Original Research

Abdominal paracentesis drainage attenuates intestinal mucosal barrier damage through macrophage polarization in severe acute pancreatitis

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Impact statement

Severe acute pancreatitis (SAP) is a gastrointestinal disease with high morbidity and mortality. Furthermore, intestinal barrier damage during SAP would aggravate the severity of SAP, leading to increased risk of multiple organ dysfunction. Abdominal paracentesis drainage (APD) is used as an effective treatment for SAP and related enteral damage. The present study demonstrated that APD improved the intestinal mucosal barrier damage through increasing the portion of M2 phenotype macrophages in the intestine via inhibiting the ASK1/JNK pathway in rats with SAP.

Abstract

Abdominal paracentesis drainage (APD), as an effective treatment of severe acute pancreatitis (SAP) in clinical settings, can ameliorate intestinal barrier damage and the overall severity of SAP. However, the mechanism underlying therapeutic effects of APD on damaged intestinal mucosal barrier during SAP is still unclear. Here, SAP was induced by injecting 5% Na-taurocholate retrograde into the biliopancreatic duct of rats to confirm the benefits of APD on enteral injury of SAP and further explore the possible mechanism. Abdominal catheter was placed after SAP was induced in APD group. As control group, the sham group received no operation except abdominal opening and closure. By comparing changes among control group, sham group, and APD group, APD treatment obviously lowered the intestinal damage and reduced the permeation of intestinal mucosal barrier, which was evidenced by intestinal H&E staining, enteral expression of tight junction proteins, intestinal apoptosis measurement and detection of serum diamine oxidase, intestinal fatty acid binding protein and D-lactic acid. Furthermore, we found that APD polarized intestinal macrophages toward M2 phenotype by the determination of immunofluorescence and western blotting, and this accounts for the benefits of APD for intestinal injury in SAP. Importantly, the protective effect against intestinal injury by APD treatment was mediated through the inhibited ASK1/JNK pathway. In summary, APD improved the intestinal mucosal barrier damage in rats with SAP through an increasing portion of M2 phenotype macrophages in intestine via inhibiting ASK1/JNK pathway.

Keywords: Severe acute pancreatitis, abdominal paracentesis drainage, macrophage polarization, intestinal mucosal barrier, apoptosis, ASK1/JNK pathway

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Introduction

Severe acute pancreatitis (SAP) is a lethal disease with a high mortality rate, 10% of which is associated with sterile pancreatic necrosis, while 25% is associated with infected pancreatic necrosis.[1–3] SAP with persistent organ failure (>48 h) is associated with a markedly increased mortality of up to 50%.[4] What's more, the most common cause of death in the late process is multiple organ dysfunction (MOD) secondary to infectious pancreatic necrosis. Of note, the intestine is thought to have a pivotal role in the

development of MOD syndrome (MODS) in critical illness, in that intestinal mucosal injury might account for the onset of MODS.[5] The secondary infection in SAP is often caused by the translocation of bacteria and endotoxins from the damaged intestinal mucosal barrier to the systemic circulation.[6] Consistent with the above, the intestinal tract is reported to contribute to extraintestinal harmful effects and increased severity and mortality of disease through "Intestinal leakage" mediated by increased intestinal permeability, or through producing and releasing harmful cytokines or toxins.[7]

SAP can be aggravated by pancreatitis associated ascitic fluid (PAAF), which contains a variety of mediators that can cause MOD and upregulate inflammatory cytokines.[8–10] Removal of PAAF by abdominal lavage can produce systemic anti-inflammatory effects, lower serum levels of inflammatory cytokines, attenuate SAP severity, and increase the survival rate of AP animal models.[11] Our previous studies have shown that abdominal paracentesis drainage (APD) can alleviate the severity of SAP and the pathological conditions of affected multiple organs, including intestine, by removing PAAF without increasing infection risk.[12–15] Although APD, as an effective curative method for SAP, has been studied and applied clinically for years, the underlying mechanisms in treating SAP and improving SAP intestinal injury have not yet been fully elucidated.

Macrophage polarization plays important roles in pathological mechanisms of intestinal injury and is involved in the therapeutic mechanism under many treatments of intestinal diseases.[16,17] Macrophages are a class of immune cells that can be activated into M1 and M2 phenotype, playing pro-inflammatory and anti-inflammatory roles, respectively, at specific time points in specific microenvironments. This process is known as macrophage polarization, which is commonly seen in inflammatory processes.[18] M1 and M2 can transform to the other phenotype due to changes of the microenvironment.[19] Macrophages are involved in maintaining intestinal homeostasis. Also, more macrophages will be induced to the intestinal tract due to inflammatory response and then participate in the pathological process.[20] Apoptotic signaling regulated kinase 1 (ASK1) was suggested to be required by infiltration and activation of macrophages.[21] ASK1 is a mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK) that can activate its downstream factors, c-jun n-terminal kinase (JNK) and P38.[22] What's more, inhibition of ASK1 suppressed M1-associated genes while it augmented M2-associated genes under sustained-inflammatory conditions.[23] Therefore, for intestinal changes in SAP, macrophages are likely to participate in its pathological process, and macrophage polarization may be one of the mechanisms accounting for SAP intestinal injury. It can be inferred that ASK1 may influence and determine the polarization of intestinal macrophages. Thus, we wonder if macrophage polarization participates in SAP intestinal changes mediated by ASK1?

Based on the above consideration, we investigated the influence of APD treatment on intestinal injury in rats with SAP and determined whether intestinal macrophages and ASK1/JNK signaling play a crucial role during this treatment process. All these could bring new insights into the mechanism of APD in alleviating SAP intestinal injury.

Here we hypothesized that APD influences the polarization of macrophages in the intestinal tissue through inhibiting the ASK1/JNK pathway in the intestine through removing PAAF, and thus reduces the inflammatory condition of the intestine, finally exerting beneficial effects in the treatment of SAP.

Materials and methods

Animals and surgical models

Adult male SD rats (200–250 g) used in this experiment were purchased from Chengdu Dossy Experimental Animals Co., Ltd. (Chengdu, China). Standard laboratory food and water were supplied for experiment animals, while SD rats were fasted but had free access to water 24 h before the experiment. The experimental procedure was approved by the animal protection and use organization committee of General Hospital of Western Theater Command and in accordance with established international guidelines for animal research.

