

Original Article

Abdominal paracentesis drainage attenuates intestinal barrier dysfunction via upregulating ZO-1 expression in rats with severe acute pancreatitis

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Abstract: Background: Our previous reports found that abdominal paracentesis drainage (APD) benefits the clinical outcome in patients with severe acute pancreatitis (SAP). However, the effect of APD on SAP-associated intestinal injury remains unclear. Objective: We aimed to determine whether APD could attenuate intestinal barrier dysfunction and bacterial translocation in rats with SAP. Methods: Sprague-Dawley rats were divided into sham operation, SAP and APD groups after labeling with green fluorescent protein-expressing *Escherichia coli* (GFP-E. coli). The SAP group was induced with sodium taurocholate. Rats in the APD group had a drainage tube inserted following SAP. 24 hours after the operation, the samples of blood, intestine, pancreas and mesenteric lymph nodes (MLN) were taken for histological, bacteriological, and serum analysis. Results: APD decreased the translocation of bacteria to the MLN and pancreas. Furthermore, we found that APD could exert a protective role in intestinal barrier function, which was exhibited by the decreased pathological scores and the serum levels of iFABP, DAO and D-lactate. Importantly, APD upregulated the expression of ZO-1 in intestine, thus maintaining the integrity of intestinal barrier. Conclusion: Together, our results indicate that APD reduces bacterial translocation and intestinal barrier dysfunction, thereby resulting in a protective role in SAP-associated intestinal injury.

Keywords: Abdominal paracentesis drainage, severe acute pancreatitis, bacterial translocation, intestinal barrier

Introduction

Severe acute pancreatitis (SAP) is one of the most critical diseases with high morbidity and mortality, which often leads to pancreatic necrosis and distant organ failure [1, 2]. Studies show that the intestine is frequently vulnerable to injury in the progression of SAP. Intestinal edema, ascitic fluids, bacterial translocation, and intestinal barrier dysfunction can be observed in SAP [3]. Despite continuous advances in therapeutic modalities and intensive care, the mortality rates of SAP are still high, and improvements in SAP-associated intestinal injury are required [4-6].

The intestinal barrier function plays an important role in maintaining intestinal function, which can prevent the invasion and systemic spread of bacteria and toxins in the intestinal

lumen [7]. During SAP, the intestinal mucosal structure is destroyed, resulting in bacterial translocation from the intestinal tract to the mesenteric lymph nodes and/or distant organs occur subsequently in the course of SAP [8]. Evidence from animal studies indicates that when the intestinal barrier is improved, the translocation of bacteria is reduced in rats with SAP [9-11]. Therefore, it is clinically imperative to develop effective strategies to maintain the structural integrity of intestinal epithelium and to prevent against the infection caused by SAP. In our recent retrospective study, abdominal paracentesis drainage (APD) was found to effectively relieve or control the severity of SAP, shorten the length of the hospital stay, and not increase the probability of abdominal infection [12, 13]. Experimental evidence also showed the effectiveness of APD in rats with SAP [14, 15]. However, the effect of APD on gut barrier

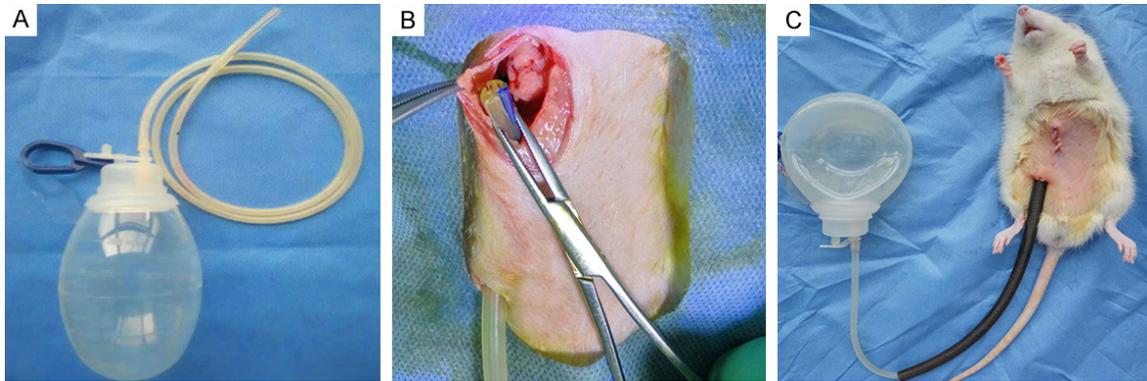


Figure 1. APD treatment in rats with SAP. A. APD device with a negative-pressure drainage ball; B. APD device was inserted in the lower right abdomen of the rats; C. Ascites drained by APD.

dysfunction and bacterial translocation in SAP patients remains unclear.

In the present study, we aimed to investigate the effect of APD treatment on intestinal barrier function and bacterial translocation in rats with SAP and determined whether the tight junction-associated protein ZO-1 is involved in the beneficial effects of APD.

