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The role of intestinal mucosa oxidative stress in gut barrier dysfunction of severe acute pancreatitis

R. TIAN, J.-T. TAN, R.-L. WANG, H. XIE, Y.-B. QIAN, K.-L. YU

Intensive Care Unit, First People's Hospital, Shanghai Jiaotong University, Shanghai, China

Abstract. – BACKGROUND: Severe acute pancreatitis (SAP) is a serious systemic disease with a sustained high mortality rate. Extensive evidence has shown that gut barrier dysfunction plays a critical role in the pathophysiology of SAP.

AIM: Investigating the role of intestinal mucosa oxidative stress in gut barrier dysfunction of SAP.

MATERIALS AND METHODS: Twenty-four BALB/c mice were randomly divided into two groups with twelve mice each group. The SAP group mice received six intraperitoneal injections of cerulein (50 μg/kg) at 1-hour intervals, then given one intraperitoneal injection of 10 mg/kg lipopolysaccharide (LPS from *E. coli*) for inducing SAP. Normal saline was given to the mice of control group. The animals of each group were averaged to two batches. Four and eight hours after the final injection, respectively, mice were anesthetized and blood and tissue samples were harvested for examination. The pathological changes of pancreas and gut were observed and scored. The serum levels of diamine oxidase (DAO), amylase and tumor necrosis factor-alpha (TNF-α) were measured. The contents of malondialdehyde (MDA) and reduced glutathione (GSH) and activity of superoxide dismutase (SOD) and xanthine oxidase (XO) in gut mucosa were detected. In gut mucosa, the caspase-3 activity was measured and the cell apoptosis and apoptosis index (AI) were determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. The data were analyzed by ANOVA and *t*-test.

RESULTS: At four and eight hours after SAP induction, the SAP group mice had significantly higher pancreatic and gut pathological scores ($p < 0.01$) and increased serum levels of amylase ($p < 0.05$), DAO and TNF-α ($p < 0.01$) and increased MDA contents and XO activity of gut mucosa ($p < 0.01$) compared with those of control mice. There were significantly lower GSH contents ($p < 0.05$) and SOD activity ($p < 0.01$) of gut mucosa in the SAP mice. It was also observed that the gut mucosa cells of SAP mice had significantly higher caspase-3 activity and apoptosis index ($p < 0.01$).

CONCLUSIONS: In SAP, waterfall-style release of inflammatory factors such as TNF-α led to ischemia-reperfusion injury of gut mucosa which resulted in serious oxidative stress and activation of caspase-3 pathway and severe apoptosis of gut mucosa. Therefore, intestinal mucosal oxidative stress may play an important role in the mechanism of gut barrier dysfunction.

Key Words:
Ischemia-reperfusion injury, Reactive oxygen species, Oxidative stress, Severe acute pancreatitis, Gut barrier dysfunction.

Introduction

Gut mucosa serves as a major anatomic and functional barrier preventing the entrance of potentially harmful intestinal malignant bacteria and endotoxin into extraintestinal tissues and the systemic circulation[1]. Gut mucosal barrier possesses the largest mass of lymphoid tissue in the human body and it is an important component of the body's immune syste[2]. However, under critical illness such as severe acute pancreatitis (SAP), the structure and function of intestinal mucosa are damaged and lead to gut barrier dysfunction[3]. Impaired gut mucosa barrier in SAP will lead to increased intestinal permeability and translocation of intestinal lumen-derived bacteria and endotoxins, as well as release of inflammatory mediators and cytokines, and initiation of systemic inflammatory response syndrome (SIRS), and multiple organ dysfunction syndrome[4,5]. The excessive release of inflammatory mediators such as tumor necrosis factor-alpha (TNF-α) during SAP is a main cause of gut mucosal injury. TNF-α causes accumulation and activation of polymorphonuclear granulocytes and release of oxidant and proteolytic enzymes. Meanwhile, TNF-α aggravates the intestinal inflammatory reaction and microcirculation disturbance of SAP. Micro-

Corresponding Author: Ruilan Wang, MD; e-mail: wangyusun@hotmail.com

circulation disturbance will result in gut barrier dysfunction through reactive oxygen species (ROS) production by the xanthine oxidase (XO) and hypoxanthine accumulated in intestinal tissue. SAP also increases epithelial apoptosis of intestinal mucosa[4]. In this study, using mice as an *in vivo* model, we have investigated the roles of inflammatory factors releasing, intestinal mucosal oxidative stress, and cell apoptosis in gut barrier dysfunction in SAP.

