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Magnesium isoglycyrrhizinate alleviates liver injury in obese rats with acute necrotizing pancreatitis

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ABSTRACT

Objective: For patients with acute necrotizing pancreatitis (ANP), a high body mass index (BMI) increases the likelihood of acute hepatic injury (AHI). In the current study, we explored whether magnesium isoglycyrrhizinate (MgIg) could alleviate ANP-induced liver injury in obese rats.

Methods: Sprague-Dawley (SD) rats were selected for the present study, and the ANP model was established by retrograde injection of 5% sodium taurocholate into the biliary-pancreatic duct. Thirty-six SD rats were randomly assigned to six groups: the normal (N), standard rat chow (SRC) normal (S—N), SRC ANP (S-ANP), high-fat diet (HFD) normal (H–N), HFD ANP (H-ANP), and MgIg pretreatment HFD ANP (H-ANPT) groups. The rats in the H-ANPT group were treated with MgIg (30 mg/kg) intragastrically for 7 days before the ANP model was established. The rats were sacrificed 12 h after ANP was established, and the blood and pancreatic and liver tissues were collected. Differences in the physiology, pathology and cellular and molecular responses of the rats in each group were examined.

Result: Analyses of serum amylase lipase, alanine aminotransferase and aspartate aminotransferase indicated that obesity aggravated ANP-induced hepatic injury and that MgIg improved liver function. The superoxide dismutase, malondialdehyde, M1 macrophage, M2 macrophage, neutrophil, NF-κB, IL-1β and caspase-3 levels in liver tissue showed that MgIg attenuated H-ANP-induced hepatic injury by inhibiting oxidative stress and inflammation.

Conclusion: Obesity aggravated ANP-induced liver injury *via* oxidative stress and inflammatory reactions. MgIg alleviated oxidative stress and decreased the inflammatory reaction, protecting the liver against the AHI induced by ANP in obese rats.

1. Introduction

Acute pancreatitis (AP) is a common type of inflammatory disease in clinic, and its incidence has increased in recent years. The aetiology of AP has been determined and includes alcoholic consumption, a biliary aetiology, hyperlipidaemia, drug consumption, toxins, metabolic and/or endocrine disorders, among others [1]. AP is usually a mild and self-limiting disease, and approximately 20% of cases may develop into severe acute pancreatitis (SAP), which has a mortality rate of 10–20%, owing to early systemic inflammatory response syndrome (SIRS) and systemic organ failure syndrome (SODS) [2].

The prevalence of obesity is rising worldwide, and the WHO has confirmed that obesity is a global epidemic. Approximately 30% of the world's population is overweight or obese, and few countries have had decreases in the incidence of obesity in 33 years [3]. In recent years, obesity has been associated with metabolic syndrome, dyslipidaemia, diabetes, hypertension, stroke and cardiovascular disorders, impacting on public health [4]. Additionally, obesity is accompanied by an increased accumulation of peripancreatic fat, microcirculatory changes and a low-grade inflammatory status, which may influence AP processes [5].The increase in the incidence of obesity over the past decades has been noticed as a well-known risk factor for AP and linked to a more severe course and worse outcomes [6]. Furthermore, a previous study suggested that the Acute Physiology and Chronic Health Examination (APACHE)IIscoring system provided a more accurate assessment of AP severity when an obesity score was included [7].

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Due to its special location in the gastrointestinal circulation and its role in metabolic processes, the liver is a vital site of extra-pancreatic organ injury during acute necrotizing pancreatitis (ANP) [8]. A Study has reported that liver injury occurs in approximately 80% of AP patients, and its severity is positively responsible for the severity of pancreatitis and prolongs pancreatitis [9]. At present, the specific mechanisms of liver injury with SAP are not fully known, but reactive oxygen species (ROS) and inflammatory factors play critical roles in liver injury [8]. Classically activated macrophages (M1) and neutrophils play an important role in inflammatory responses and are related to tissue injury. A study reported that obesity was strongly correlated with an increase in M1 and neutrophil populations in adipose tissue [10]. Additionally, a previous report indicated that hepatic injury was more severe in obese rats than in lean rats [11].

Magnesium isoglycyrrhizinate (MgIg), a magnesium salt of 18α-glycyrrhizic acid and a stereoisomer of glycyrrhizic acid, was found to have an anti-inflammatory action and improve hepatic function [12]. Additionally, MgIg can clear oxygen free radicals *in vivo*, showing a potent hepatoprotective effects [13]. However, few studies have been performed to evaluate the effects of MgIg on ANP-induced liver injury in obese rats. The purpose of the present study was to investigate the protective effects of MgIg on ANP-induced hepatocyte injury in obese rats and to further explore the potential molecular mechanisms.

2. Materials and methods

2.1. Materials and reagents

The high-fat diet (HFD, D12492) chow was purchased from Beijing Huafukang Bioscience CO.,; standard rat chow (SRC) was provided by the experimental animal centre of Wuhan University Renmin Hospital; malondialdehyde (MDA) and superoxide dismutase (SOD) reagent kits were purchased from the Nanjing Jiancheng Bioengineering Institute; MPO (GB11224), CD11C (GB11059), CD206 (GB11062), NF-κBp65 (GB11142) and IL-1β (GB11113) antibodies were purchased from Wuhan Google Biotechnology; and CD68 (ab955) and active caspase-3 (ab2302) antibodies were purchased from Abcam Co.