The 18 SD rats were randomly assigned to three groups: SAP group, APD group, and Sham group ($n = 6$ rats per group). The SAP models in the SAP group and the ADP group were established by injecting 5% Na-taurocholate (Sigma Chemical Co., St. Louis, MO, USA) retrograde into the biliopancreatic duct at a rate of 12 ml/h through a microinfusion pump (0.01 ml/100 g rat body weight) under standard pressure. After the biliopancreatic duct was clipped and kept under pressure for 5 min, the artery clip and puncture needle were removed. In the APD group, a drainage tube with a vacuum bulb was implanted in the right lower abdomen immediately after the induction of acute pancreatitis. The sham group received no operation except abdominal opening and closure. Anesthesia was performed with the isoflurane anesthetic. After successful modeling, the rats were sacrificed at 24 h after modeling. Blood samples, pancreatic and intestinal tissues were collected.

Measurements of serum inflammatory factors and serum amylase

Expression levels of TNF-α, IL-1β, endotoxin, DAO (diamine oxidase), iFABP (intestinal fatty acid binding protein) and D-lac (D-lactic acid) in serum were detected using the enzyme-linked immunosorbent assay (ELISA) kit (Nanjing Jiancheng Institute of Biological Engineering, China) in accordance with the manufacturer's manual. The serum amylase levels were detected according to the product information sheet of the automatic biochemical analyzer (Taikang Technology Co. Ltd, Jiangxi, China).

H&E staining and histological scoring

After fixation with paraformaldehyde for 24 h, the intestinal tissues and pancreatic tissues were paraffin-embedded and then cut into 4 μm tissue sections. The sections were

stained with hematoxylin and eosin after being dewaxed and rehydrated. Stained tissue sections were observed under an optical microscope. The pathologic score was assessed by two investigators using a double-blind method, and pancreatic and small enteral histological changes were evaluated according to the Schmidt[24] and Siegmund[25] standards, respectively.

Immunofluorescence staining

The small intestine was paraffin embedded and cut into 4 μm sections. The sections were dewaxed and rehydrated. Antigen retrieval was performed using citrate buffer (Beijing Solarbio science and Technology, Inc.), heating in microwave oven. After the sections cooled down and were washed with PBS three times x 5 min (Beijing Solarbio science and Technology, Inc.), the sections were incubated in 1% bovine serum for 30 min. The sections were incubated with primary antibody overnight at 4 °C. Single staining antibodies were Claudin-1 (1:1000, ab15098, Abcam, USA) and Occludin (1:100, GB111401, Servicebio Technology Co. Ltd., Wuhan, China). Double staining antibodies were CD68 (1:100, GB11067), CD163 (1:100, GB11340-1), and iNOS (Inducible nitric oxide synthase) (1:200, GB11119) from Servicebio Technology Co. Ltd (Wuhan, China) and Arg-1 (Arginase-1) (1:200, 16001–1-AP, Proteintech, China). After washing with PBS (Beijing Solarbio science and Technology, Inc.) three times x 5 min, fluorescein labeled secondary antibodies (1:300, GB21303, Servicebio Technology Co. Ltd., Wuhan, China) were added to incubate tissue sections for 50 min, and then the secondary antibodies were washed with PBS three times x 5 min. Nuclei were stained with dibutyl hydrochloride (DAPI) for 10 min in the dark and then sections were washed with PBS three times. An appropriate amount of anti-fluorescence quenching agent was added, and finally the slides were sealed with resin sealing agent. The sections were observed and images were taken under a fluorescence microscope, and the image analysis was done using Image J software.

Immunohistochemistry staining

The expression of TJP (tight junction proteins), ZO-1 (zonula occluden-1), and apoptotic proteins, Bax (B-cell lymphoma 2 (Bcl-2) associated X protein) and Cleaved-Caspase 3 (cysteine aspartic acid protease 3) in the small intestine was detected by IHC. Paraffin sections of small intestine were dewaxed and rehydrated. Antigen retrieval was performed using citrate buffer in a microwave oven. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min. After sealing with goat serum, primary antibody of ZO-1(1:200, ab96587, Abcam, USA), Bax (1:200, ab32503, Abcam, USA), and cleaved-caspase3 (1:200, #9662S, CST, USA) was added to incubate the sections overnight at 4 °C. After washing with PBS for three times, secondary antibody (1:200, GB1213, GB1214, Servicebio Technology Co. Ltd., Wuhan, China) was added to tissue sections to incubate at room temperature for 50 min. After washing three times with PBS, DAB was added dropwise. Nuclei were stained blue with hematoxylin. After gradient alcohol dehydration and xylene immersion, the tissue sections were sealed with neutral gum and photographed under a microscope. Brownish yellow staining indicated positive expression.

TUNEL staining

The cellular apoptosis in small intestinal paraffin sections was detected by the terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) assay (TUNEL Apoptosis Detection Kit III, FITC, MK1023, Boster Biological Technology co. ltd, Wuhan, China) according to the manufacturers' instructions. All nuclei were stained blue with DAPI and TUNEL-positive cells were green. The number of TUNEL-positive cells was calculated as a percentage of total villus numbers.