Materials and Methods

Ethical Approval

This study was approved by the Local Animal Ethical Committee.

Animal Model

Male BALB/c mice weighting 25 g were supplied by Shanghai Laboratory Animal Center, Chinese Academy of Science. Mice were maintained on a standard rat chow and fasted for 12 hours before the experiment with tap water allowed *ad libitum*. The twenty-four mice were randomly allocated to two groups (12 for each group). The SAP group mice received six intraperitoneal injections of cerulein (obtained from Bachem AG, Vubendorg, Switzerland, 50 μg/kg) at 1 hour intervals, then one intraperitoneal injection of 10 mg/kg lipopolysaccharide (LPS from *E. coli*, obtained from Sigma-Aldrich, St. Louis, MO, USA) for inducing severe acute pancreatitis[6]. Two ml of normal saline were given to the control group mice. The animals of each group were averaged to two batches. Four and eight hours after the final injection, respectively, mice were laparotomized under anaesthesia and blood and tissue samples were harvested for examination. Three to five cm of terminal ileac segments without mesentery and ileac contents were harvested and fixed in formalin solution. The precisely dissected mucosal tissue was immersed in liquid nitrogen for subsequent assays.

Histopathologic Score Criteria

The specimens of pancreas and gut were fixed in formalin solution and embedded in paraffin. Sections were routine chipped and stained with hematoxylin and eosin (HE) dye for morphologic analysis by light microscopy. Severity of pancreatitis was scored as previously described criteria [7] with a score range of 0-16, including edema, acinar necrosis, hemorrhage and fat necrosis, inflammatory and perivascular infiltration. Meanwhile, the score of gut tissues was based on the changes of mucosal cells and structure, interstitial hemorrhage and inflammatory cell infiltration according to pathological score criterion (Table I).

Table I. Pathological score criterion of intestine.

Score	Score criterion
0	No damage
1	Partial atrophy and necrosis of gut mucosa, interstitial hemorrhage and partial inflammatory cell infiltration
2	Splinter ecclasis and hemorrhage of gut mucosa, splinter inflammatory cell infiltration, intact mucosal structure
3	Massive necrosis and ecclasis of gut mucosa, inflammatory cell infiltration, damage of mucosal structure
4	Widespread necrosis of gut mucosal, disappearance of mucosal structure, splinter hemorrhage, inflammatory cell infiltration

Serum Sample Test

1. Amylase level was measured by CNPG3 Kits (Shanghai Kehua Dongling Diagnostic Products Co, Shanghai, China).
2. Diamine oxidase (DAO) level was determined using spectrophotometry (standard DAO from Sigma-Aldrich, St. Louis, MO, USA).
3. TNF-α level was tested with ELISA Kits (Diaclone SAS, Besancon, France).

Measurement of Concentration of Malondialdehyde (MDA) and Reduced Glutathione (GSH) and Activity of Superoxide Dismutase (SOD) and Xanthine Oxidase (XO) in Gut Mucosa

Gut mucosa was triturated in pre-cooling mortar, and then ice-cold saline was added in a low temperature homogenate machine for tissue homogenate. After centrifugation of the tissue homogenate, the supernatant was collected for the measurement of GSH content and activity of SOD and XO of gut mucosa according to the reagent kit introductions[8]. Determination of MDA content was monitored following the TBA-spectrophotometry method[9]. (Kit purchased from Nanjing Jiancheng Bioengineering Institute).

Detection of Apoptosis

Caspase-3 activity assay. Tissue sample (20 mg), excised from the gut mucosa, was lysed with 200 μl of lysis buffer and then homogenized on ice. After 10 min centrifuge at 4°C, the supernatant was collected in pre-cold centrifuge tube and incubated for 2 hours at 37°C. The protein concentration was detected using the Bradford, then the caspase-3 activity was measured according to the reagent kit introductions (Kits purchased from Beyotime Co, Jiangsu, China).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. According to standard procedures (Kits purchased from Roche Diagnostic, Mannheim, Germany), the specimens of gut mucosa was embedded in paraffin and chipped. After dewaxing, hydration and digestion with proteinase K, TUNEL and POD (peroxidase) were applied sequentially. After developing of DAB (3,3'-diaminobenzidine) substrate solution at room temperature, sections were stained with hematoxylin, rinsed and dehydrated. The positive cells presented a brown nucleus under light microscope. For the apoptosis index (AI) determination, the number of apoptotic cells in every 100 epithelial cells from five optional views of each slide was recorded.

