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Original Article

Spatial and temporal differences of HMGB1 expression in the pancreas of rats with acute pancreatitis

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Abstract: We aimed to investigate the spatial and temporal differences in expression between HMGB1 and early-stage inflammatory cytokines (IL-1, IL-6 and TNF-α) in pancreas tissue in rats with acute pancreatitis. SD rats (BW 350 ± 30 g, n = 48) were randomly divided into the experimental group (n = 36) which were injected with 5% sodium taurocholate into the bilipancreatic duct retrogradely to produce acute necrotic pancreatitis (ANP) rat models, and the sham-operated (SO) group (n = 12) injected with equal dose of saline. The rats were sacrificed at different time points at 0 h, 3 h, 6 h, 12 h, and 24 h post modeling, respectively. The peripheral blood amylase and different inflammatory factors in ANP rats at different time points were detected by ELISA, and the expression of HMGB1 in the pancreatic tissue was detected by immunohistochemistry, Western blot and Q-PCR methods. Results showed that the serum amylase in the ANP model rats was significantly higher than the sham-operated group ($P < 0.05$). The early inflammatory factors (IL-1, TNF-α and IL-6) increased quickly at 3 h after the model induction, reached the peak level at 6 h (higher than SO group, $P < 0.05$), then decreased at 12 h, and at 24 h the levels were lower than those at 12 h ($P < 0.05$). The HMGB1 level in the pancreatitis tissue did not change significantly at 3 h and 6 h ($P > 0.05$), however, it increased remarkably at 12 h, and maintained up to 24 h ($P > 0.05$). As a late inflammatory factor, the expression of HMGB1 in acute pancreatitis was obviously later than the early inflammatory factors IL-1, TNF-α and IL-6. HMGB1 may play a key role in maintaining the development of the acute pancreatitis.

Keywords: Acute pancreatitis, HMGB1, early inflammatory factor

Introduction

Acute pancreatitis (AP) is a common acute abdomen disease. The inflammatory factors involved in the systemic inflammatory response play an important role in the pathogenesis of pancreatitis [1, 2]. It is found that in the course of the inflammatory response, there exists a late-acting proinflammatory factor, namely the high mobility group protein B1 (HMGB1). This factor is usually expressed late in the inflammation, and persists with the inflammation. Therefore, HMGB1 is considered as the key inflammatory cytokine which persists with the inflammation and its further development [3]. This study investigated HMGB1 and early inflammatory factors in the pancreas of acute pancreatitis rat, elucidating the expression and significance of HMGB1 expression in acute pancreatitis.

Materials and methods

Materials and reagents

Forty-eight healthy male SD rats, weighting 350 ± 30 g, were provided by the Experimental Animal Center of Third Xiangya Hospital. After regular feeding, they were fasted at 12 h before the experiment, but free to drink water. Sodium taurocholate was purchased from Sigma company; ELISA kits for IL-1β, IL-6, TNF-α and HMGB1 were from ImmunoWay; Rabbit anti-HMGB1 primary antibody from Abcam; Goat anti-rabbit secondary antibody was from Jackson; Color pre-stained protein marker was from Fermentas; Bradford protein concentration assay kit was from the China Jiangsu Beyotime Institute of Biotechnology; Trizol agent from Invitrogen; and SYBR Green qRCR Mix from Toyobo company.

Table 1. ELISA detection of serum amylase, IL-1β, IL-6, TNF-α and HMGB1 ($\bar{x} \pm s$, unit: pg/ml)