Materials and methods

Construct of a visible model of intestinal bacterial translocation in rats

Luria-Bertani (LB) plate culture medium was used for monoclonal screening after successful heat shock transformation of an ampicillin resistance eGFP plasmid (pBluescript II KS(+)-eGFP, Shanghai Jiran Biology, Inc.) into *E. coli* DH5 α competent cells (TAKARA Biotechnology (Dalian). Co. Ltd). Then, LB liquid culture medium was used to amplify a large amount of GFP-*E. coli*. Culture of the stool from male SD rats (approximately 200-250 g, 9 weeks, Chengdu Dossy Experimental Animals Co. Ltd) on LB plate culture medium was performed to identify animals with ampicillin-resistant bacteria in their stool. The rats were fed 300 mg/L of an ampicillin solution as their daily drinking water for intestinal preparation for 3 days. From the 4th day to the 6th day, the rats were administered GFP-*E. coli* bacteria (10 mL/kg, 1/d) daily at 1 min after the gavage of 1 mL of 1.5% sodium bicarbonate solution. From the 7th day to the 9th day, the rats were fed ampicillin solution. On the 10th day, their stool was cultured using 100 mg/L ampicillin in LB culture medium. Ampicillin-resistant bacteria were isolated from the stool, which suggested successful coloniza-

tion of the GFP-*E. coli* bacteria within the gut, which was used as a visualization model of intestinal bacterial translocation in rats [16, 17].

Animal grouping and model preparation

Seventy-five male SD rats were randomly divided into three groups: a sham operation group (sham group), an SAP group and an APD group. After colonization in the gut, 100 mg/L ampicillin solution was administered freely before and after the operation but was withheld from food 12 h before and after the operation. The animal models were prepared as follows. In the sham group, the pancreas of each rat was exposed for as long as in the other two groups. In the SAP group, 5% sodium taurocholate (American Sigma company) was injected at a rate of 12 mL/h into the pancreatic duct using a micro infusion pump (0.1 mL/100 g rat weight), and the abdomen was then closed according to the classical method of Aho [18]. In the APD group, a "+" was placed onto the indwelling drainage tube (Fr16, Shandong Baiduoan Co. Ltd) in the lower right abdomen of the rats with a negative-pressure drainage ball (Figure 1) according to the methods of Chen [14] and Zhou [15], after the establishment of a SAP model. All rats were injected with 37°C saline (4 mL/100 g) in the back after the operation.

Harvesting of tissue specimens

All rats were anesthetized 24 h after their operations, and all subsequent procedures were performed under strict aseptic conditions. Following euthanasia, as much abdominal aortic blood as possible was collected after opening the abdomen, and the mesenteric lymph

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nodes and pancreatic tissues were harvested as a 200 mg specimen. Sterile saline, 1 mL, was added to the specimens, and then the specimens were homogenized on ice and stored at 4°C for future analysis.

Histological examinations and scores

The remaining cut pancreatic tissues and approximately 1.5 cm of ileum tissue at the end of the ileum 3 cm from the appendix were placed in 4% neutral formaldehyde for a 24 h fixation, embedded in paraffin, cut into 4- μ m-thick sections and mounted on slides. Then, the slides were normally stained with hematoxylin/eosin (H&E), sealed, and finally observed under light microscopy. The biopsy was performed according to the standard procedure by Chiu, and the average score of 10 points of view was regarded as the final score of each slice [19, 20].

Detection of serum-related parameters

Serum iFABP, DAO, D-Lactate, CRP, TNF- α , and IL-1 β was detected using an ELISA kit (Wuhan Eli Reiter Biological Technology Co. Ltd) according to the manufacturer's instructions. A full automatic enzyme standard instrument (American Thermos Company) was used to detect the previously mentioned serum factors.

Bacterial culture and identification

A homogenate sample volume of 0.2 mL was well distributed on LB agar plates containing 100 mg/L ampicillin, and then the plates were cultured at 37°C for 16 h. Finally, bacterial colony counts were performed, and the bacterial translocation rate was calculated. A sterile toothpick was used to pick a single colony and then inoculate 3 mL of LB culture medium containing 100 mg/L ampicillin. Bacterial culture was performed at 180 rpm oscillation under 37°C for 12 h, followed by identification of bacteria. A volume of 5 μ L of bacterial culture was placed onto a slide with a cover slip, and the fluorescence image of bacteria was obtained with an inverted phase contrast fluorescence microscope (Olympus IX81). Image-Pro Plus 5.1 software was used to obtain the fluorescence image.

