Original Article
Association between glucose-regulated protein and neutrophil apoptosis in severe acute pancreatitis

Lan-Tao Xu[1], He-Ling Xu[2], Ming-Sheng Fu[3]

[1]Department of Gastroenterology, Ruijin Hospital North Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai 201801, China; [2]Soochow University, Suzhou, 215006, China; [3]Department of Gastroenterology, The Fifth people's Hospital of Shanghai, Fudan University, Shanghai 200240, China

Abstract: Objective: This study aimed to investigate the role of glucose regulated protein 78 (GRP-78) in the apoptosis of neutrophils in rats with severe acute pancreatitis. Methods: A total of 54 SD male rats were randomly assigned into 2 groups: sham group (n=24) and pancreatitis group (n=30). Severe acute pancreatitis was induced by retrograde cholangiopancreatography injection of sodium taurocholate. Rats were sacrified at 3 h, 6 h and 12 h after injection. In control group, rats received laparotomy, but the pancreates remained intact. The serum amylase was detected at different time points, and flow cytometry was done to detect the apoptosis of neutrophils. Proteins were extracted from neutrophils and subjected to detection of GRP78 and Mcl-1 expression by Western blot assay. HE staining was performed for pathological scoring of the pancreas. Results: The serum amylase in pancreatitis group increased markedly when compared with control group (*P*<0.01). In SAP group, the serum amylase increased gradually over time (*P*<0.01). HE staining showed a lot of inflammatory cells and infiltration of red blood cells and the apoptosis rate of neutrophils reduced gradually (*P*<0.01). Western blot assay showed the protein expression of GRP-78 and Mcl-1 increased in neutrophils over time. Conclusion: In rats with SAP, the apoptosis rate of neutrophils reduced over time, which may be associated to the stress induced expression of GRP78 and subsequent activation of Mcl-1 resulting in suppression of neutrphil apoptosis over time.

Keywords: 78 kDa glucose-regulated protein, myeloid cell leukemia 1, neutrophils, phosphatidylinositol kinase-3/serine-threonine protein kinase pathway, severe acute pancreatitis, cell apoptosis

Introduction

Severe acute pancreatitis is a disease with high mortality [1]. To clear the neutrophils in the inflammatory foci via the apoptosis pathway is a mechanism of restricting tissue damage [2]. Neutrophils are a group of important cells in the defense system and also a group of major inflammatory cells involved in the inflammatory injury. The 78 kDa glucose regulated protein is a stress protein and its expression increases significantly following stress to maintain the stability of endoplasmic reticulum and protect cells [3]. In the present study, serum biochemistry, flow cytometry, Western blot assay and pathological examination were performed to investigate the association between GRP78 expression and apoptosis of neutrophils in rats with SAP. Our findings may provide evidence for the investigation of pathogenesis, prevention and therapy of SAP.

Materials and methods

Materials

A total of 54 male SD rats (specific pathogen free) weighing 260-310 g were purchased from the Experimental Center of the 6th People Hospital in Shanghai. Sodium taurocholate (Sigma, USA), neutrophil separation medium LZS1091 (Tianjin Haoyang Biological Products Co., Ltd, China), BCA protein quantification kit, Annexin V-FITC cell apoptosis detection kit, SDS-PAGE kit, erythrocyte lysate (Beyotime Biotech Co., Ltd), ECL chemiluminescence kit, protein marker (Thermo Scientific, USA), PVDF membrane (Biosharp, China), rabbit anti-rat GRP-78 and Mcl-1, β-actin (epitomics, CA, USA), and horseradish peroxidase conjugated goat anti-rabbit IgG (Hangzhou Hua'an Biotech Co., Ltd, China) were used in the present study.

Table 1. Serum concentration of amylase at different time points (U/L)

Group	3 h	6 h	12 h
SO	1532.00±214.812	1606.00±205.557	1608.38±231.369
SAP	3573.00±424.317[a,b]	5183.70±761.834[a,b]	6246.00±602.931[a,b]

Footnotes: [a]$P<0.01$ vs. SO group at different time points in; [b]$P<0.01$ at different time points in SAP group.