Western blotting

The intestinal tissue (1 cm small intestinal tissue from the same region of each rat) was placed in Ripa lysate buffer with protease inhibitor, phosphatase inhibitor, and PMSF (Total protein extraction kit, Beijing Solarbio science and Technology, Inc.), and the tissue was cut into pieces with ophthalmic scissors and then homogenized with homogenizer according to the instructions. The tissue homogenate was centrifuged at 12,000 g for 30 min at 4 °C, and then the supernatant was collected. After protein concentration was determined by using nanodrop, the supernatant liquid was mixed with loading buffer (5× loading buffer, Beijing Solarbio science and Technology, Inc.) at a ratio of 4:1, then boiled at 100 °C for 10 min for protein denaturation, and stored at –80 °C after separation. After being separated by SDS-PAGE (Beijing Solarbio science and Technology, Inc.), the protein was transferred to PVDF membrane (0.45 μm, IPVH00010, Millipore), blocked in 5% nonfat milk for 1 h at room temperature (22 ± 3 °C), and then incubated with primary antibody, ZO-1 (1:1000, ab96587, Abcam, USA), Occludin (1:1000, GB11149-2, Servicebio Technology, China), Claudin-1 (1:1000, ab15098, Abcam, USA), Bax (1:200, ab32503, Abcam, USA), cleaved-caspase3 (1:200, #9662S, CST, USA), CD163 (1:1000, GB11340, Servicebio Technology, China), iNOS (1:1000, GB13495, Servicebio Technology, China) and Arg-1 (1:1000, 16001–1-AP, Proteintech, USA), ASK1 (1:1000, 8662S, CST, USA), p-ASK1 (1:1000, PA5105027, Invitrogen, USA), JNK (1:1000, ab179461, Abcam, USA), p-JNK (1:1000, ab124956, Abcam, USA), GAPDH (1:5000, Proteintech, China) at 4 °C overnight. The membranes were then washed with Tris Buffered Saline with Tween-20 (TBST) (Beijing Solarbio science and Technology, Inc.) three times and incubated with secondary antibody (1:10,000, TA130023, Origene, USA) for 1 h at room temperature. After the secondary antibody was washed three times with TBST, the protein bands were visualized by enhanced chemiluminescence (ECL) (IMMOBILON WESTERN CHEMILUM HRP SUBSTRATE, Millipore) in a biological imaging system.

Statistical analysis

Statistics and graphs were performed using GraphPad Prism™ 8.0 (GraphPad Software Inc., USA). All data were presented as the means ± SD. One-way ANOVA was used as the statistical method for the comparison among three groups, and Tukey's multiple comparisons test was used to evaluate the significance between two groups. A P value of < 0.05 was considered statistically significant.

Results

Effects of APD on SAP in rats

APD can alleviate the severity of SAP. As shown in Figure 1 (a), the pancreatic tissue of the Sham group showed a normal structure with almost no pathological changes in H&E staining sections under light microscope. Meanwhile in rats with SAP, significant morphological injury in the form of edema, acinar cell necrosis, and inflammatory infiltration were observed ($P < 0.001$). SAP rats treated with APD exhibited reduced tissue damage in comparison with the SAP group. Also, the histology score of pancreatic injury was higher in the SAP group than those in the APD group and the Sham group (Figure 1b).

In addition, curative effects were also observed in the serum index. The serum amylase and endotoxin, known as a biomarker of SAP and of systematic inflammation, respectively, increased in the SAP group but then decreased with APD treatment (Figure 1c and d). Thus, APD was confirmed to ameliorate SAP. This result could also be supported by the lower expression of pro-inflammatory cytokines, TNF-α and IL-1β, which are able to cause systematic inflammation in SAP, in APD than in SAP (Figure 1e and f).

APD ameliorates intestinal mucosal barrier damage in rats with SAP

APD can alleviate the severity of SAP intestinal injury. As shown in Figure 2(a), the intestinal mucosal barrier was severely damaged with edema, infiltration of inflammatory cells, and shedding of some villi in the SAP group compared with the Sham group. In contrast, the structural damage of intestinal tissue induced by SAP was much reduced by the treatment of APD. This is consistent with the lower histological score of intestines in Sham and APD groups (Figure 2b).

As the physiological functions of the intestinal mucosal barrier are crucial for maintaining intestinal homeostatic balance and SAP severity, we therefore measured the indicators of intestinal mucosal barrier function in intestinal tissues of rats. Results showed that the levels of serum endotoxins, DAO, iFABP and D-lac in the SAP group were not only higher than those in the Sham group, but were also higher than those of the APD group (Figure 2c to e). These results indicated that APD could restore the impaired intestinal mucosal barrier function of SAP.

APD improved the expressions of tight junction proteins in SAP intestinal tissue

TJPs, as important components of the seal between adjacent epithelial cells, play a vital role in the maintenance of intestinal barrier functions.[26,27] We therefore investigated the effects of APD on the expressions of TJPs, including Occludin, claudin-1, and ZO-1. After SAP was induced, the three TJPs were all decreased compared with the Sham group, as indicated by the results of immunofluorescence and immunohistochemistry, while APD treatment could increase the expressions of these TJPs (Figure 3a to c). In addition, we performed WB to reexamine the