Statistical Analysis

Statistical analyses were performed with the software package PASW (Predictive Analytic Software) Statistics 18.0 and evaluated using ANOVA and *t*-test. Data were expressed as Mean ± SD and p values < 0.05 were considered to be statistically significant.

Results

Pancreatic Histopathology and Serum Amylase Level

At 4 and 8 hour after SAP induction, pancreatic splinter hemorrhage and necrosis, calcified spots were observed by visual inspection in SAP mice. Meanwhile, partial hemorrhage and necrosis of the pancreas, infiltration of inflammatory cells, glandular swelling and spacing broadening were also observed by light microscopy in SAP mice and no pathological changes were shown in control mice. The pathological scores of pancreas in SAP mice were significantly higher compared with that of the control mice ($p < 0.01$) at both of the two time points. Serum amylase level was significantly increased in SAP mice ($p < 0.05$). These changes showed the successful establishment of SAP *in vivo* model (Figure 1).

Intestinal Histopathology and Serum DAO Level

At 4 and 8 hour after SAP induction, significant congestion, hemorrhage, infiltration of inflammatory cells, gland atrophy, massive necrosis and ecclasis of mucosal cells were observed in SAP mice. By comparison, only slight congestion, mild ecclasis of mucosal cells were observed in control mice. The pathological scores of gut mucosa and serum DAO level in SAP mice were remarkably elevated in comparison to that of the control mice ($p < 0.01$) at both of the two time points (Figure 2).

Serum TNF-α Level

At 4 and 8 hour after SAP induction, serum TNF-α level was significantly higher in SAP mice ($p < 0.01$) (Figure 3).

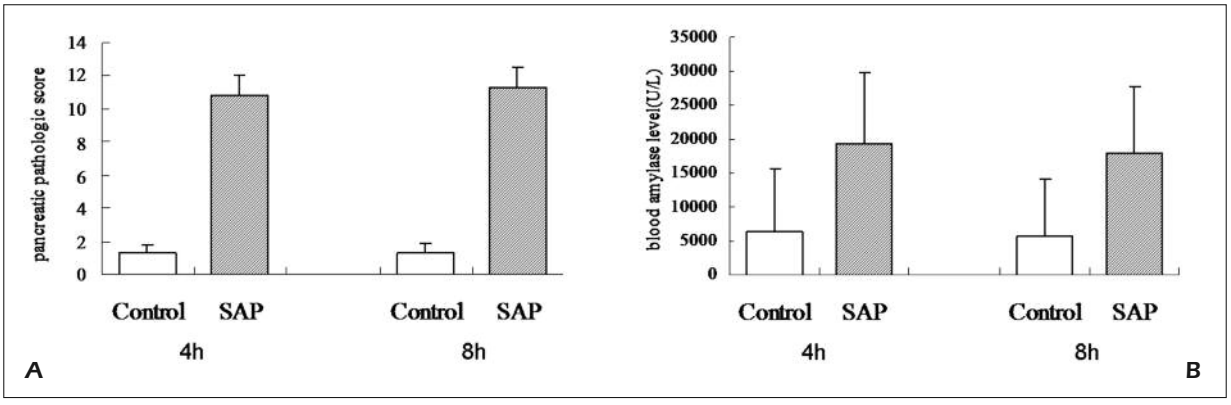

Figure 1. Pancreatic pathological score and blood amylase level (Mean ± SD). ***A,*** At 4 and 8 hour after SAP induction, the pathological scores of pancreas in SAP mice were significantly higher compared with that of the control mice ($p < 0.01$). ***B,*** At 4 and 8 hour after SAP induction, blood amylase level was significantly increased in SAP mice compared with control mice ($p < 0.05$).