Establishment of animal model

SD rats were housed for 1 week and fasted for 12 h before surgery. Animals were anesthetized with 1% ketamine hydrochloride at 1 ml/100 g intraperitoneally. Rats were fixed in a surgery table, and abdominal skin was sterilized. A midline incision was made at the abdomen (2 cm in length) and the abdominal cavity was exposed. A transparent pancreaticobiliary tract was exposed along the stomach and duodenum connection. A swab was used to gently separate the pancreaticobiliary tract from the tissues surrounding the stomach and duodenum connection and the lower end of the pancreaticobiliary tract was exposed. The upper end of the pancreaticobiliary tract was clamped with an arterial clamp. A 24-gauge needle was retrogradely inserted 1 cm along the pancreaticobiliary tract through an avascular zone of the duodenum and then fixed. The core was extracted and the needle was connected to a syringe, followed by slow injection of 5% sodium taurocholate at 0.1 ml/100 g. Two minutes later, the clamp was released, and the wound was closed. After surgery, rats received food deprivation (but no water deprivation). At 3 h, 6 h and 12 h after surgery, rats were intraperitoneally anesthetized and 6-8 ml of blood was collected from the femoral vein. The pancreatic tissues at the same site were harvested for following experiments. In control group, the pancrease was exposed, but not injected with sodium taurocholate.

Detection of serum amylase

In brief, 1 ml of blood was collected and centrifuged, and the concentration of amylase was detected by using an automatic biochemical analyzer.

Separation of neutrophils

Neutrophils were separated by density gradient centrifugation. In brief, 4 ml of heparin treated blood was mixed with whole blood and solution for tissue homogenation at a ratio of 1:1. The mixture was allowed to stay at room tempera-ture for 30 min and the supernatant was harvested onto solution A (1:1), followed by centrifugation at 1500 r/min for 20 min. Four layers were observed in the centrifuge tube, and the third layer contained a large amount of neutrophils. The third layer was harvested and transferred into a tube containing 4-5 ml of washing buffer, followed by centrifugation at 1500 r/min for 15 min. This was repeated once. When there were red blood cells, 5 volumes of erythrocyte lysate were added, followed by incubation for 2 min. After centrifugation at 400 g for 5 min, the supernatant was removed, and the remaining cells were neutrophils. The concentration of neutrophils was adjusted to $1×10^6$/L. Detection showed more than 95% of PMN was intact and trypan blue staining revealed that more than 99% of cells were viable.

Detection of apoptotic neutrophils

In brief, $5\text{-}10×10^4$ cells were collected and centrifuged at 1000 g for 5 min. The supernatant was removed. Then, cells were re-suspended in annexin V-FITC binding buffer (195 μl), followed by addition of Annexin V-FITC (5 μl). Incubation was done in dark at room temperature for 10 min, followed by centrifugation at 1000 g for 5 min. The supernatant was removed, and annexin V-FITC binding buffer (190 μl) was used to re-suspend these cells. After addition of 10 μl of propidium iodide staining solution, incubation was done in dark on ice, and this cell suspension was used for flow cytometry.

HE staining for pathological examination

The pancreatic tissues were fixed in formalin, followed by routine embedding with paraffin and sectioning. The sections were then subjected to deparaffinization and HE staining. Experienced pathologists who were blind to this study were asked for pathological examination according to the Schmids method [4]. The pancrease was observed under a light microscope.