	Group	3 h	6 h	12 h	24 h
Amylase	SO group	62.81 ± 8.05	65.81 ± 6.31	70.81 ± 8.57	78.81 ± 6.46
	ANP group	734.13 ± 65.30	1206.76 ± 83.27	2006.24 ± 119.81	1825.90 ± 105.14
IL-1β	SO group	7.44 ± 3.04	6.94 ± 2.01	6.93 ± 4.07	6.64 ± 5.01
	ANP group	93.88 ± 6.04	163.42 ± 19.63	95.73 ± 8.87	60.11 ± 9.14
IL-6	SO group	6.13 ± 2.07	5.57 ± 3.17	6.08 ± 1.87	5.38 ± 2.27
	ANP group	92.78 ± 9.55	168.98 ± 19.97	93.95 ± 11.06	60.92 ± 18.38
TNF-α	SO group	37.43 ± 3.33	39.43 ± 5.31	42.39 ± 5.15	50.14 ± 5.07
	ANP group	572.33 ± 52.57	1438.60 ± 94.78	904.01 ± 55.25	575.35 ± 35.48
HMGB1	SO group	15.52 ± 2.06	18.79 ± 6.06	17.52 ± 3.09	16.62 ± 4.47
	ANP group	52.46 ± 34.82※	192.46 ± 34.29	426.46 ± 44.08	598.50 ± 37.07

Comparison between ANP group and SO group of amylase, IL-1β, IL-6 and TNF-α contents at different time points, $P < 0.05$. Comparison of HMGB1 between ANP group at 3 h and SO group at 3 h, ※, $P > 0.05$; but for other time points, $P < 0.05$. For the ANP group, comparison with the preceding group, $P < 0.05$.

Establishment of animal models and experimental groups

Before experiment the rats were fasted for 24 h, free to access water. They were anesthetized by intraperitoneal injection (0.3 ml/100 g) with 10% chloral hydrate. The ventral midline was incised for entry into the abdominal cavity to expose the general bile duct and the pancreatic duct. The biliopancreatic duct was occluded with vascular clip for entry to the distal duodenum, and for the experimental group it was infused slowly with 5% sodium taurocholate (0.1 ml/100 g) by 1 ml syringe at the proximal end, for the SO control group with normal saline in equal quantity. After injection, the needle was maintained motionless for 10 min to prevent the drug refluxing in large amount. After modeling, the rat skin was disinfected, and the peritoneum was closed with continuous single-layer suture, the rest with full-layer simple-interval suture. After the abdomen was closed, the rat was injected with 5 ml saline at the inside position of the lower limb muscle to supplement the water lost in the operation. Experimental group (ANP group) rats were sacrificed at 3 h (n = 8), 6 h (n = 8), 12 h (n = 8), and 24 h (n = 8) after modeling, and the sham-operated group (SO group) rats were sacrificed at 3 h (n = 3), 6 h (n = 3), 12 h (n = 3) and 24 h (n = 3), respectively.

Specimen collection and preservation

When the rats were sacrificed, about 5 ml blood was collected from the heart, placed for half an hour, centrifuged at 1000 × *g* for 15 min, and the supernatant was pipetted and preserved at -80°C. The pancreatic tissue was taken and placed in cryogenic tubes, and put into liquid nitrogen quickly.

Detection of the serum inflammatory cytokine by ELISA assay

The contents of amylase, IL-1β, IL-6, TNF-α and HMGB1 in the serum were detected by ELISA kits according to the kit manuals.

Detection of the HMGB1 expression in the pancreatic tissue by immunohistochemistry method

The wax slices (5 μm thick) of the pancreatic tissue were dewaxed and hydrated, and rinsed by PBS (pH = 7.4) 3 × 5 min; boiled in 10 mM citrate buffer (pH = 6.0) with high pressure, rinsed by distilled water and then by PBS 3 × 5 min, and immersed; treated with 3% hydrogen peroxide at room temperature for 10 min, and rinsed by PBS 3 × 5 min; blocked by 5% fetal calf serum for 1 h, and rinsed by PBS for 5 min; added with anti-HMGB1 antibody, 4°C overnight; rinsed by PBS 3 × 5 min, and each slice was dropped with biotinylated secondary antibody in the kit, incubated and rinsed in a humidified chamber; each slice was dropped with HRP-conjugated streptavidin, incubated and rinsed in a humidified chamber, added with coloring solution, observed under microscope, when the color was appropriate the reaction was terminated by washing; and finally, dropped