Immunohistochemical examinations

The slides were deparaffinized and rehydrated in an ethanol gradient and then incubated in

3% H₂O₂ for 10 min. Then, the samples were added with rabbit polyclonal anti-bodies against ZO-1 (Abcam, USA, 1:100) and incubated at 4°C overnight. Subsequently, the sections were incubated with biotinylated mouse anti-rabbit antibodies (Boster, China, 1:400) and peroxidase-conjugated avidin (Santa Cruz Biotechnology, 1:200) at 37°C for 30 min after being washed in PBS. The color of the solution became brown 5 min after the addition of DAB (3, 3'-diaminobenzidine). Finally, the specimens were counterstained with hematoxylin. We also prepared appropriate positive and negative control slides as above.

Western blotting of tight junctional proteins

Tissue from rat were homogenized by homogenizer in RIPA buffer (Biyuntian Shanghai, China). The tissue suspension was centrifuged at 12,000 rpm for 10 min at 4°C and the supernatants were collected and stored at 80°C. Proteins in the supernatants were extracted via a Whole-Cell Lysis Assay kit according to the manufacturer's instructions (KeyGEN BioTECH, Nanjing, China). The protein concentration was determined with a commercial BCA protein assay kit (Biyuntian Shanghai, China). Each protein sample was separated via electrophoresis in a 6% SDS-polyacrylamide gel, and the proteins were transferred to PVDF nitrocellulose membrane. Blots were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS)-0.1% Tween for 1 h at room temperature and washed 3 times in TBS, and then incubated with a primary antibody for ZO-1 (Abcam, Cambridge, MA, diluted to 1:2000) and an anti-GAPDH antibody (1:2000, Abcam, Cambridge, MA) in TBS-0.1% Tween overnight at 4 °C. The membranes were washed three times in TBS-Tween and then incubated with a goat anti-rabbit IgG secondary antibody (Bio-Rad, Hercules, CA) for 1 h at room temperature. The protein expression was detected using a chemiluminescent reagent (Amersham Biotechnology Pharmacia, Piscataway, NJ) and Quantity One software (Bio-Rad, Hercules, CA).

Statistical methods

SPSS 13.0 software was used for statistical analysis. The data were expressed as the mean plus or minus the standard deviation, and the normally distributed data were analyzed by ANOVA. The abnormally distributed data were analyzed by the rank sum test and U-test.