Detection of GRP78 and Mcl-1 expression by western blot assay

In brief, $50\text{-}100×10^4$ neutrophils were mixed with 150 μl of RIPA lysis buffer (900 μl RIPA + 100 μl PMSF), followed by incubation. Then, cell lysate was centrifuged at 12000 g for 5
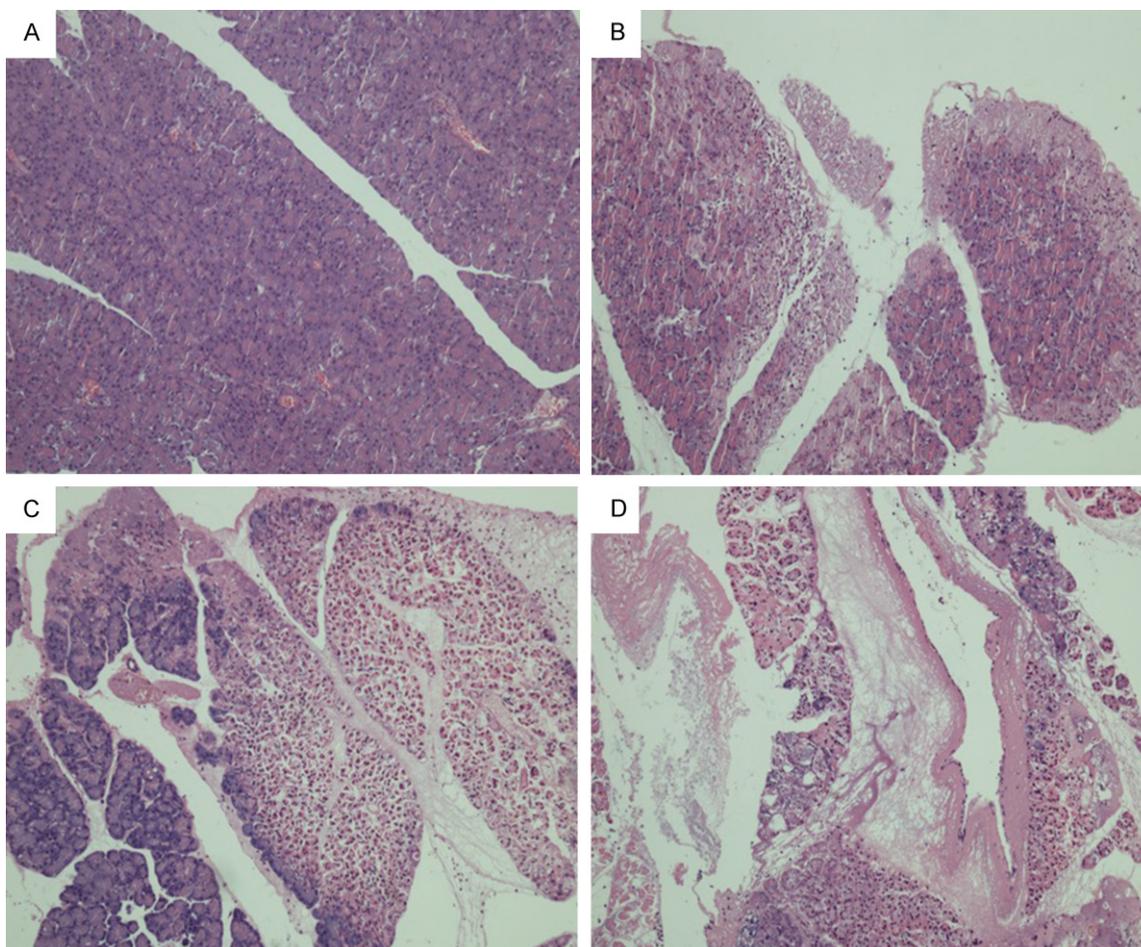
Figure 1. HE staining of the pancreatic in different groups at different time points (×100; A: 3 h SO; B: 3 h SAP; C: 6 h SAP; D: 12 h SAP).

Table 2. Pathological score of the pancreas in different groups

Group	3 h	6 h	12 h
SO	0.1±0.30	0.1±0.23	0.2±0.21
SAP	5.4±1.32[a,b]	7.5±1.34[a,b]	9.2±1.42[a,b]

Footnotes: [a]$P<0.01$ vs. SO group at different time points; [b]$P<0.01$ at different time points in SAP group.

min, and the supernatant was collected for protein quantification with BCA method. Then, 40 µg of proteins per sample were loaded onto gel for SDS-PAGE at 80 V for 30 min and then 120 V for 90 min. The proteins were transferred onto PVDF membrane at 250 mA for 60 min which was then blocked in 5% non-fat milk for 1 h. This membrane was incubated with rabbit anti-rat GRP78 antibody, β-actin antibody (1:3000) or Mcl-1 antibody (1:1000) at 4°C over night. After washing in T-BST, the membrane was incubated with HRP conjugated anti-rabbit IgG (1:3000) for 1 h. After washing, chemiluminescence method was used for visualization, and Image Quant LAS4000 min was used for detect the protein bands.

Statistical analysis

Data were expressed as mean ± standard deviation (x ± s) and analyzed with analysis of variance. Statistical analysis was done with SPSS version 17.0. Intragroup comparisons of data at different time points were done with t test, and independent sample t test was employed for intergroup comparisons of data at the same time point. A value of $P<0.05$ was considered statistically significant.