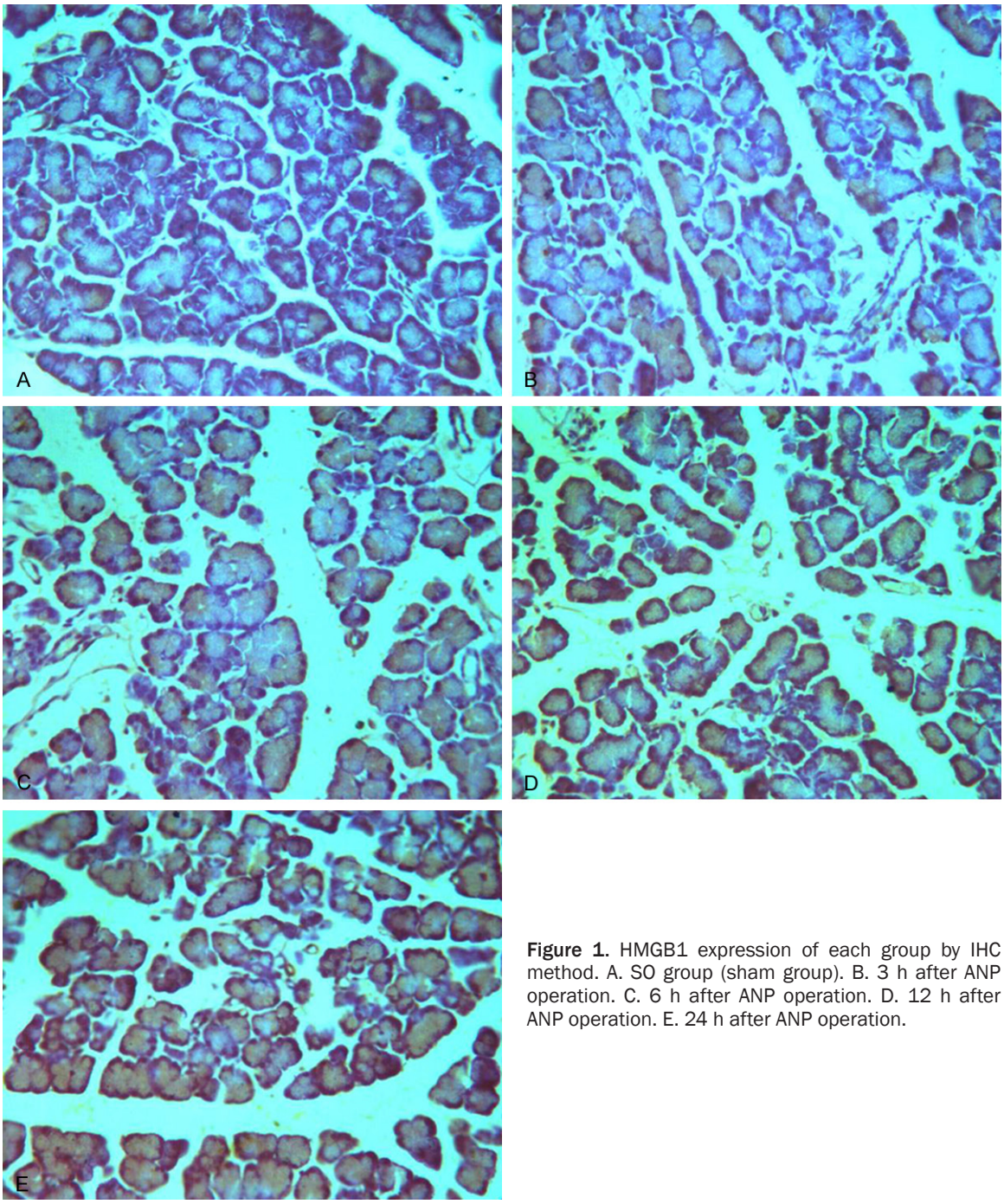

Figure 1. HMGB1 expression of each group by IHC method. A. SO group (sham group). B. 3 h after ANP operation. C. 6 h after ANP operation. D. 12 h after ANP operation. E. 24 h after ANP operation.

with hematoxylin, rinsed by water, dehydrated by alcohol gradients, immersed in xylene to make transparent, dropped with resin to mount.

