successfully prepared; (2) the results of the pathology of ileum and the serum diamine oxidase indicated that EEN could improve the intestinal mucosal barrier; (3) To unveil the mechanism, the EEN could improve the intestinal mechanical barrier including the tight junction ZO-1, Occludin and Claudin-1 proteins, and the intestinal immune barrier including the intraepithelial CD8⁺ and TCR γ δ ⁺ lymphocytes. (4) The results of the serum amylase and endotoxin and the pathology of pancreas and lung indicated that EEN could not improve the pancreas and lung injury, but the decreasing trend was found.

In the process of SAP, the intestinal mucosal ischemia and hypoxia resulted in the failure of intestinal mucosal barrier. The bacteria, endotoxin and related toxic factors translocated from the intestinal lumen to mesenteric lymph nodes, systemic circulation and non-intestinal organs, such as lung and pancreas. The infected pancreatic necrosis and acute lung injury were extremely severe complication [17]. Enteral nutrition could directly provide nutrients needed to intestinal mucosa, and the enteral nutrient solution directly touched intestinal mucosa, which contributed to the proliferation of intestinal mucosal epithelium, the increase of blood supply to the intestinal mucosa and the improvement of intestinal mucosal barrier [18]. The intestinal mucosal barrier could be reflected by the pathology of intestine and the content of serum diamine oxidase [19]. Our results showed that pathology score of the intestine and the content of serum diamine oxidase were significantly lower in EEN group than TPN group, which indicated that EEN could improve the intestinal mucosal barrier. Our results showed that the serum amylase and endotoxin and the pathology of pancreas and lung indicated that EEN could not improve the pancreas and lung injury, but the decreasing trend was found. Qin et al. [20] indicated that EEN significantly improved the endotoxin and the bacterial translocation, but not the pathology of pancreas compared with TPN in the dog model of SAP. These results were consistent with our study. Fukatsu et al. [21] results showed that enteral nutrition could significantly reduce the vascular permeability index expressed by count ¹²⁵I-labeled albumin activity, but not in the wet weight/dry weight ratio and myeloperoxidase activity of the lung compared with TPN in the rat with superior mesenteric artery occlusion. Wu et al [22]. results showed that enteral nutrition including high-lipid significantly improved intestinal mucosal barrier and partially lung inflammation (tumor necrosis factor- α and macrophage inflammatory protein-2), but not in oxidation and pathology of the lung in the rats with hemorrhagic

shock/resuscitation. The pathological mechanism of lung injury in SAP was multiple and may be involved with pancreatic necrosis, bacteremia, intestinal barrier failure, activation of inflammatory cascades and diffuse alveolar damage [23]. Just as our result, the EEN could improve the intestinal mucosal barrier, but not in lung injury.

The tight junction, also known as zonula occludens, was a multifunctional complex with dynamic changes composed of a variety of proteins. It was located at the top of the side of adjacent cells of the intestinal mucosal epithelium and banded around the cells [6]. Tight junctions form two types of barriers: a paracellular one that regulates selective paracellular permeability and an intramembrane one that restricts exchange of membrane components between the apical and basolateral cell surface domains. The tight junctions were mutated, reduced or deleted, which resulted in the increase of intestinal permeability and translocation of bacteria and endotoxins. The ZO-1 belonged to cytoplasmic protein, which was the first identified tight junction. The Occludin and Claudin-1 were transmembrane protein. The PDZ domain of ZO-1 was connected respectively with the c-terminus of Occludin and Claudin-1 and they were anchored to the cytoskeleton, which played a major role in intestinal barrier [24]. Our results showed that the expression of the tight junction ZO-1, Occludin and Claudin-1 proteins were significantly higher in EEN group than TPN group. Sun et al. [25] indicated that TPN treatment significantly decreased the expression of the tight junction ZO-1, Occludin and Claudin-2, 7, 15 proteins compared with free diet in rats. Wu et al. [26] indicated that EEN treatment significantly increased the expression of the tight junction ZO-1, Occludin and Claudin-1 proteins compared with TPN in the rat model of intestinal ischemia/reperfusion injury. These results are similar to the results of our study.