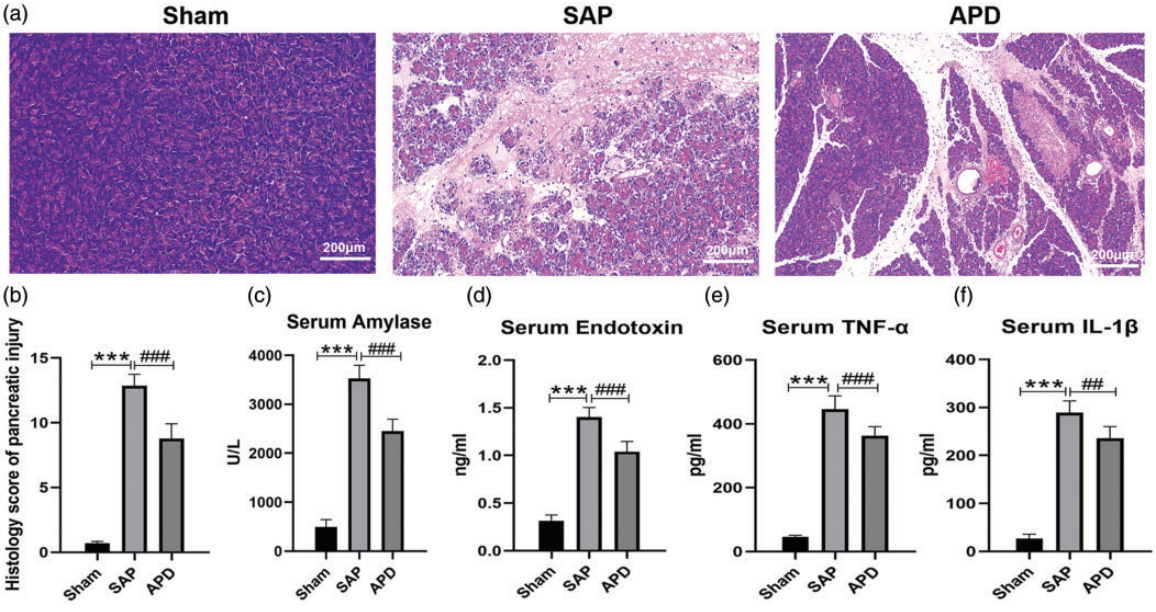

Figure 1. APD alleviated the pancreatic injury and systematic inflammatory severity of SAP. (a) H&E staining sections of pancreatic tissue from three groups. (b) Histological score of pancreatic injury. (c) and (d) Serum levels of amylase and endotoxin. (e) and (f) Serum levels of inflammatory factors (TNF-α and IL-1β). All data are presented as mean ± SD (n = 6). Sham, Sham operation; SAP, severe acute pancreatitis; APD, SAP + APD. $\alpha = 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Sham, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. SAP. (A color version of this figure is available in the online journal.)

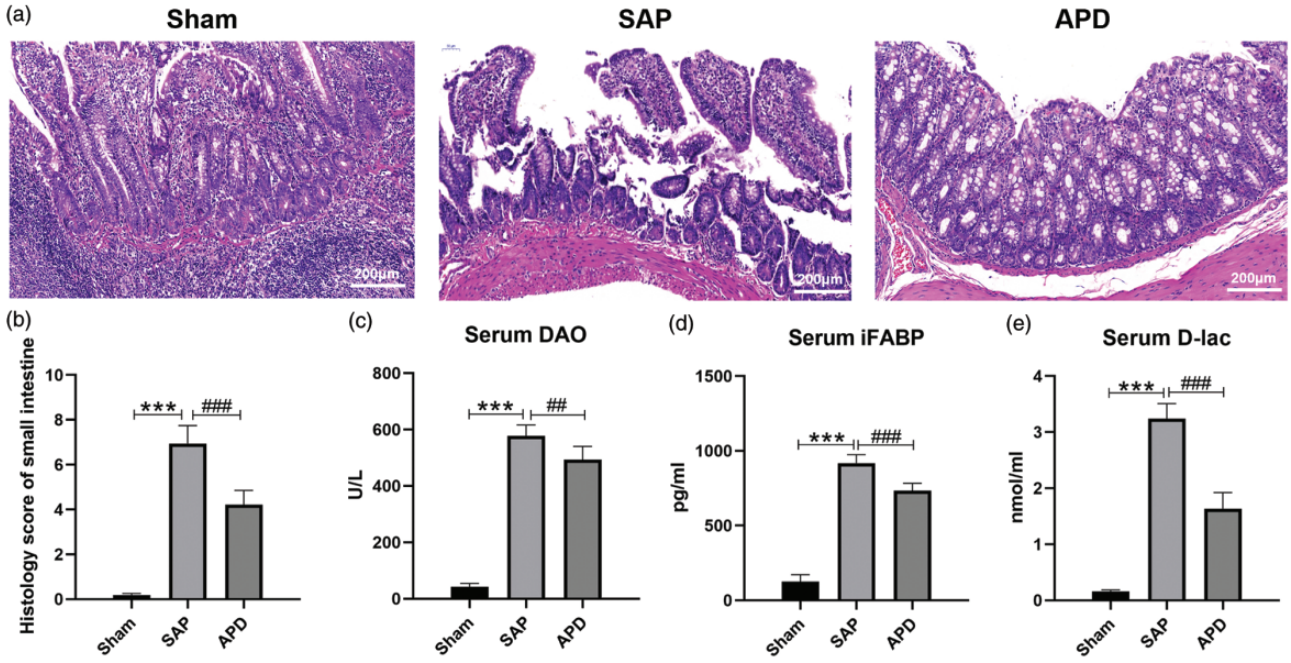

Figure 2. APD ameliorated damaged intestine and reduced increased permeation of intestinal mucosal barrier in SAP. (a) H&E staining sections of small intestinal tissue from three groups. (b) Histological score of small intestine. (c) to (e) Serum levels of indicators of intestinal mucosal barrier integrity and functions (DAO, iFABP, and D-lac). All data are presented as mean ± SD (n = 6). Sham, Sham operation; SAP, severe acute pancreatitis; APD, SAP + APD. $\alpha = 0.05$, *p < 0.05, **p < 0.01, ***p < 0.001 vs. Sham, #p < 0.05, ##p < 0.01, ###p < 0.001 vs. SAP. (A color version of this figure is available in the online journal.)