Detection of the inflammatory cytokines in the pancreatic tissue by Western blot method

The pancreatic tissues from different time points were collected in a clean homogenizer, and added with 200 μl protein lysis solution per 20 mg tissue. The total protein lysate was collected to determine the protein content by Bradford assay. After running the polyacrylamide gel electrophoresis (PAGE), the proteins were electrically transferred to PVDF membrane. After blocked by 5% skim milk, the PVDF membrane was shaken overnight at 4°C with primary antibody, and incubated with HRP-labeled goat anti-rabbit secondary antibody in a shaker at

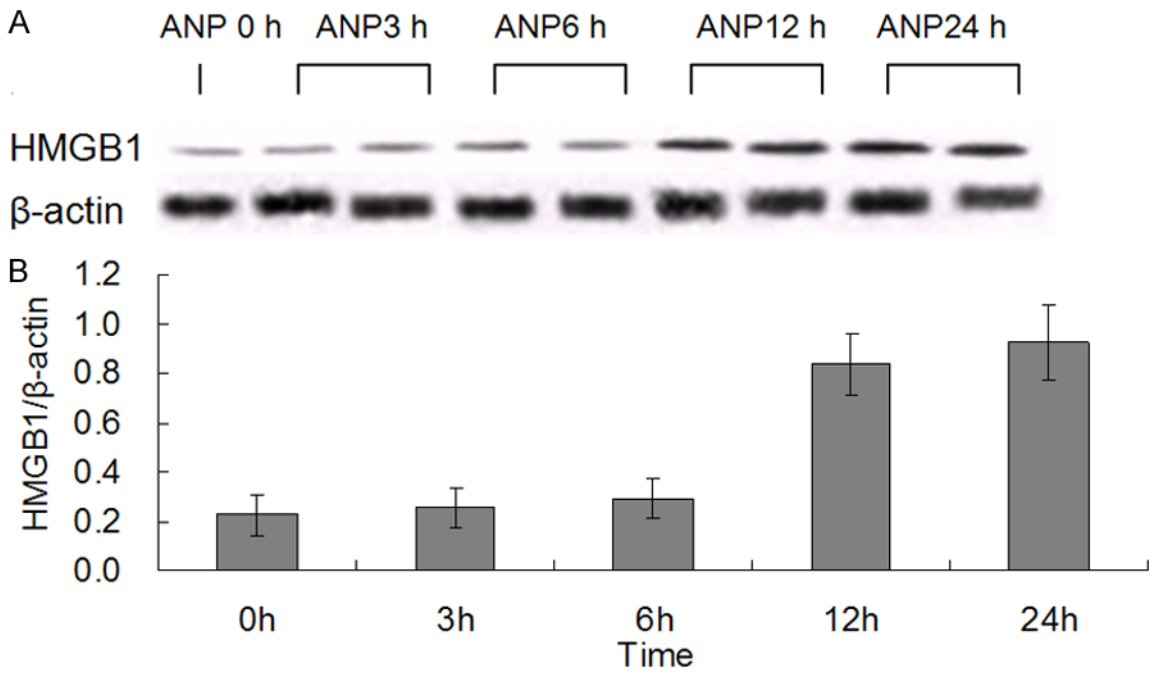

Figure 2. The HMGB1 protein expression in pancreatic tissue of ANP group at different time points. A. The protein bands by Western blot method. B. Histogram of the HMGB1 expression.

Table 2. HMGB1 immunohistochemistry densitometry and analysis (mean ± standard deviation)

Group	3 h	6 h	12 h	24 h
SO group	3.72 ± 0.86	3.59 ± 0.96	3.62 ± 0.91	3.68 ± 0.87
ANP group	3.76 ± 0.82	4.86 ± 0.99	12.46 ± 0.88※,◆	18.50 ± 0.87※,◆

Compared with SO group, the expression had no change in the ANP 3 h group, slightly increased in the ANP 6 h group (but $P > 0.05$). ※, $P < 0.05$, compared with SO group; ◆, $P < 0.05$, compared with the previous group.

room temperature for 1 h. And then the PVDF membrane was reacted with ECL solution in a dark room for light exposure to a film, which was then washed to dry. The PVDF membrane was cleaned with eluent to remove the antibodies, and then hybridized with anti-β-actin antibody and corresponding secondary antibody, and then imaged by the methods described above. The films were scanned into computer, the optical density values of the HMGB1 and β-actin (internal control) bands were measured with Image-Pro Plus *IPP6.0* image analysis system, and the ratios were obtained for semi-quantitative analysis.