Results

Serum concentration of amylase (U/L)

In SAP group, the serum concentration of amylase increased gradually and reached a peak at

Table 3. Apoptosis rate of peripheral neutrophils in rats of different groups at different time points

Group	3 h	6 h	12 h
SO	7.452±0.4621	7.125±0.5148	6.900±0.5682
SAP	4.800±0.8340[a,b]	3.030±0.4923[a,b]	1.300±0.5812[a,b]

Footnotes: [a]$P<0.01$ vs. SO group at different time points; [b]$P<0.01$ at different time points in SAP group.

12 h (6246.00±602.931). The amylase concentration in SAP group at different time points after surgery was significantly higher than that in SO group ($P<0.01$). In SO group, the serum concentration of amylase remained at a low level (**Table 1**).

Macroscopic findings of the pancrease

In SO group, there was a small amount of white fluid and the pancrease was intact. At 3 h after SAP, there was a small amount of light red ascites, the pancrease was edema. At 6 h after SAP, the ascites increased. At 12 h after SAP, a large amount of red ascites was observed, and the pancrease became hemorrhagic and necrotic.

Pathological findings of the pancrease

HE staining showed no hemorrhage and normal structure of the pancrease under a light microscope in control group. The pancreatic injury in SO group deteriorated over time. The mild pancreatic injury was characterized by alveolar interstitial edema, infiltration of inflammatory cells including neutrophils and lymphocytes, and scattered hemorrhagic and necrotic foci, and the change in blood vessels was not obvious. The severe pancreatic injury was characterized by evident hemorrhage in the pancrease, necrotic alveolar cells, and lobular destruction and disordered lobular structure; there was a large amount of red blood cells and inflammatory cells in the lobular septum (**Figure 1**; **Table 2**).

Apoptosis rate of neutrophils (%)

After establishment of animal model, the apoptosis rate of neutrophils in SAP increased gradually over time and reached a peak at 12 h (1.300±0.5812). At the corresponding time points, the apoptosis rate in SAP group was significantly higher than that in SO group ($P<0.01$; **Table 3**; **Figure 2**).

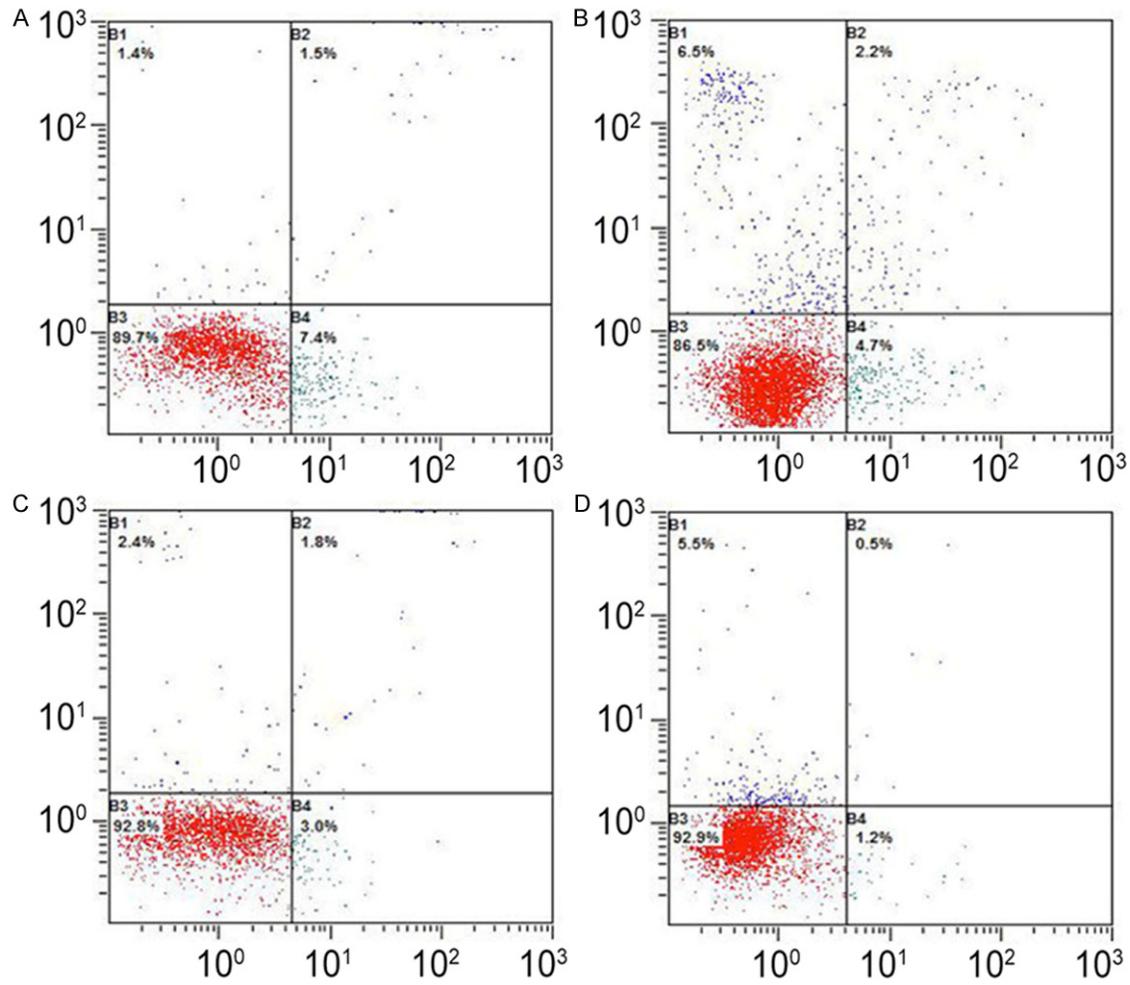*Protein expression of GRP78 and Mcl-1 in neutrophils*