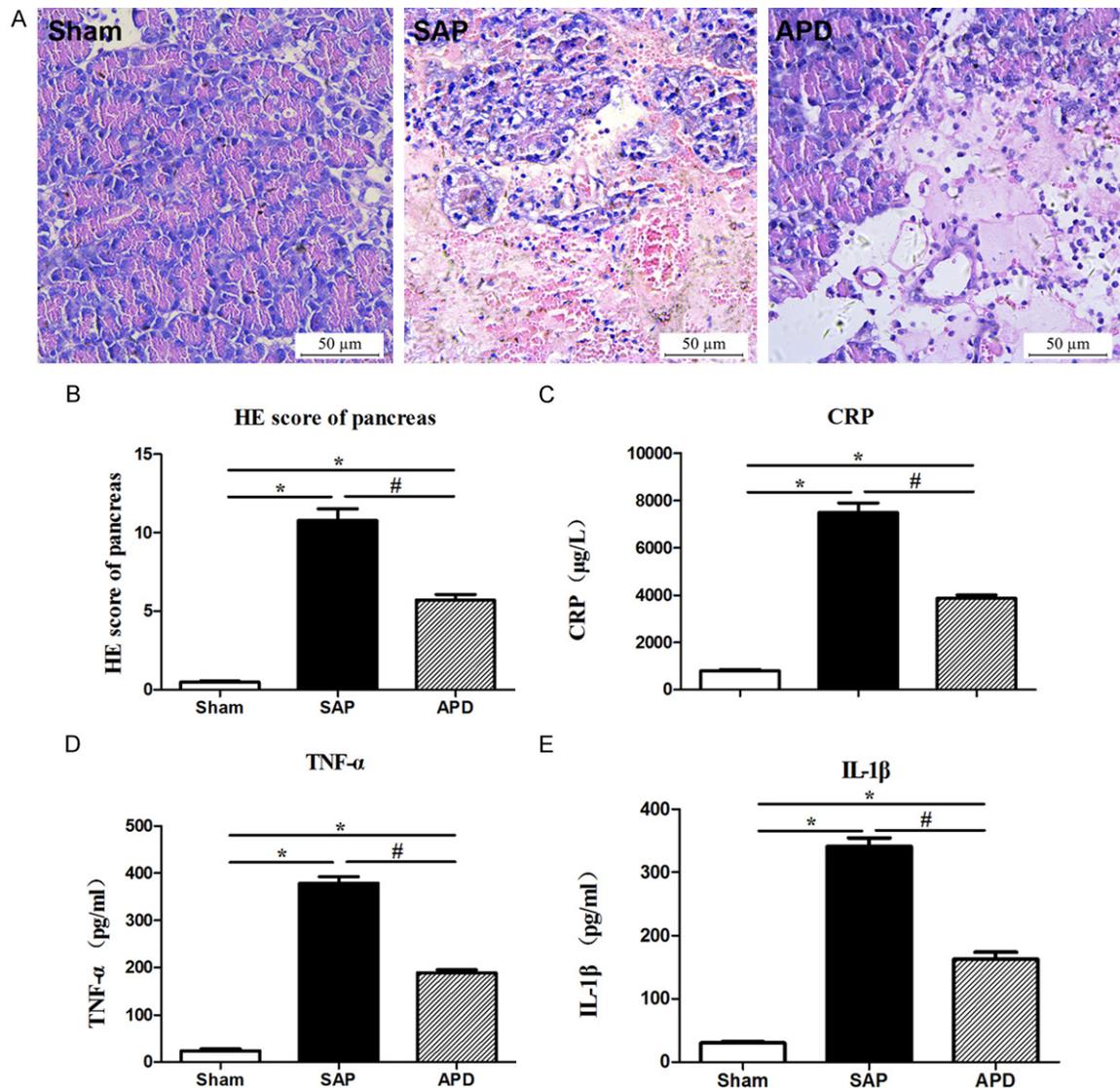


Figure 2. APD ameliorated the severity of SAP. A. Histological changes of the pancreas after 24 h of treatment (HE staining). The structure of the pancreas is generally normal in the sham group. The necrosis of pancreatic acinar cells and inflammation were both clearly noted in the pancreatic tissues in the SAP and APD groups. However, the severity of the pathological damage decreased more in the APD group than in the SAP group. B. There was a significant difference in the histological score in the SAP group and the APD group compared with the sham group ($P<0.05^*$), while the histopathological scores for the APD group were significantly lower when compared with the SAP group ($P<0.05^{\#}$). C-E. The serum levels of CRP, TNF- α and IL-1 β significantly increased in the SAP group and APD group than in the sham group ($P<0.05^*$). When compared with the SAP group, a significant decrease in the serum levels of CRP, TNF- α , and IL-1 β was observed in the APD group ($P<0.05^{\#}$).

$P<0.05$ was considered statistically significant.

Results

APD ameliorated the severity of pathological damage in rats with SAP

The 24 h survival rate after SAP induction was 48% (12/25) in the SAP group, whereas it was

80% (20/25) in the APD group and 100% (25/25) in the sham group. These rates were significantly different ($P<0.05$), indicating that APD treatment increased the survival rate of rats with SAP.

The effect of APD treatment on pathological changes of SAP was observed at 24 h after modeling by HE staining. As shown in **Figure 2A**, the structure of the pancreas is generally

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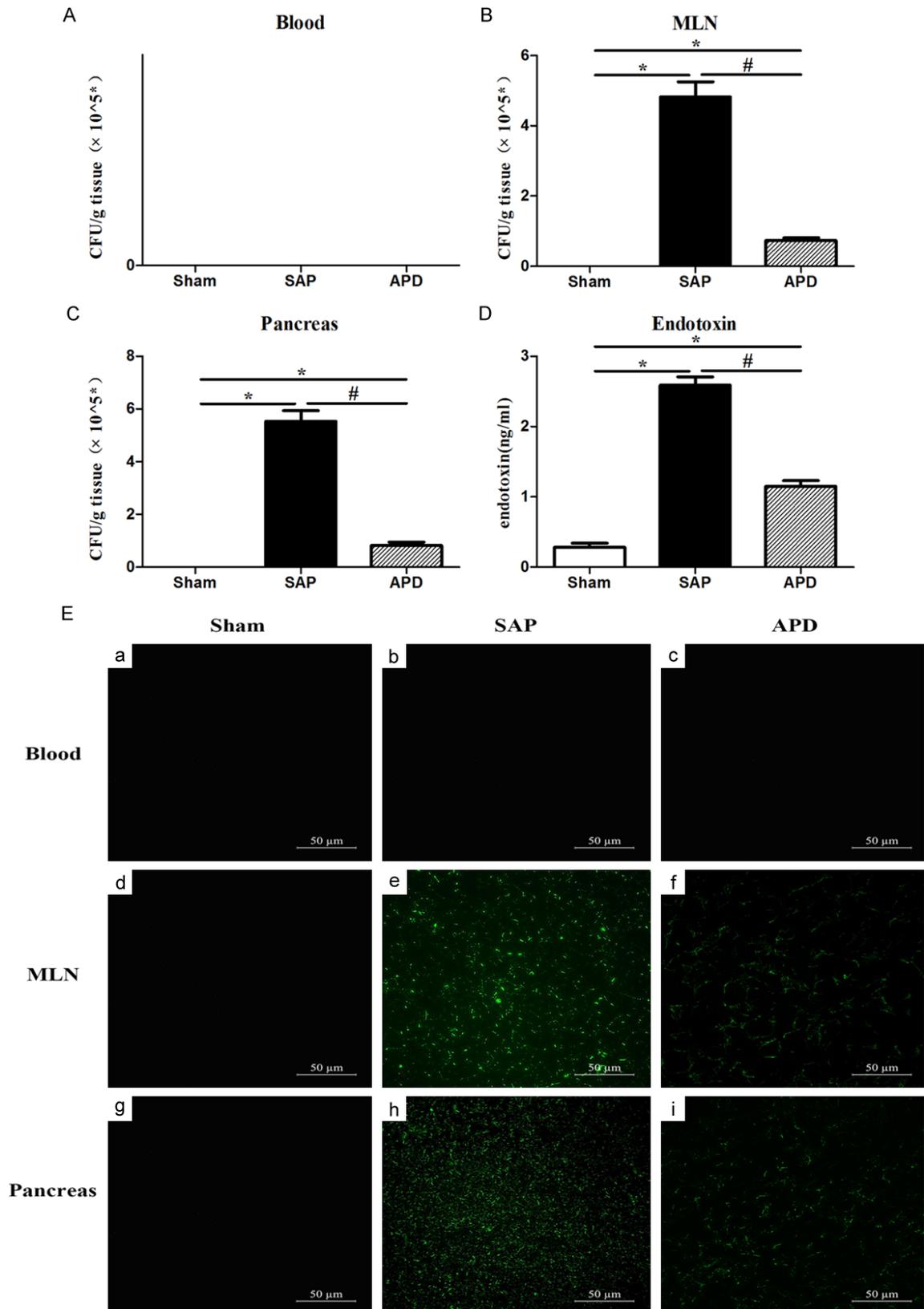