Detection of the HMGB1 expression in pancreatic tissue by Q-PCR

Total RNA was extracted using Trizol kit from pancreatic tissue from different time points (0 h, 3 h, 6 h, 12 h and 24 h) after ANP modeling. For each sample, 2.0 μg total RNA was reverse transcribed to cDNA using 1 μL oligo(dT) (0.5 μg/μL) primer. PCR primers were designed according to the gene sequences downloaded from NCBI by primer design software Primer 5.0. The PCR primers for HMGB1 were GCTCAGAGAGGTGGAAGAC (sense) and CCAATGGATAAGCCAGGAT (antisense); The PCR primers for internal reference β-actin were CCCATCTATGAGGGTTACGC (sense) and TTTAATGTCACGCACGATTTC (antisense). The PCR reaction was, (1) pre-denaturation at 95°C for 3 min, (2) denaturation at 95°C for 10 s, annealing and extension at 58°C 30 s, for total 35 cycles, and reading the fluorescence at each extension phase.

Statistical analysis

SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The parameters in each experimental group at each time point were expressed as mean ± standard deviation ($\bar{x} \pm s$) and analyzed using the Student's t-test and one-way ANOVA followed by the SNK post hoc test. P value < 0.05 was considered = significant difference.

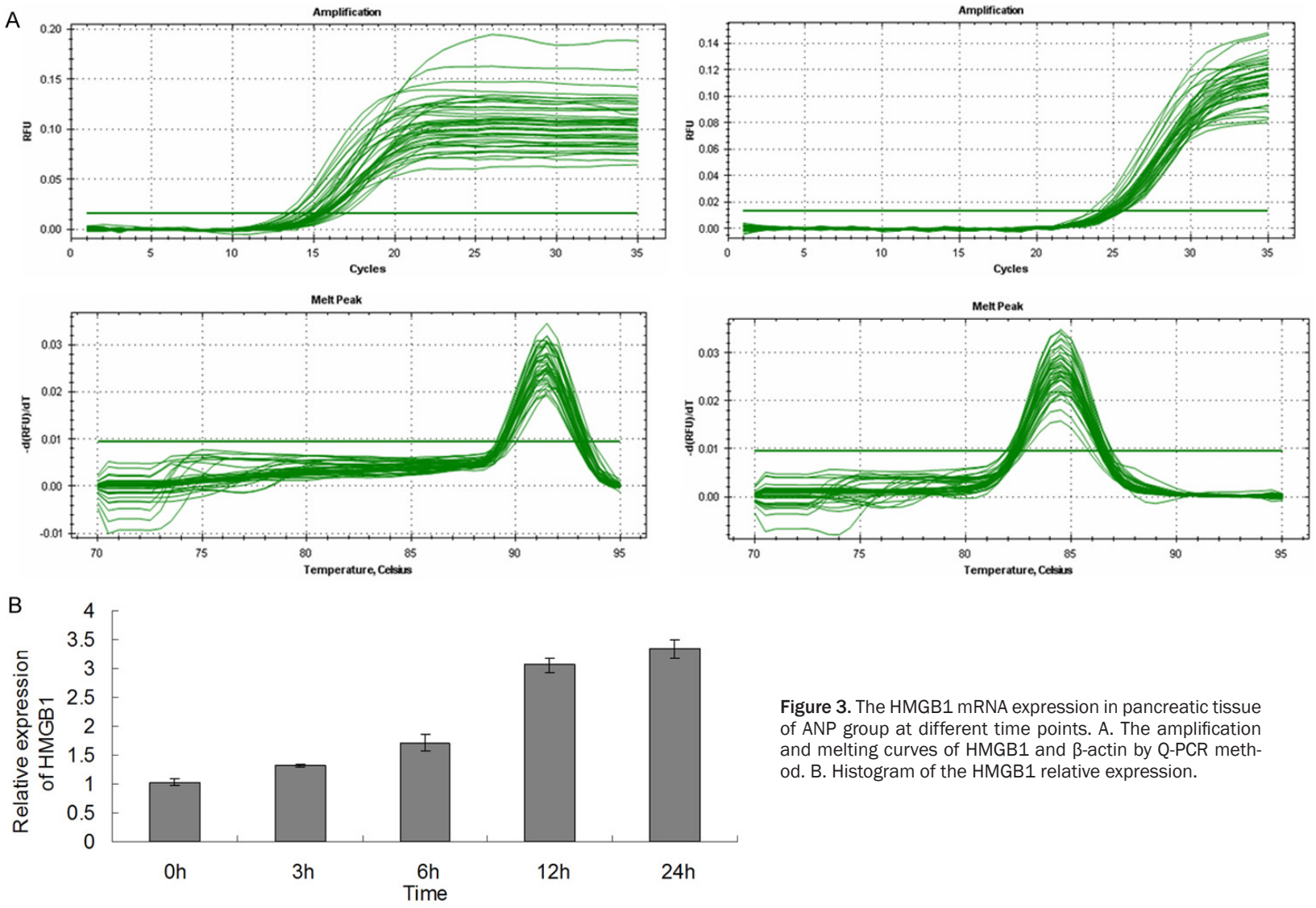

Figure 3. The HMGB1 mRNA expression in pancreatic tissue of ANP group at different time points. A. The amplification and melting curves of HMGB1 and β-actin by Q-PCR method. B. Histogram of the HMGB1 relative expression.

Results

ELISA results of serum amylase, IL-1β, IL-6, TNF-α and HMGB1

The serum amylase content in the ANP model group was significantly higher than that in the SO group, reached the peak at 12 h, and declined later on. This also indicated that the modeling was successful. The contents of IL-1β, IL-6 and TNF-α in the ANP group were also significantly higher than the SO group respectively, increased significantly at 3 h, peaked at 6 h, declined after 12 h, and continued to decline at 24 h. The HMGB1 expression in the ANP group increased with no significance at 3 h, increased significantly at 6 h (significantly higher than the SO group), and continued to increase with the progress of the inflammation (**Table 1**).