Western blot assay showed the protein expression of GRP78 and Mcl-1 in SAP was significantly higher than that in SO at corresponding time point. In addition, the protein expression of GRP78 and Mcl-1 in SAP increased gradually over time and that in SO group remained at a low level (**Figure 3**).

Discussion

Acute pancreatitis is an acute abdominal disease, has an acute onset, progresses rapidly and has a high mortality. Especially, severe acute pancreatitis (SAP) progresses more rapidly, has a complex pathogenic mechanism, is difficult to treat and has a mortality of higher than 30%. It has been confirmed that multiple factors are involved in the pathogenesis of SAP [5]. The increase in neutrophils reflects the presence of inflammatory response and neutrophils play an important role in the occurrence, development and outcome of inflammation. Apoptosis refers to programmed cell death and the change in the survival environment may affect the process of apoptosis [6]. The normal aging PMN dies in the form of apoptosis and the dead PMN is then recognized, phagocytized and cleared. In the apoptosis, there is no rupture of cell membrane and no release of cytotoxic contents after phagocytosis, which are important to promote the attenuation and restriction of inflammation. In SAP, the function cycle of PMN prolongs when the apoptosis of PMN is inhibited, and a large amount of harmful substances such as free radicals, proteolytic enzymes and inflammatory mediators are released, resulting in sustained tissue injury [7]. In the present study, results showed, at 3 h, 6 h and 12 h after SAP, the apoptosis rate of peripheral neutrophils reduces gradually and reached a minimal level at 12 h. In SO group, the apoptosis rate of neutrophils remained at a high level. Statistical analysis showed the apoptosis rate of neutrophils in SAP was significantly lower than that in SO group at corresponding time points ($P<0.01$). In addition, marked difference was also observed at any two time points in SAP group ($P<0.01$).

GRP was first identified in 1977. Shiu et al [8] found that the chicken embryo fibroblasts in

Figure 2. Apoptosis rate of peripheral neutrophils in rats of different groups at different time points. A: 3 h SO; B: 3 h SAP; C: 6 h SAP; D: 12 h SAP.
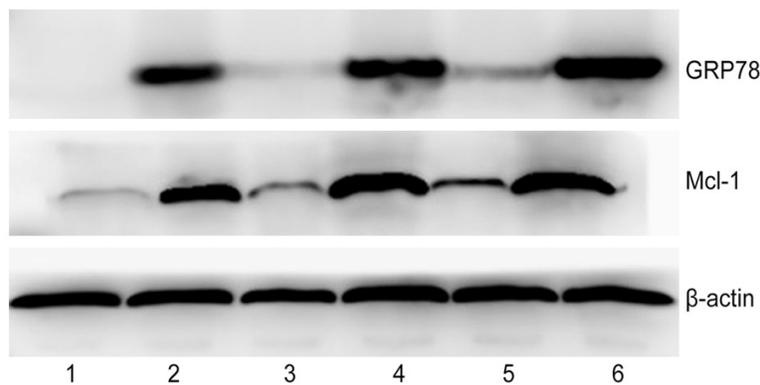
Figure 3. Western blot assay of protein expression of GRP78 and Mcl-1 in neutrophils (1, 3, 5: 3 h, 6 h and 12 h in SO; 2, 4, 6: 3 h, 6 h and 12 h in SAP group).