Figure 3. Bacterial translocation. A-C. Bacterial translocation of blood, MLN and pancreatic tissues; D. Level of serum endotoxin. Compared with the sham group, the above indicators in the SAP and APD groups were significantly higher, and the difference was statistically significant ($P < 0.05^*$). The above indicators were significantly lower in the

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APD group than in the SAP group, and the difference was statistically significant ($P < 0.05^{\#}$). E. Fluorescence detection of bacterial culture 24 h after treatment. Mesenteric lymph nodes and pancreatic tissues from sham groups as well as blood samples from all the 3 groups did not exhibit green fluorescence by GFP-E. coli. SAP group mesenteric lymph nodes and pancreatic tissues produced a large amount of GFP-E. coli. APD group mesenteric lymph nodes and pancreatic tissues produced a small amount of GFP-E. coli.

normal in the sham group. The necrosis of pancreatic acinar cells and inflammation were both clearly noted in the pancreatic tissues in the SAP and APD groups. However, the severity of the pathological damage decreased in the APD group more than that in the SAP group (**Figure 2A**). The histopathological scores of the sham group, SAP group and APD group were 0.47 ± 0.09 , $4.18 \pm 0.32^*$, $2.34 \pm 0.21^{\#}$ (compared with the sham group, $P < 0.05^*$, compared with the SAP group, $P < 0.05^{\#}$), respectively (**Figure 2B**). These differences in morphological changes were further supported by the observed serum levels of inflammatory factors. The serum levels of CRP, TNF- α and IL-1 β significantly increased in the SAP group and APD group compared to the sham group ($P < 0.05$, **Figure 2C-E**). However, when compared with the SAP group, a significant decrease in serum levels of CRP, TNF- α , IL-1 β was observed in the APD group ($P < 0.05$, **Figure 2C-E**).

APD decreased bacterial translocation in rats with SAP

Given that bacterial translocation from the intestine is an early event in SAP, we next determined whether APD played a role in bacterial translocation from the intestinal tissue. Therefore, we assessed the number of bacterial colonies (CFU/g tissue) cultivated per unit mass of blood, MLN and pancreatic tissues at 24 h after operation. In the sham group, no colonies were observed in any sample of the blood, MLN and pancreatic tissues. In contrast, bacterial colonies appeared in the SAP and APD groups. However, the number of colonies in the APD group was significantly lower when compared with that in the SAP group (**Figure 3A-C**). This result was also supported by the observed changes in the endotoxin levels. The ELISA method showed that the serum endotoxin level was significantly higher in the SAP ($2.59 \pm 0.12^*$) and APD ($1.15 \pm 0.08^{\#}$) groups than in the sham group (0.28 ± 0.06) ($P < 0.05^*$), while the level was lower in the APD group than in the SAP group ($P < 0.05^{\#}$) (**Figure 3D**).

To further evaluate the role of APD on bacterial translocation, we measured the bacterial trans-

location by culturing blood, MLN and pancreatic tissues and evaluating using a fluorescence microscope. As shown in **Figure 3E**, there was a mass of GFP-E. coli observed from the MLN and pancreatic tissues in the SAP and APD groups while nothing was observed in the sham group. However, there was a significant increase in the number of GFP-E. coli in the MLN and pancreatic tissues in the APD group than in the SAP group, suggesting that APD decreased the magnitude of translocation to the MLN.