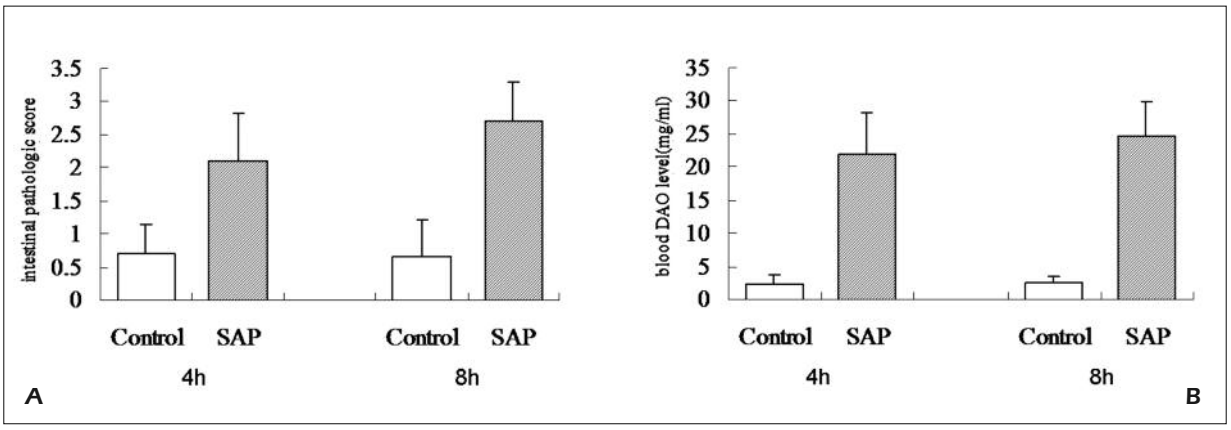

Figure 2. Intestinal pathological score and blood DAO level (Mean ± SD). ***A,*** At 4 and 8 hour after SAP induction, the pathological scores of intestinal mucosa in SAP mice were remarkably elevated in comparison to that of the control mice ($p < 0.01$). ***B,*** At 4 and 8 hour after SAP induction, blood DAO level was significantly increased in SAP mice compared with that in control mice ($p < 0.01$).

Intestinal MDA, GSH Content and SOD, XO Activity

At 4 and 8 hour after SAP induction, the SAP mice demonstrated significant increase in intestinal MDA content ($p < 0.01$) and XO activity ($p < 0.01$), and significant decrease in intestinal SOD activity ($p < 0.01$) and GSH content ($p < 0.05$). These results indicated that oxidative stress was enhanced in SAP mice (Figure 4).

Caspase-3 Activity and Apoptosis Index in Gut Mucosa Cells

At 4 and 8 hour after SAP induction, SAP mice had significantly increased caspase-3 activity and had higher apoptosis index in gut mucosa cells ($p < 0.01$) (Figure 5).

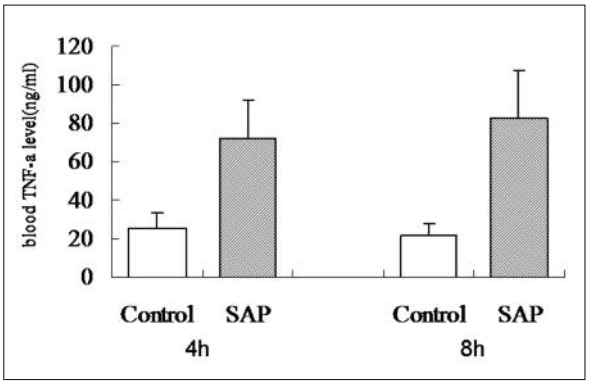

Figure 3. Blood TNF-α level (Mean ± SD). At 4 and 8 hour after SAP induction, blood TNF-α level was significantly higher in SAP mice compared with that in control mice ($p < 0.01$).

Discussion

Previous studies showed that the most important factor in determining the SAP progression is infection which is mainly derived from gut[10,11]. During SAP, structure and function of intestinal mucosa are damaged and lead to gut barrier dysfunction[3]. Impaired gut mucosa barrier in SAP will lead to increased intestinal permeability and translocation of intestinal lumen-derived bacteria and endotoxin, as well as release of inflammatory mediators and cytokines, and initiation of systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome[4,5,12]. Therefore, finding the players that cause gut barrier dysfunction in SAP and preventing the development of gut barrier dysfunction may improve the prognosis of SAP patients[4,13,14].

In line with the damage or repair in gut mucosal structure, level of DAO (diamine oxidase) was expressed the trend of raise or reduction, which indicated that DAO level is a reliable marker to reflect the changes associated with mucosal injury in gut barrier dysfunction[14,15]. In our study, compared with control mice, DAO level was significantly increased in SAP mice at 4 and 8 hour after SAP induction. In addition, massive necrosis and ecclasis of gut mucosa and high scores of gut pathology were observed in SAP mice. These results indicated that severe structure and function damage of gut mucosa occurred in SAP mice.