Detection of the HMGB1 expression in the pancreatic tissue by immunohistochemistry

As shown in **Figure 1** and **Table 2**, the HMGB1 expression had no change in the ANP 3 h group compared with SO group, slightly increased in the ANP 6 h group ($P > 0.05$). The HMGB1 expression had significant change in the ANP 12 h and 24 h groups compared with SO group ($P < 0.05$) and the expression in ANP 24 h group significantly increased compared with ANP 12 h group ($P < 0.05$).

Detection of HMGB1 expression in pancreatic tissue by Western blot

HMGB1 had no significant change between ANP-3 h and ANP-6 h groups. In the ANP-12 h group, HMGB1 expression increased significantly compared with ANP-6 h group. HMGB1 expression continued to increase at 24 h, significantly different from the ANP-12 h group (**Figure 2**).

HMGB1 mRNA expression in pancreatic tissue by Q-PCR

The HMGB1 mRNA expression in pancreatic tissue was continuously increasing with the time after modeling (**Figure 3**).

Discussion

With the deepening of the study on the occurrence and development of acute pancreatitis, it has been fully confirmed that NF-κB acts as the initiating factor in acute pancreatitis development [4]. NF-κB regulates the transcription and expression of a variety of early inflammatory factors in the development and progression of acute pancreatitis, and has become a key factor in the control and treatment of the early progression of pancreatitis. However, in the clinical treatment of acute pancreatitis, it is difficult to timely control the inflammatory response waterfall caused by acute pancreatitis. Therefore, it is particularly important for control of the progression of the disease after onset of acute pancreatitis. Meanwhile, at the late stages in the development of acute pancreatitis there must be a key factor in the maintenance and promotion of further aggravation of acute pancreatitis.