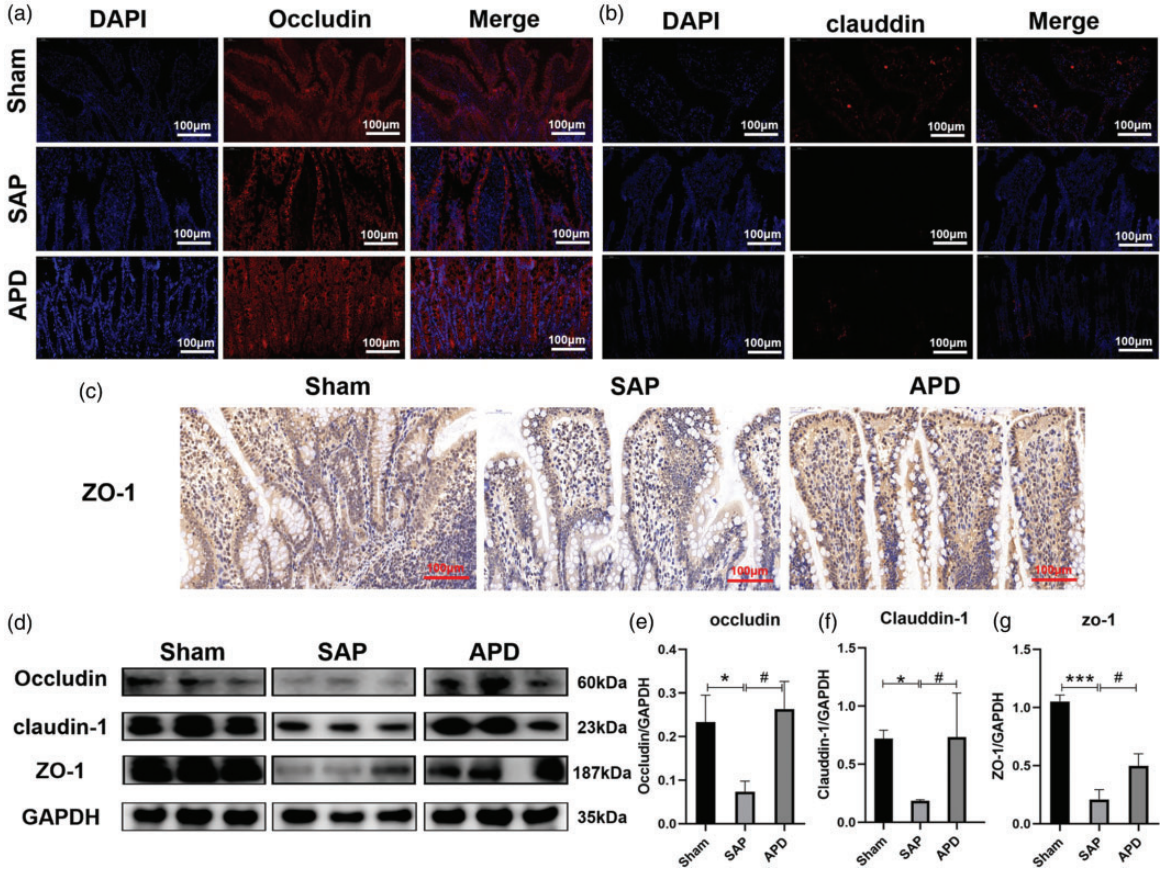

Figure 3. APD improved the reduced expression of TJPs in SAP. (a) and (b) Expression of intestinal Occludin and Claudin-1 in three groups measured by IF staining (n = 6). (c) Expression of intestinal ZO-1 in three groups measured by IHC staining (n = 6). (d) Immunoblotting of intestinal TJPs (Occludin, Claudin-1, and ZO-1) expression in three groups (n = 3). (e) to (g) Quantitative densitometric analyses of the immunoblot data of TJPs in intestinal tissue. All data are presented as mean ± SD. Sham, sham operation; SAP, severe acute pancreatitis; APD, SAP + APD. $\alpha = 0.05$, *p < 0.05, **p < 0.01, ***p < 0.001 vs. Sham, #p < 0.05, ##p < 0.01, ###p < 0.001 vs. SAP. (A color version of this figure is available in the online journal.)

expression levels of Occludin, Claudin-1, and ZO-1, and the outcomes were consistent with the above (Figure 3d to g). As a whole, these results suggested that SAP can increase the permeability of the intestine and cause the gut "leak" mediated by the decline of TJPs, while APD improved the intestinal barrier function.

APD reduced intestinal apoptosis in rats with SAP

Apoptosis, as a vital cellular phenomenon, was observed and measured in this study. Cell apoptosis in the intestine was examined by TUNEL assay. Compared with the Sham group, the cellular apoptosis was increased in the SAP group. Conversely, treatment with APD attenuated the apoptosis induced by SAP and lowered the portion of cell apoptosis (Figure 4a). Then immunohistochemistry was performed to detect the expression of apoptosis related proteins, like Bax and cleaved-Caspase 3. The results of immunohistochemistry showed that the expressions of pro-apoptotic protein Bax and cleaved-Caspase 3 were decreased in the APD group compared with the SAP group (Figure 4b). Furthermore, western blotting confirmed the results of apoptotic proteins in APD and SAP groups to be the same as shown by immunohistochemistry, including Bax, pro-Caspase 3 and cleaved-Caspase 3 (Figure 4c). These results suggested that SAP promoted apoptosis in intestine tissues, while APD treatment reduced the increased intestinal apoptosis of SAP.

Macrophages are polarized to M2 phenotype in intestine of rats with APD

Polarization of macrophages in the intestine under SAP was examined in this study. The protein expression level of M1 macrophage markers and M2 macrophage markers in the intestines of APD and SAP rats were evaluated. The results of immunofluorescence showed the number of M2 macrophages was obviously increased in the APD group compared with that in the SAP group (Figure 5a). In contrast, M1 macrophages decreased in the intestine of APD rats (Figure 5a). We further detected the expression levels of macrophage markers by western blotting. Compared with SAP rats, the expression of M2-related markers Arg-1 and CD 163 was higher in APD rats in the intestine. By contrast, M1-related marker iNOS-1 was lower in the APD group than in the SAP group (Figure 5b). These data demonstrated that intestinal macrophages can be polarized to the anti-inflammatory M2 phenotype by treating with APD.

APD alleviated intestinal damage in rats with SAP through ASK1/JNK pathway

ASK1 is reported to not only induce cell apoptosis, but is also involved in the polarization of M1/M2 phenotype. Inhibiting ASK1 can induce macrophage polarization toward M2 and decrease the M1 phenotype.[23] JNK is a known downstream target of ASK1.[28] We therefore subsequently investigated if the ASK1/JNK pathway which functions during SAP intestinal injury is mediated by regulating apoptosis and macrophage polarization. Western blotting analysis of ASK1, JNK, and their corresponding phosphorylation forms was performed. The phosphorylation of ASK1 and JNK was increased in rats with SAP, compared with the Sham group, while the increased expression of phosphorylation forms of ASK1 and its downstream target, JNK, induced by SAP, reduced after treating with APD (Figure 6a). It could be inferred from these results that the ASK1/JNK pathway is activated in SAP but suppressed by APD treatment.