In our study, TNF-α level was significantly higher in SAP mice. TNF-α, one of the most important inflammatory mediators, plays a key role

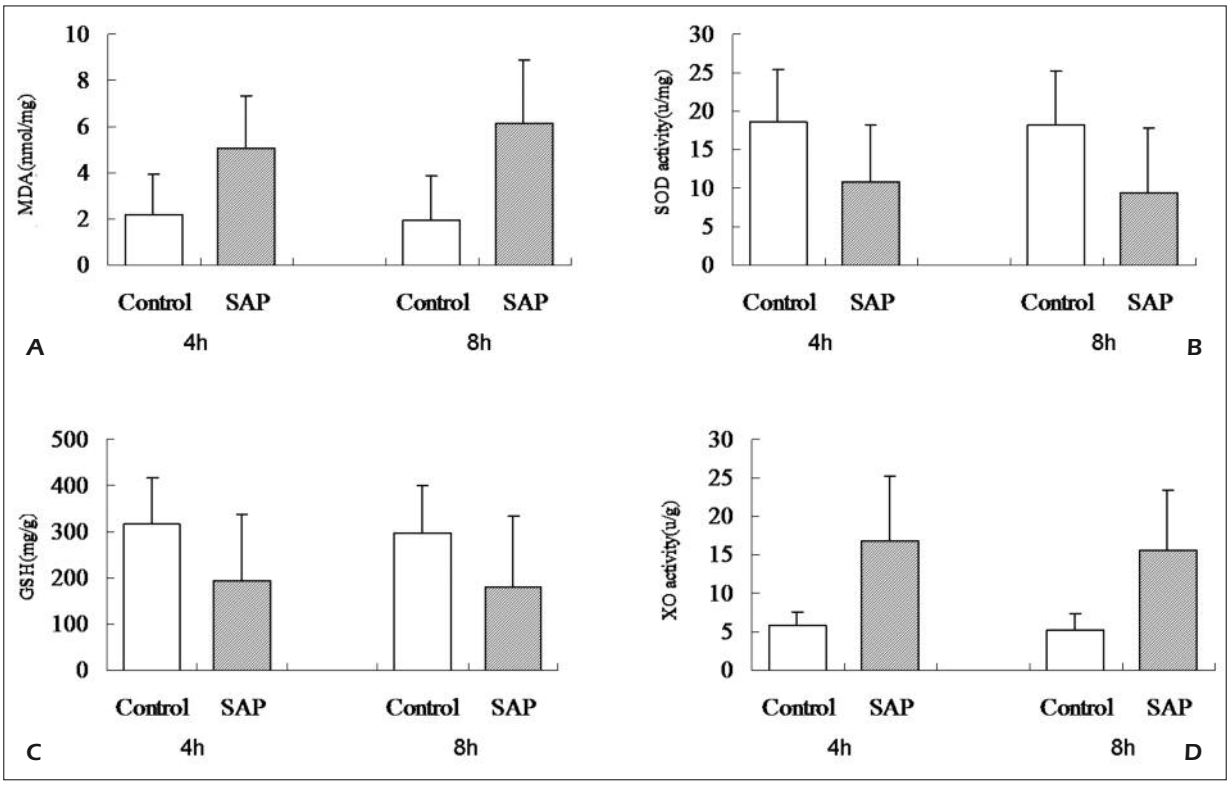

Figure 4. Intestinal MDA, GSH content and SOD, XO activity (Mean ± SD). ***A,*** At 4 and 8 hour after SAP induction, the SAP mice demonstrated significant increase in intestinal MDA content compared with control mice ($p < 0.01$). ***B,*** At 4 and 8 hour after SAP induction, the SAP mice demonstrated significant decrease in intestinal SOD activity compared with control mice ($p < 0.01$). ***C,*** At 4 and 8 hour after SAP induction, the SAP mice demonstrated significant decrease in intestinal GSH content compared with control mice ($p < 0.05$). ***D,*** At 4 and 8 hour after SAP induction, the SAP mice demonstrated significant increase in intestinal XO activity compared with control mice ($p < 0.01$).

in SIRS which occurred at early stage of SAP. The increased TNF-α leads to cascade reaction of inflammatory mediators which in turn results in ischemia-reperfusion injury of gut mucosa[16].