The intestinal epithelial lymphocytes (IEL) were mainly composed of T lymphocytes. Unlike lymphocytes in spleen, blood or lymph nodes, the IEL had unique T lymphocyte phenotypes that mainly were CD8⁺ phenotype and some TCR $\gamma\delta$ ⁺ phenotype. The IEL were distributed among intestinal mucosa columnar epithelial cells, which played the role of immune surveillance and defense [27]. Our results showed that the expression of intraepithelial CD8⁺ and TCR $\gamma\delta$ ⁺ lymphocytes were significantly higher in EEN group than TPN group. Yang et al. [28] indicated that TPN treatment significantly decreased the overall number of IEL, so does CD8⁺ lymphocytes compared with free diet in rats. Hong et al. [29] indicated that enteral nutrition treatment significantly increased the overall number of IEL, so does CD8⁺ lymphocytes in the patients with long-term fasting intestinal fistula. Wan et al. [30] indicated that partial enteral nutrition significantly decreased the ratio of *Bacteroidetes* and *Tenericutes* compared with parenteral nutrition. David et al. [31] indicated that intestinal microorganisms significantly affect the distribution, migration and energy metabolism of intestinal TCR $\gamma\delta$ ⁺ lymphocytes. Therefore, we speculated that EEN treatment may affect the intraepithelial TCR $\gamma\delta$ ⁺ lymphocytes through changing intestinal microorganisms.

There are two major limitations in this study. Firstly, we only performed a short-term survival analysis in SAP animals. Our previous study had showed that the major of deaths occurred within 48h after finished model [13]. Therefore, the survival observation was ended at 72h after finished model in the present

study. Secondly, we demonstrated that the EEN could not improve the pathology of pancreas and lung in the rat model of SAP, but the underlying mechanisms need to be explored in our future studies.

Conclusion

In summary, this experimental study demonstrated that EEN could significantly improve the intestinal mucosal barrier and endotoxin. To unveil the mechanism, the EEN could significantly improve the intestinal mechanical barrier including the tight junction ZO-1, Occludin and Claudin-1 proteins, and the intestinal immune barrier including the intraepithelial CD8 + and TCR $\gamma\delta$ + lymphocytes. But the EEN could not improve the pancreas and lung injury in the rat model of SAP. These findings may be useful in the development of early enteral nutrition and a more personalized treatment to restore the intestinal barrier protective functions in the patients with SAP.

Abbreviations

SAP: Severe acute pancreatitis; MODS: Multiple organ dysfunction syndrome; TPN: Total parenteral nutrition; EEN: Early enteral nutrition; GALT: gut-associated lymphoid tissue

Declarations

Acknowledgments

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Authors' contributions

XGL and JBS designed this study; JBS, LBZ, XK, YLZ and YLY performed animal experiments; JBS, LBZ, YLZ and WY performed biochemical and histological analysis; JBS, WXG and YS collected data and performed data analysis; JBS wrote this manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data are available from the corresponding author upon request.

Ethics approval and consent to participate

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals and was approved by the Animal Use and Care Committee of Nanjing University of Chinese Medicine.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

The contents of serum amylase (A), diamine oxidase (B) and endotoxin (C). *P <0.05 compared with SO group; #P <0.05 compared with TPN group.

Figure 2

The pathology of pancreas, lung and ileum. (A): Images of sections stained with hematoxylin and eosin from the pancreas (200'), lung (200') and ileum (100'); (B): The pathology score of pancreas, lung and ileum tissues. *P <0.05 compared with SO group; #P <0.05 compared with TPN group.

Figure 3

The expression of the tight junction ZO-1, Occludin and Claudin-1 proteins. (A): Images of immunohistochemistry from the ileum (200'); (B): The average optical density of expression of ZO-1,

Occludin and Claudin-1. *P <0.05 compared with SO group; #P <0.05 compared with TPN group.

Figure 4

The expression of the tight junction ZO-1, Occludin and Claudin-1 proteins. (A): Images of immunofluorescent from the ileum (200 $\times$); ZO-1 polyclonal antibody (red); Occludin polyclonal antibody or Claudin-1 polyclonal antibody (MH25) (green); DAPI (blue). (B): The immunoreactivity score of expression of ZO-1, Occludin and Claudin-1. *P <0.05 compared with SO group; #P <0.05 compared with TPN group.

Figure 5

The expression of the tight junction ZO-1, Occludin and Claudin-1 proteins. (A): Images of western blot from the ileum; (B): The ratio of cumulative optical density of expression of ZO-1, Occludin and Claudin-1. *P < 0.05 compared with SO group; #P < 0.05 compared with TPN group.

Figure 6

The expression of intraepithelial CD8⁺ and TCR $\gamma\delta$ ⁺ lymphocytes. (A): Images of immunofluorescent from the villi (200 $\times$); Anti-CD8 antibody or Anti-TCR $\gamma\delta$ antibody (red); Anti-laminin antibody (green); DAPI (blue). (B): The number of expression of intraepithelial CD8⁺ and TCR $\gamma\delta$ ⁺ lymphocytes. Data were from 50 villi each group. *P < 0.05 compared with SO group; #P < 0.05 compared with TPN group.