the glucose free medium could produce 2 proteins with the relative molecular weight of 78 kDa and 94 kDa, respectively. Thus, they named these two proteins as GRP78 and GRP94. Pathological states such as hypoxia, low glucose, low calcium or viral infection may induce the production of a large amount of unfolded proteins in the endoplasmic reticulum, and then induce a series of adaptive responses which are known as a unfolded protein response (UPR) [9]. UPR has three branches to control the transcription and expression of molecular chaperone proteins including PRK like protein kinase (PERK), inositol requiring enzyme 1 (IRE1) and activating transcription

factor 6 (ATF6) [10-14]. GRP78 is also known as immunoglobulin heavy chain binding protein and is a calcium binding protein. Under normal conditions, GRP78 binds to the sensors on the endoplasmic reticulum including PERK, IRE1 and AFT6, to inhibit the signaling pathways related to UPR [15]. In the presence of endoplasmic reticulum stress, the unfolded proteins or misfolded proteins release GRP78 from the sensors and the GRP78 expression increases significantly. Then, GRP78 binds to misfolded or unfolded proteins to restore the normal conformation. Thus, proteins can be normally synthesized under stress state, which maintain the calcium stability in the endoplasmic reticulum and the homeostasis [16]. Thus, the rapid increase in GRP78 expression has been regarded a sensitive marker of endoplasmic reticulum stress (ERS) [17]. In SAP, the low calcium and toxic substances may increase the GRP78 expression and initiate ERS. In the present study, at 3 h, 6 h and 12 h after SAP, the GRP78 expression increased gradually in the peripheral neutrophils of SAP rats and reached a peak at 12 h. In addition, the GRP78 expression in SAP group was significantly higher than that in SO group at corresponding time point.

GRP78 signaling pathways include Ras/MAPK, PI3-kinase/Akt, and PAK2 dependent signaling pathways. The increase in GRP78 expression may activate above signaling pathways [18-21]. Which pathway is involved in the regulation of Mcl-1 expression in neutrophils is still unclear. There is evidence showing that PI3k/Akt signaling pathway may phosphorylate Akt in the help of 3-phosphate inositol dependent protein kinase 1 (PDK-1) and then increase the activity of Akt, which may influences the expression of apoptosis related proteins (such as BAD, Mcl-1), exerting anti-apoptotic effect [22]. Currently, studies focus on the key molecules in the PI3k/Akt signaling pathway which may become new targets for the anti-tumor therapy. However, no studies have been conducted on the apoptosis of neutrophils in the SAP [23]. Mcl-1 is a anti-apoptotic protein of Bcl-2 family and can bind to the pro-apoptotic proteins of Bcl-2 family to block the change in mitochondrial permeability and the release of pro-apoptotic factors from the mitochondria, exerting anti-apoptotic effect [24, 25]. In the present study, at 3 h, 6 h and 12 h after SAP, the Mcl-1 expression increased gradually in the peripheral neutrophils of SAP rats and reached a peak at 12 h. In addition,

the Mcl-1 expression in SAP group was significantly higher than that in SO group at corresponding time points. Semi-quantification showed Mcl-1 expression was negatively associated with the apoptosis rate of neutrophils. However, the specific mechanism is required to be further studied.

Our results showed the GRP78 expression increased over time, but the apoptosis rate of neutrophils reduced gradually in rats with sodium taurocholate induced SAP. These indicate that GRP78 can activate PI3K/Akt signaling pathway to induce the expression of Mcl-1 (a pro-apoptotic protein), resulting in delayed apoptosis of peripheral PMN and deterioration of inflammation in SAP rats. Thus, GRP78 and Mcl-1 may become new targets for the therapy of injury due to neutrophil apoptosis, and blocker of PI3K/Akt signaling pathway may promote the apoptosis of neutrophils and then attenuate the tissue injury and inflammation. However, our results are required to be further confirmed in future studies.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. He-Ling Xu, Soochow University, Suzhou, 215006, China. E-mail: xuheling0001@126.com; Dr. Ming-Sheng Fu, Department of Gastroenterology, The Fifth people's Hospital of Shanghai, Fudan University, Shanghai 200240, China. E-mail: mingshengfu@yahoo.com

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