In summary, SAP caused the activation of the ASK1/JNK pathway in intestinal tissue, and then subsequently increased cell apoptosis, inflammatory cell infiltration, and macrophage polarization to the M1 phenotype in the intestine. After APD treatment, the activated ASK1/JNK pathway was inhibited, and its downstream phenomenon was also changed, manifesting as decreased intestinal apoptosis and conversion of M1 macrophage to M2. Ultimately, APD alleviated the intestinal damage caused by SAP.

Discussion

The present study, for the first time, provided evidence that APD alleviates intestinal injury in rats with SAP by influencing the intestinal macrophage phenotypes. The main results are that: (1) by removing PAAF, APD could ameliorate SAP and reduce intestinal mucosal barrier damage; (2) APD contributes to the polarization toward the M2 phenotype of intestinal macrophages; (3) APD might function through the ASK1/JNK pathway. These findings might be helpful to investigate the mechanisms underlying the effective role of APD in the treatment of SAP.

Our previous studies have shown that APD could ameliorate varied pathological aspects of SAP through various pathways,[15,29–31] without increasing the risk of infection.[13] APD is an invasive treatment to effect drainage of PAAF from the abdominal or pelvic cavity through percutaneous puncture and catheterization.[12] There are high concentrations of many pro-inflammatory cytokines in PAAF, including IL-6, IL-8, IL-1β, and TNF-α.[32] PAAF could worsen the intestinal mucosa ischemia damage, and toxic substance in it can directly stimulate the intestine.[33] By removing PAAF, APD can not only alleviate the injured intestinal mucosa and improve microcirculation of intestine in SAP rats,[14] but can also improve the tolerance of enteral nutrition in AP patients.[29] Therefore, these high concentrations of inflammatory cytokines in ascites might be responsible for the AP associated intestine damage. The results of H&E staining showed that APD alleviated SAP and related enteral injury in rats. Through measuring enteral TJPs and serum indicators including DAO, iFABP and D-lac, our results, which are in line with previous related studies, showed that APD reduced the intestinal mucosal barrier damage.

Apoptosis, as a highly regulated form of cell death, can influence and reflect the severity of SAP.[34] In AP, cell apoptosis, which is positively correlated with enteral permeability, is increased in intestinal tissue.[35] In this study, an increased number of apoptotic cells were observed in the intestinal tissue in rats with SAP compared with the Sham group through TUNEL staining. This increase in apoptotic cells was reduced in the APD group compared with the

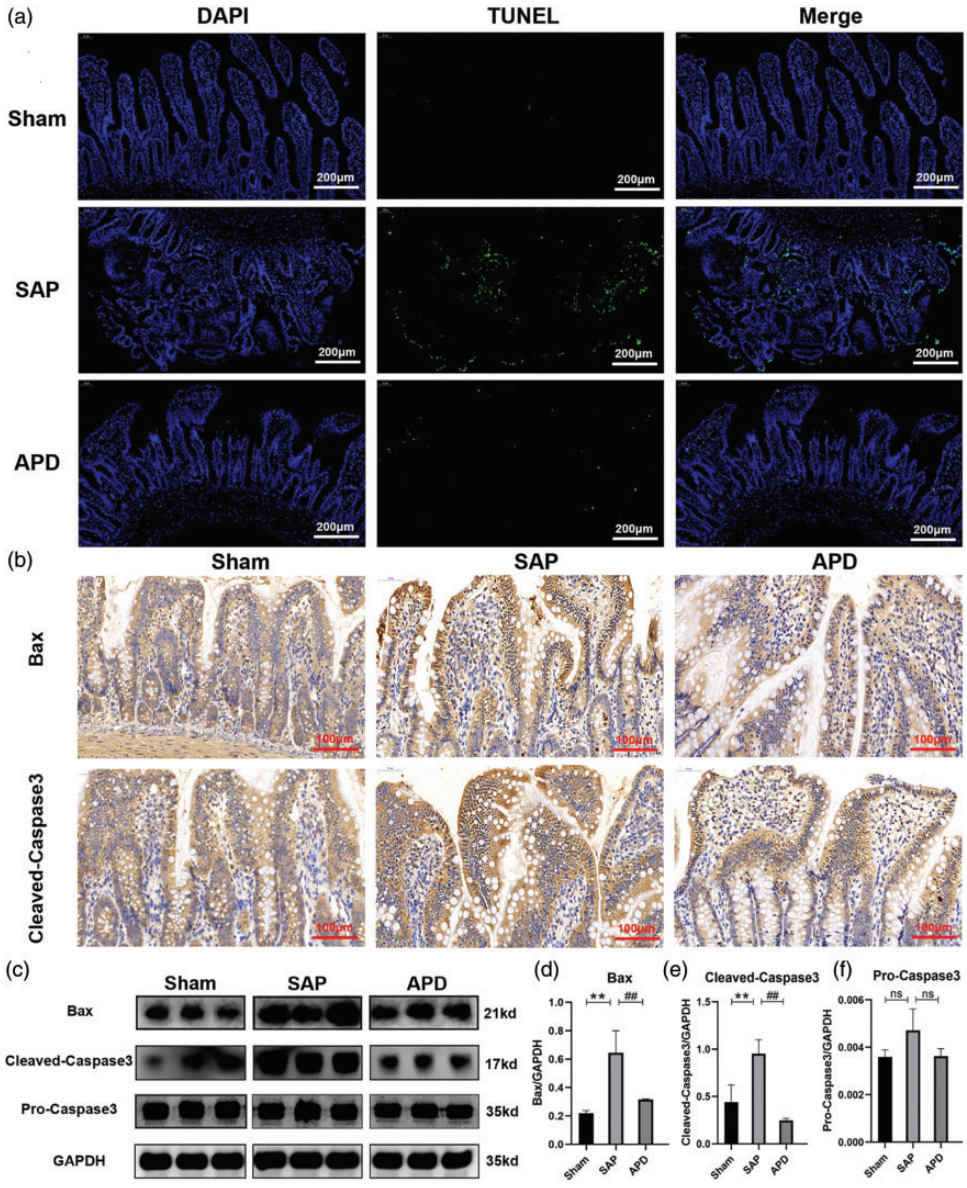

Figure 4. APD reduced cellular apoptosis in intestine of rats with SAP. (a) Intestinal cellular apoptosis in three groups measured by TUNEL staining (n = 6). (b) Expression of enteral pro-apoptosis proteins (Bax and Cleaved-Caspase3) measured by IHC staining of three groups (n = 6). (c) Immunoblotting of intestinal Bax, Pro-Caspasse3, and Cleaved-Caspase3 expressions in three groups (n = 3). (d) to (f) Quantitative densitometric analyses of the immunoblot data of apoptosis-related proteins in enteral tissue. All data are presented as mean ± SD. Sham, sham operation; SAP, severe acute pancreatitis; APD, SAP + APD. $\alpha = 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Sham, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. SAP. (A color version of this figure is available in the online journal.)