HMGB1 is a class of non-histone proteins, mainly existing in the nucleus, widely distributed in the lymph, liver, lung, pancreas and other tissues, and involved in nucleosome stability, cell differentiation, DNA repair, DNA reorganization, steroid hormone regulation and other effects. However, when cell is damaged under inflammatory conditions, HMGB1 may be actively secreted outside the cell and thus participates in inflammation and cell damage. HMGB1 stimulates the monocytes, neutrophils and dendritic cells to secrete IL-1α, IL-1β, IL-6, IL-8, TNFα, etc., playing an important role in extending and maintaining the inflammatory response [5]. As a late inflammatory factor currently acknowledged, HMGB1 has been found up-regulated in a variety of acute and chronic inflammation, including sepsis, acute lung injury, rheumatoid arthritis, Sjögren's syndrome and so on [6-9].

In the study of acute pancreatitis, Yasuda et al. [10, 11] reported that the expression levels of HMGB1 and its receptor (sRAGE) in the serum of severe acute pancreatitis patients were related to the severity of the disease, and showed a significant positive correlation with lactate dehydrogenase (LDH), C-reactive protein (CRP) and total bilirubin and other indicators. At the same time, the antioxidants (pyrrolidine dithiocarbamate and ethyl pyruvate) can protect the pancreatic tissue from being damaged in acute pancreatitis, and it's very likely that they play the roles by inhibiting HMGB1

[12, 13]. Similarly, HMGB1 blockade also has a significant protective effect on acute pancreatitis injury [14]. While the research on HMGB1's role in acute pancreatitis is still going on, the temporal and spatial characteristics of HMGB1 expression in acute pancreatitis is still not clear. We found that IL-1, TNF-α, IL-6 and other inflammatory factors reached the peak in a short period of time after the onset of acute pancreatitis. In about 12 hours after the occurrence of acute pancreatitis, serum inflammatory factors began to down-regulate. However, inflammation caused by the acute pancreatitis continued, and in theory, it can not be explained for all the roles of these inflammatory factors in the development of acute pancreatitis. By experiments, we further confirmed that after the expression of early inflammatory factors decreased, HMGB1 as a late inflammatory factor was persistently up-regulated in the serum and pancreatic tissue of rats with acute pancreatitis. Although it was not completely consistent with the findings by Yasuda et al. [9], this result further defined that differently from the early inflammatory factors such as IL-1, TNF-α and IL-6, HMGB1 was up-regulated at the late stages of the development of acute pancreatitis.

In the exploration of the acting mechanism of HMGB1, we successfully made acute pancreatitis model by injecting 5% sodium taurocholate retrogradely into the biliopancreatic duct to carry out further study. The results showed that HMGB1 expression in the peripheral blood of acute pancreatitis rat was obviously different from early inflammatory factors, TNF-alpha, IL-1 and IL-6, mainly reflected in the emergence of the peak and the sustained time. The expressions of TNF-α, IL-1 and IL-6 reached the peak at 6 h after modeling and then decreased, but HMGB1 had no significant expression at early stages after modeling, and when other inflammatory factors went down, HMGB1 began to increase significantly. Analysis of HMGB1 expression in pancreatic tissue at different time points in ANP group by Western blot and Q-PCR methods found that HMGB1 expression had no significant change at 3 h and 6 h after modeling, increased significantly only at 12 hours, and still maintained a high level at 24 h, showing a time difference with early inflammatory factors TNF-alpha, IL-1 and IL-6 [13]. The experiments suggested that the HMGB1 expression in pancreatic tissue was later than its existence in the serum, probably due to that at the onset of acute pancreatitis, the systemic inflammatory response induced by acute pancreatitis caused other organs or tissues to secrete HMGB1 which was released into the blood. In summary, our study has indicated that HMGB1 plays a key role in the maintenance and promotion of the development of acute pancreatitis.

The study is still inadequate in that the acute pancreatitis modeled rats with 5% sodium taurocholate injected retrogradely into the biliopancreatic duct had shorter survival time and high mortality, making it difficult to further research on the change of inflammatory factors in the pancreatic tissue at 48 h or 72 h after the modeling. Meanwhile, we still need to further explore the expression of above early inflammatory cytokines after blocking the HMGB1 expression, elucidating the existence relationship between HMGB1 as a late inflammatory factor and other early inflammatory cytokines. These explorations provide a guideline to further improve our experimental model and follow-up research on the role of HMGB1 as key point in the development of acute pancreatitis.