APD improved intestinal mucosal barrier function in rats with SAP

Given that intestinal mucosal barrier dysfunction is one of the main promoters of bacterial translocation in SAP, we speculated that APD could improve the intestinal mucosal barrier function and thus lead to reduced translocation. Therefore, we first performed histological examinations of intestinal tissues in the three groups. As demonstrated in **Figure 4A**, the morphological structure of intestinal mucosa was generally normal with orderly, continuous and complete villi in the sham group. In the SAP group, there were clearly shortened and defected villi with partial disintegration and inflammatory cell infiltration within intestinal mucosa. However, a significant improvement in the structure of the intestinal mucosa was noted in the APD group, which was also supported by the results of the histopathological scores. The histopathological scores of the small intestine were significantly higher in the SAP ($4.18 \pm 0.32^*$) and APD ($2.34 \pm 0.21^{\#}$) groups than in the sham group (0.46 ± 0.11) ($P < 0.05^*$), while they were lower in the APD group than in the SAP group ($P < 0.05^{\#}$) (**Figure 4B**).

Considering that iFABP is an early marker of intestinal barrier dysfunction, we assessed the serum concentration of iFABP. As shown in **Figure 4C**, the level of serum iFABP was significantly elevated in the SAP and APD groups compared with the sham group. However, the serum iFABP level was significantly lower in the APD group than that in the SAP group. In agreement with the different level of serum iFABP, the levels of DAO and D-lactate were also