The major consequence of ischemia-reperfusion injury is oxidative stress which leads to toxic reactive oxygen species (ROS) generation. Xanthine oxidase (XO) pathway is the most im-

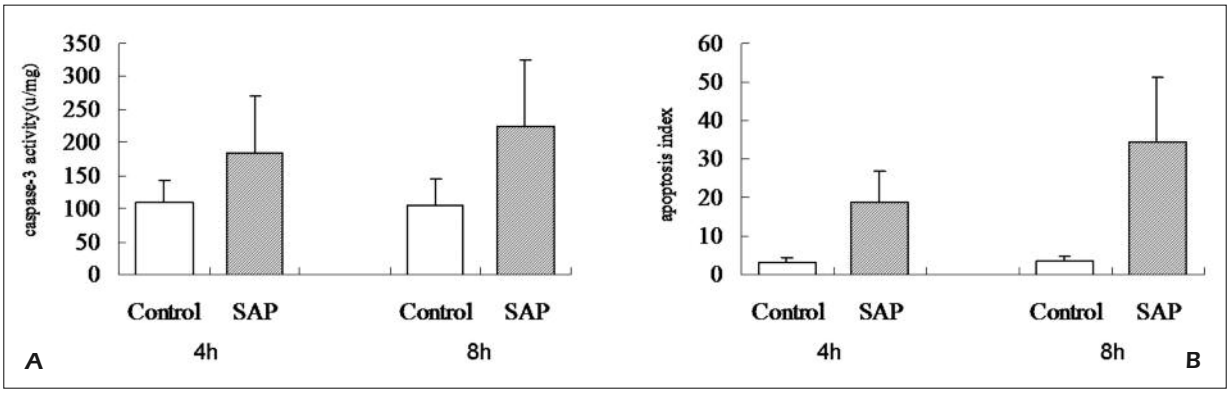

Figure 5. Intestinal mucosa cell caspase-3 activity and apoptosis index (Mean ± SD). ***A,*** At 4 and 8 hour after SAP induction, SAP mice had significantly increased caspase-3 activity in intestinal mucosa cells compared with control mice ($p < 0.01$). ***B,*** At 4 and 8 hour after SAP induction, SAP mice had significantly higher apoptosis index in intestinal mucosa cells compared with control mice ($p < 0.01$).

portant source of ROS in gut during ischemia-reperfusion injury[17,18]. Therefore, the gut mucosal cells, which are abundant in XO[19], will show severe oxidative stress when ischemia-reperfusion injury occurs. In addition, MDA, the product of lipid peroxidation[20] induced by ROS, is regarded as an indicator of cellular toxicity[21,22]. Reduced glutathione (GSH), a major antioxidant of cellular defense system *in vivo*, is depleted after it being oxidized to oxidized GSH (GSSG) during oxidative stress. Superoxide dismutase (SOD), as a natural antioxidant, can scavenge ROS which are induced by metabolism. GSH and SOD represent the important cellular antioxidants in the body. In our study, we found that XO activity and MDA content in SAP mice were significantly elevated, whereas SOD activity and GSH level were significantly decreased. These results suggested that there were extensive ROS generation and decrease of antioxidant defense system in gut mucosa during SAP.

The activity of effector caspases represents an irreversible phase of apoptosis[23]. Meanwhile, a key protease caspase-3 of the conjunctive downstream caspases of apoptosis in mammalian cellular apoptosis is the main effector of apoptosis and the most important executive[24]. There is evidence showing that caspase family is sensitive to oxidative stress[25]. Several reports revealed that caspase-3 can be immediately activated by ROS in diabetes[26,27]. Our study showed that oxidative stress of gut mucosa in SAP mice was associated with the significantly higher activity of caspase-3 and elevated apoptosis index. These results demonstrated that severe oxidative stress and activated caspase-3 pathway occurred in gut mucosal cells, which aggravated apoptosis of gut mucosal cells and led to gut barrier dysfunction in SAP.

Conclusions

Our study suggested that SAP could induce the ischemia-reperfusion injury of gut mucosa by cascade release of inflammatory factors such as TNF-α, which led to massive generation of ROS, depletion and inhibition of antioxidant system. Therefore, the severe oxidative stress developed in gut mucosa and activated caspase-3, which aggravated apoptosis of gut mucosal cells, was an important mechanism of gut barrier dysfunction in SAP.

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