SAP group. The results of apoptosis related proteins, as measured by western blotting, were also in line with this point of view. These findings further confirmed that cellular apoptosis is augmented in the intestine during SAP, while APD, as a therapeutic method, decreases intestinal apoptosis.

There are a large number of macrophages in the lamina propria beneath the intestinal epithelium,[36] implying that macrophages might account for enteral damage in SAP. M1 activation, also known as classical activation, is mediated by pro-inflammatory cytokines and M1 can also produce inflammatory factors.[37] M2 activation, also known as alternative activation, is mediated by anti-inflammatory factors, producing high doses of IL-10 and low doses of IL-12, which have anti-inflammatory and tissue repair effects.[38] We therefore wonder if benefits of APD on SAP intestinal damage were conducted through influencing enteral macrophages. By measuring specific surface markers in our study, we found that intestinal macrophages were polarized toward the M1 phenotype in SAP accompanied by a decreased portion of M2 phenotype. However, the proportion of M2 was increased along with a decreased portion of M1 phenotype after the treatment of APD. It is commonly known that macrophage polarization can occur in response to the inflammatory microenvironment during inflammatory disease, and TNF-α, which is of high concentration in PAAF, is a major anti-M2 factor.[18] All these pieces of evidence support our assumption that macrophage polarization is involved in the pathological mechanism of intestinal damage in SAP, and APD might improve the intestinal

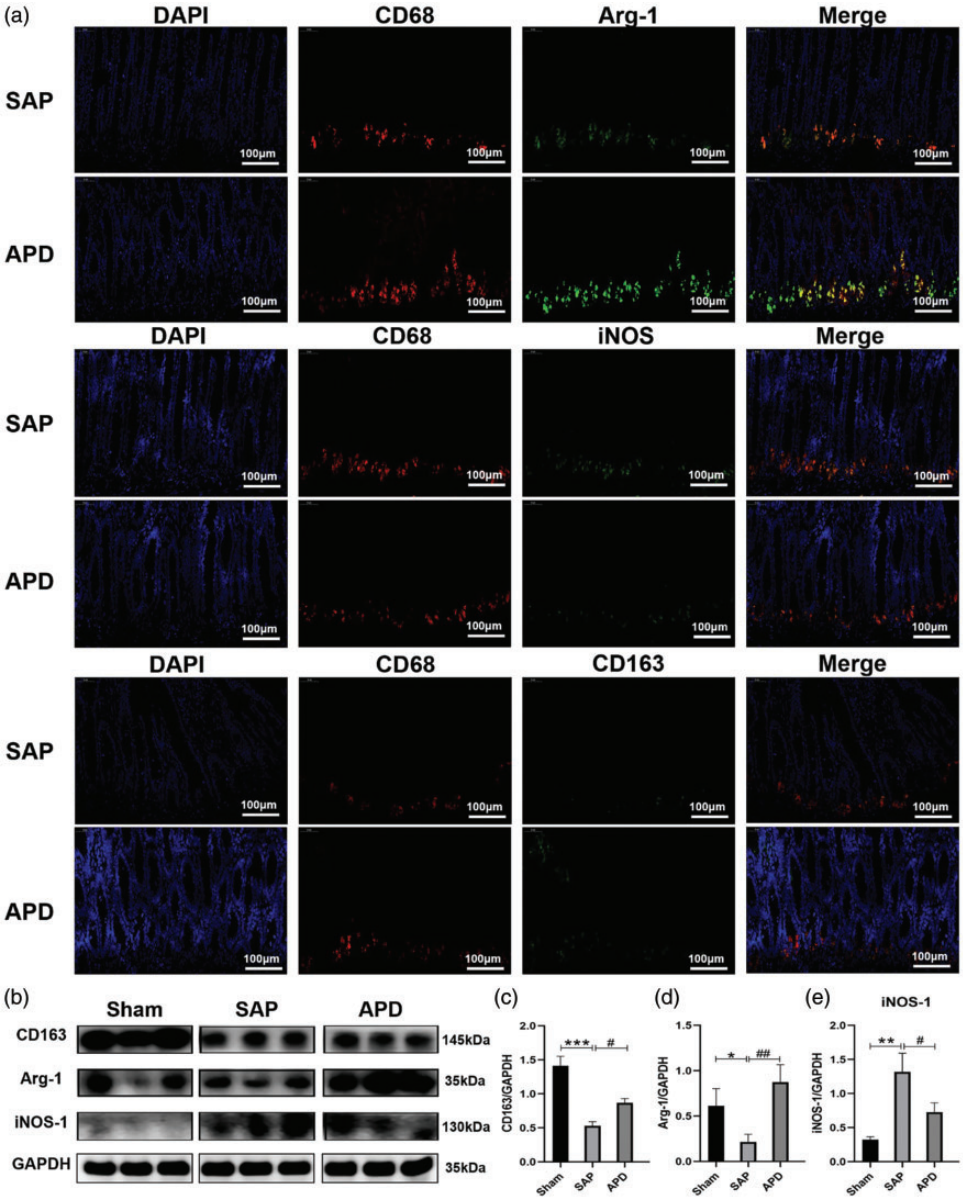

Figure 5. APD promoted enteral macrophage polarization toward M2 phenotype. (a) Double-stained macrophage markers of CD68 and Arg-1, CD68 and iNOS, CD68 and CD163 of intestinal tissue section by IF in SAP and APD groups ($n = 6$). (b) Immunoblotting of intestinal expression of CD163, Arg-1, and iNOS in three groups ($n = 3$). (c) to (e) Quantitative densitometric analyses of the immunoblot data of macrophage markers in enteral tissue. All data are presented as mean ± SD. Sham, sham operation; SAP, severe acute pancreatitis; APD, SAP + APD. $\alpha = 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Sham, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. SAP. (A color version of this figure is available in the online journal.)

inflammatory microenvironment and further influence the activation status of intestinal macrophages by removing PAAF.