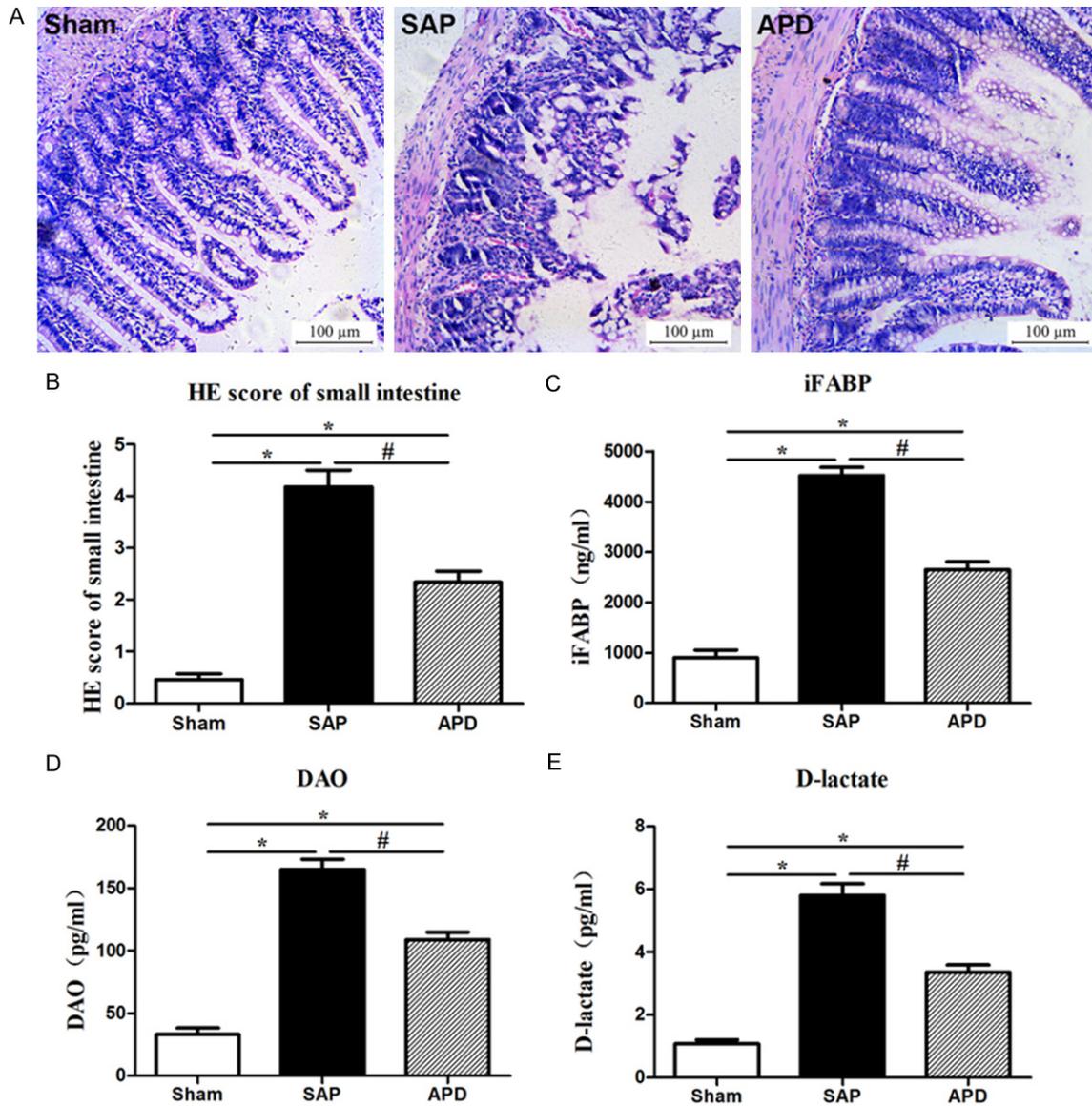


Figure 4. Intestinal barrier function. A. Histological changes of the small intestine 24 h after treatment. In the sham group, the morphological structure of the intestinal mucosa is generally normal with orderly, continuous and complete villi. In the SAP group, there were clearly shortened and defected villi with partial disintegration and inflammatory cell infiltration within the intestinal mucosa. However, a significant improvement in the structure of intestinal mucosa was noted in the APD group. B shows the intestinal pathology score of the three groups of rats; C-E show the serum iFABP, DAO and D-lactate levels. The above indicators were significantly higher in the SAP and APD groups than in the sham group, and the difference was statistically significant ($P < 0.05^*$). The indicators were significantly lower in the APD group than in the SAP group, and the difference was statistically significant ($P < 0.05^{\#}$).

remarkably reduced in the APD group compared with those in the SAP group ($P < 0.05$, **Figure 4D, 4E**). Together, these results indicate that APD protected the intestinal barrier function in rats with SAP.

APD upregulated ZO-1 expression in the intestinal tissues

Given that the tight junction protein ZO-1 is important for the structural integrity of the

intestinal mucosa epithelium, we examined the expression of ZO-1 protein in the intestinal tissues via immunohistochemistry staining and western blotting. As shown in **Figure 5**, strong immunostaining of ZO-1 protein was observed in normal intestinal tissue in the sham group. In contrast, the SAP group displayed significantly weaker expression of the ZO-1 protein in the intestine compared with that in the sham group. However, the APD group exhibited stronger

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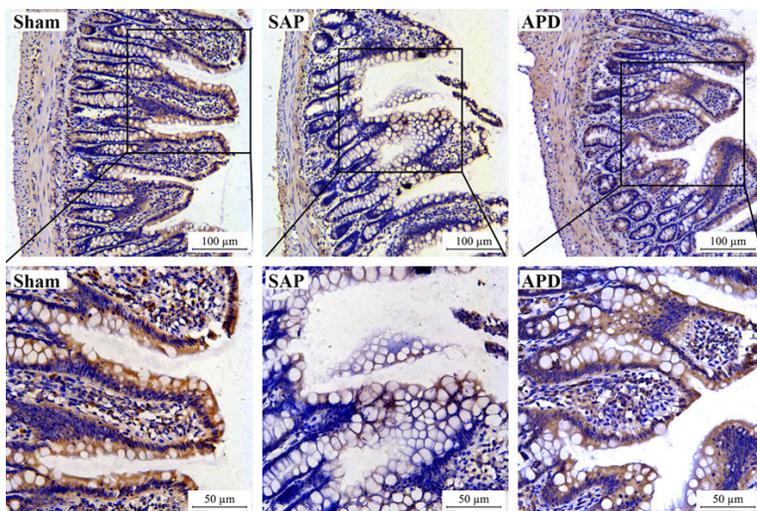


Figure 5. Representative immunohistochemical analyses of the intestinal tight junction protein ZO-1. Strong immunostaining was noted in the sham group. The SAP group displayed significantly weaker expression of the ZO-1 protein in the intestine compared with the sham group. The APD group exhibited stronger ZO-1 expression in the intestine than that in the SAP group.

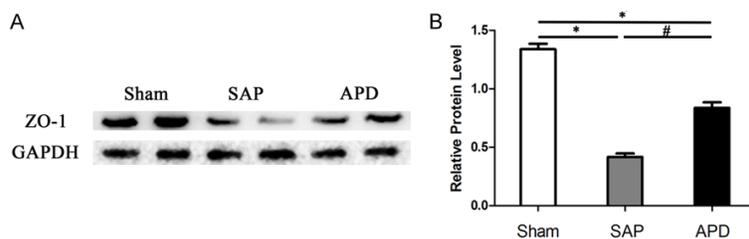


Figure 6. WB analyses of the intestinal tight junction protein ZO-1. A large number of the ZO-1 protein was observed in the sham group. The SAP group displayed significantly weaker expression of the ZO-1 protein ($P < 0.05^*$), while the APD group exhibited stronger ZO-1 expression in the intestine than that in the SAP group ($P < 0.05\#$).

ZO-1 expression in the intestine than that in the SAP group. Western blot analysis further showed that the protein levels of ZO-1 in intestinal tissues were significantly higher in the APD group compared to the SAP group (**Figure 6**). The results indicated that APD could upregulate the expression of ZO-1 in the intestinal tissues from rats with SAP.