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Disclosure of conflict of interest

None.

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References

[1] Yu X, Li YG, Huang SF, Yang ZL, Chen DJ, Li XR, Zhang Y and Xu M. Relationship between the

pancreatic acinar cell activity in acute necrotizing pancreatitis and the blood inflammatory cytokines. Chinese Journal of General Surgery 2005; 20: 793-795.

[2] Yu X, Li YG, He XW, Li XR, Din BN, Gan Y and Xu M. Hyperbaric oxygen reduces inflammatory response in acute pancreatitis by inhibiting NF-kappaB activation. Eur Surg Res 2009; 42: 130-135.

[3] Biscetti F, Ghirlanda G and Flex A. Therapeutic potential of high mobility group box-1 in ischemic injury and tissue regeneration. Curr Vasc Pharmacol 2011; 9: 677-681.

[4] Sah RP and Saluja A. Molecular mechanisms of pancreatic injury. Curr Opin Gastroenterol 2011; 27: 444-451.

[5] Deveraux QL and Reed JC. IAP family proteins-suppressors of apoptosis. Genes Dev 1999; 13: 239-252.

[6] Tanida I, Tanida-Miyake E, Komatsu M, Ueno T and Kominami E. Human Apg3p/Aut1p homologue is an authentic E2 enzyme for multiple substrates, GATE-16, GABARAP, and MAP-LC3, and facilitates the conjugation of hApg12p to hApg5p. J Biol Chem 2002; 277: 13739-13744.

[7] Nemoto T, Tanida I, Tanida-Miyake E, Minematsu-Ikeguchi N, Yokota M, Ohsumi M, Ueno T and Kominami E. The mouse Apg10 homologue, an E2 like enzyme for Apg12p conjugation, facilitates MAP-LC3 modification. J Biol Chem 2003; 278: 39517-39526.

[8] Komatsu M, Tanida I, Ueno T, Ohsumi M, Ohsumi Y and Kominami E. The C-terminal region of an Apg7p/Cvt2p is required for homodimerization and is essential for its E1 activity and E1-E2 complex formation. J Biol Chem 2001; 276: 9846-9854.

[9] Yasuda T, Ueda T, Shinzeki M, Sawa H, Nakajima T, Takeyama Y and Kuroda Y. Increase of high-mobility group box chromosomal protein 1 in blood and injured organs in experimental severeacute pancreatitis. Pancreas 2007; 34: 487-488.

[10] Yasuda T, Ueda T, Takeyama Y, Shinzeki M, Sawa H, Nakajima T, Ajiki T, Fujino Y, Suzuki Y and Kuroda Y. Significant increase of serum high-mobility group box chromosomal protein 1 levels in patients with severe acute pancreatitis. Pancreas 2006; 33: 359-363.

[11] Kocsis AK, Szabolcs A, Hofner P, Takács T, Farkas G, Boda K and Mándi Y. Plasma concentrations of high-mobility group box protein 1, soluble receptor for advanced glycation end-products and circulating DNA in patients with acute pancreatitis. Pancreatology 2009; 9: 383-91.

[12] Zhang ZW, Zhang QY, Zhou MT, Liu NX, Chen TK, Zhu YF and Wu L. Antioxidant inhibits HMGB1 expression and reduces pancreas injury in rats with severe acute pancreatitis. Dig Dis Sci 2010; 55: 2529-2536.

[13] Yang ZY, Ling Y, Yin T, Tao J, Xiong JX, Wu HS and Wang CY. Delayed ethyl pyruvate therapy attenuates experimental severe acute pancreatitis via reduced serum high mobility group box 1 levels in rats. World J Gastroenterol 2008; 14: 4546-4550.

[14] Sawa H, Ueda T, Takeyama Y, Yasuda T, Shinzeki M, Nakajima T and Kuroda Y. Blockade of high mobility group box-1 protein attenuates experimental severe acute pancreatitis. World J Gastroenterol 2006; 12: 7666-7670.