Increased ASK1 and JNK exacerbate SAP by affecting the polarization of macrophages. Wu *et al.*[39] and Yu *et al.*[40] found that the expression of ASK1 and JNK increased in SAP, and that the increased ASK1/JNK could increase the release of TNF-α. However, the severity of SAP and TNF-α could be reduced through inhibiting ASK1/JNK. A great deal of studies also revealed that apoptosis can be induced by the activation of the ASK1/JNK pathway.[41,42] Cheon SY *et al.*[23] found that inhibiting ASK1 can reduce pro-inflammatory factors and increase anti-inflammatory mediators, reduce the migration and infiltration of macrophages, and affect the polarization of macrophages, resulting in increased M2 macrophages accompanied by the decrease of M1 macrophages. Thus, we infer that ASK1/JNK might polarize the enteral macrophages phenotype from M2 to M1 during SAP, and be involved in the pathogenesis of SAP intestinal damage, based on the information above. We indeed found in this study that SAP increased the p-ASK1/ASK1 and p-JNK/JNK activity in the intestine, indicating that an active form of ASK1 and JNK increased in SAP. The proportions of phosphorylated ASK1 and JNK were reduced after treating with APD, which supports our assumptions. Besides, TNF-α is the main upstream activator for ASK1.[43] We therefore supposed that perhaps increased serum TNF-α caused by

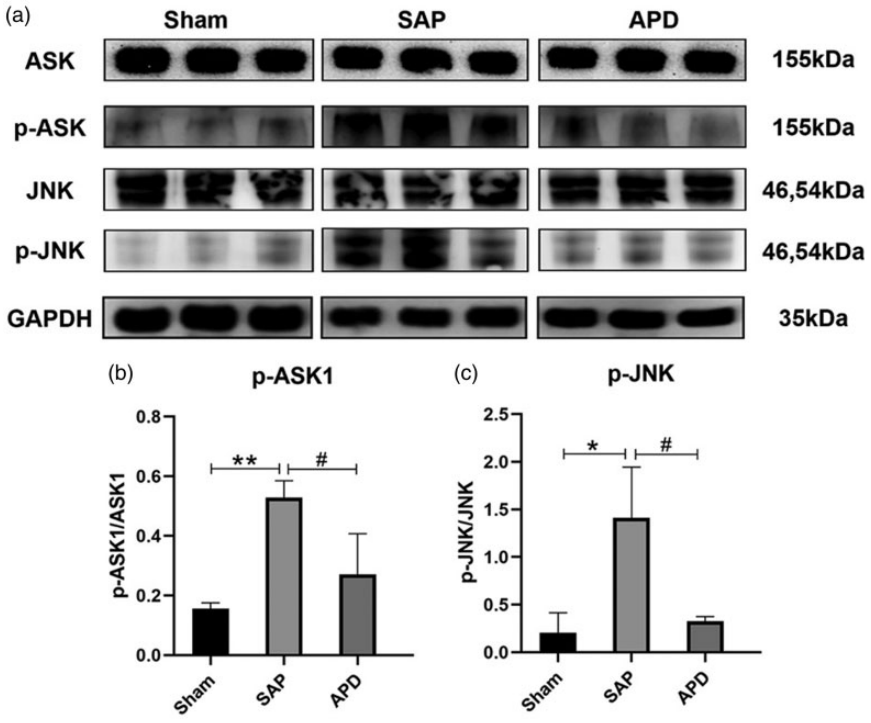

Figure 6. APD inhibited intestinal ASK1/JNK pathway which increased by SAP. (a) Intestinal protein expression of ASK1, JNK and their corresponding phosphorylation forms (p-ASK1 and p-JNK) measured by immunoblotting. (b) and (c) Quantitative densitometric analyses of the immunoblot data of proteins in ASK1/JNK proteins in enteral tissue. All data are presented as mean ± SD (n = 3). Sham, sham operation; SAP, severe acute pancreatitis; APD, SAP + APD. $\alpha = 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Sham, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. SAP.

PAAF activates the ASK1/JNK pathway to aggravate intestinal injury in SAP through regulating macrophage polarization, and that APD ameliorates SAP and its associated intestinal mucosa barrier damage through macrophage polarization toward M2, mediated by inhibiting the ASK1/JNK pathway.

Of note, there are several limitations in our study that need to be addressed in order to clarify the precise underlying mechanism in the future. Firstly, we did not reconfirm the direct influence of PAAF on the ASK1/JNK pathway and its downstream phenomenon by the refusion of PAAF. Secondly, we did not find out the exact toxic substance which activates the ASK1/JNK pathway. Thirdly, we did not reconfirm the regulatory role of the ASK1/JNK pathway on the downstream phenomenon, apoptosis and macrophage polarization, by up/downregulating the ASK1/JNK pathway.

In brief, our findings suggest that APD suppressed the ASK1/JNK pathway in the intestine by removing PAAF, leading to macrophage polarization toward the M2 phenotype, ultimately facilitating the amelioration of the intestinal mucosal barrier damage in SAP. These findings bring new insights into the therapeutic mechanism of APD.

AUTHORS' CONTRIBUTIONS

HYS and LJT designed and executed the experiments. XHY, CL established Sham, SAP, and APD rat models. WL and XG sacrificed rats and XHY, CL, JW took and collected serum, pancreas, and intestine and then processed and stored them properly. JW performed H&E and TUNEL staining. WL and XG also performed ELISA serum amylase detection. XHY and BW performed immunofluorescence. CL and SL performed immunohistochemistry. CL performed western blot. XHY analyzed all these data. The manuscript was written by XHY and CL. All authors contributed to the article and approved the submitted version.

DECLARATION OF CONFLICTING INTERESTS

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