Discussion

APD has been reported by our group to exert beneficial effects in patients with SAP [12, 13]. However, the role of APD in SAP-associated intestinal injury has not yet been studied [1, 6]. In the present study, we report for the first time, that APD reduces bacterial translocation and intestinal barrier function in rats with SAP.

Furthermore, we discovered that APD upregulates the expression of ZO-1 in intestinal tissues, thus maintaining the integrity of intestinal mucosal barrier, and ultimately plays a protective role in intestinal injury of SAP. Our findings provide new insight into understanding the effectiveness of APD, which might strengthen the clinical efficacy of APD for treating SAP.

SAP is one of the most critical diseases with a high mortality rate [21, 22]. Systemic inflammatory and infectious complications, such as pancreatic necrosis and distant organ failure, frequently occur, which make SAP management complicated [23]. Although some progress has been made in SAP management, specific treatments for these diseases remains limited [1, 24]. In our recent study, we discovered that APD could benefit patients with SAP [12, 13], which has also been confirmed in experimental animals [14, 15]. In the present study, we also demonstrated that APD treatment could significantly alleviate the pathological damage to pancreatic tissues and re-

duce the serum levels of inflammatory factors, including CRP, TNF- α and IL-1 β , in rats with SAP, suggesting that APD improves the systemic inflammatory response and ameliorates the severity of SAP.

Bacterial translocation from the intestine is widely considered as a primary mechanism underlying the progression of pancreatic infections and necrosis [9, 25]. For example, Samel *et al.* demonstrated that GFP-labeled bacteria gradually translocate from the intestinal cavity to the mucosa, muscularis serosa and finally pancreatic tissues in SAP rats [16]. Likewise, in our study, the overall incidence of bacterial translocation to the mesenteric lymph nodes and pancreases was 100% in both SAP groups, and the serum endotoxin levels were signifi-

cantly higher in the SAP group than in the sham group. Importantly, we found that APD reduced bacterial translocation from the intestinal cavity to the mesenteric lymph nodes and pancreas, which was supported by the finding of significantly decreased bacterial colonies and serum endotoxin levels in mesenteric lymph nodes and pancreatic tissues. There were significant reductions in the number of bacterial colonies cultivated from the MLN and pancreatic tissues and endotoxin levels in the APD group. Of note, this finding is the first evidence that APD reduces bacterial translocation from the gut in SAP.

Because bacterial translocation is considered one main cause of pancreatic infections and necrosis, therapeutic strategies that reduce bacterial translocation have been investigated in several studies [5, 26]. For example, Peng *et al.* demonstrated that early enteral nutrition could improve the intestinal immune barrier function, thus reducing intestinal bacterial and endotoxin translocation and improving the survival rate of SAP rats [11]. Sun *et al.* showed that melatonin could improve intestinal barrier dysfunction and reduce bacterial translocation, thus reducing the risk of pancreatitis-associated infection and early mortality [27]. In this study, we utilized GFP-E. coli as a tracer and directly observed the bacterial translocation from the gut to the MLN and pancreatic tissues in SAP rats, which was significantly improved after early APD.

The intestinal barrier function can prevent harmful substances, such as bacteria and endotoxin from the gut, from infecting extraintestinal tissues and organs [3]. In a previous study, the impairment of intestinal barrier function was widely considered to occur in the early course of acute pancreatitis [8, 28]. Once intestinal barrier dysfunction occurred in SAP, bacteremia and endotoxemia follow resulting in SIRS (systemic inflammatory response syndrome, SIRS), MODS (multiple organ dysfunction syndrome, MODS) and other serious systemic complications [28]. Thus, intestinal mucosal barrier dysfunction is accepted as a main promoter of bacterial translocation in SAP. In our study, we also observed that rats exhibited intestinal barrier dysfunction 24 h after induction by 5% sodium taurocholate, which is consistent with previous reports. Notably, we found that APD significantly improved intestinal barrier function, which was reflected in the decreased pa-

thological scores of intestinal tissues and serum levels of iFABP, DAO and D-lactate, and the higher expression of ZO-1 in the intestinal tissues. This study is the first to document that APD plays a protective role in intestinal barrier dysfunction in SAP, which has important significance for the improvement of SAP treatment.

This study has several limitations. Currently, the route of bacterial translocation remains an open question. Previous reports showed that the translocation might be migration from lymph and/or systemic circulation to the pancreas [29]. In our study, we did not observe bacterial translocation in the blood during the early stage of SAP, suggesting that systemic circulation may not be the very first route of bacterial translocation. Additionally, in future studies, we should further explore the molecular pathway that regulates bacterial translocation from the gut to the MLN and pancreatic tissues in SAP rats after APD treatment.

In conclusion, APD treatment significantly reduces bacterial translocation from the gut, improves intestinal barrier function, and ultimately exerts a protective role in SAP-associated intestinal injury. Our findings offer new insight into the effectiveness of APD, which supports this strategy as an acceptable clinical therapy for SAP.

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Disclosure of conflict of interest

None.

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