2.2. Animals and model

A total of thirty-six Sprague-Dawley(SD)adult male rats(200–220 g) were bought from the experimental animals Co. of Hunan SJA (Changsha, Hunan, China). Rats were randomly allocated into six groups: normal (N, n = 6), SRC normal (S—N, n = 6), SRC ANP (S-ANP n = 6), HFD normal (H–N, n = 6), HFD ANP (H-ANP, n = 6) and MgIg pretreatment for HFD ANP (H-ANPT, n = 6) groups. After 8 weeks feeding on SRC or the HFD, the rats were euthanized at 12 h after the operation. The ANP model was established as previously described [14]. The H-ANPT group was administered a dose of 30 mg/kg MgIg *via* intraperitoneal injection once daily for 7 days before ANP was established. After closure, the rats were subcutaneously supplemented with normal saline (1 ml/100 g weight) for fluid loss.

2.3. Collection samples of blood and tissue

Rats were sacrificed and blood samples were acquired from the inferior vena cava. Blood samples were centrifuged for 10 min at 12,000 *g*, and serum were preserved at −80 °C. Pancreases and livers tissue were immediately frozen in liquid nitrogen and preserved at −80 °C for the detection of relative aspects.

2.4. Enzyme assay

The levels of amylase (AMY), lipase (LIP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride (TG) and total cholesterol (TC) in serum were detected by the Automatic Biochemistry Analyzer (Olympus Corporation, Tokyo, Japan) under standard procedures.

2.5. Histopathologic analysis

The paraffin-embedded pancreatic and hepatic specimens were sectioned at 4um and stained by HE. The morphological study were performed under a light microscope (original magnification, 200×, Olympus Optical Ltd, Tokyo, Japan) by two professional knowledge investigators who were blinded to the experiments. The pancreatic histopathology changes were measured and classified according to the Schmidt score, which included the degree of edema, inflammation, vacuolization, hemorrhage and necrosis [15]. The hepatic histopathology changes were examined and classified based on a point-counting method as describe by Camargo [16].

2.6. Oxidative stress

The MDA content and SOD activity in the liver were measured with reagent kits provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All steps were performed based on the experimental protocol recommended by the manufacturer.

2.7. Immunofluorescence assay

The expression of CD68, CD11C, CD206, IL-1β and MPO was determined by immunofluorescence analysis. Paraffin-embedded tissue sections were dewaxed with xylene and hydrated with gradient ethanol. The paraffin sections were immersed in 10 mM citrate buffer (pH 6.0) and boiled for 4 min at 121 °C in a pressure cooker for antigen retrieval. Then, the slides were allowed to cool slowly at room temperature, immersed in the 10 mM citrate buffer and rinsed with a proper amount of phosphate-buffered saline (PBS). Non-specific sites were blocked *via* incubation with 10% donkey serum (Jackson Immuno Research, West Grove, USA) diluted with 0.01% PBS. The sections were incubated overnight at 4 °C with the following different primary antibodies: mouse anti-CD68 (1:250), rabbit anti-CD11C (1:300), rabbit anti-CD206, rabbit anti-MPO (1:300) and rabbit anti-IL-1β (1:300). Then, the sections were incubated with secondary antibody (1:200, Abcam, Cambridge, UK) for 1.5 h at room temperature in the dark. The nuclei were visualized by Fluoroshield Mounting Medium with DAPI (Abcam, Cambridge, UK) staining. Images of the slides were observed and photographed under a fluorescence microscopy (Olympus, Tokyo, Japan). Immunofluorescence staining was analysed using Image Pro-Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) for quantitative analysis.

2.8. Immunohistochemistry assay

The expression of NF-κBp65 and caspase-3 was measured by immunohistochemical analysis. Paraffin-embedded tissue sections were dewaxed with xylene and hydrated with gradient ethanol. The paraffin sections were immersed in 10 mM citrate buffer (pH 6.0) and boiled for 4 min at 121 °C in a pressure cooker for antigen retrieval. Then, the slides were allowed to cool slowly at room temperature, immersed in the 10 mM citrate buffer and rinsed with the proper amount of PBS. Endogenous peroxidase activity was terminated by treatment with 3% H_2O_2 at room temperature for 15 min. Non-specific sites were blocked *via* incubation with 5% normal goat serum diluted with 0.01% PBS: the primary antibodies were rabbit anti-NF-κBp65 (1:300) and anti-caspase-3 (1:300). Sections were cultured with the primary antibody at 4 °C overnight, and secondary antibody was then added and incubated at room temperature for 0.5 h. The immunohistochemical staining was visualized using 3, 5-diaminobenzidine (DAB, Fuzhou, Maxim), and images of the slides were observed and photographed under a light microscope. Immunohistochemical staining was measured with Image

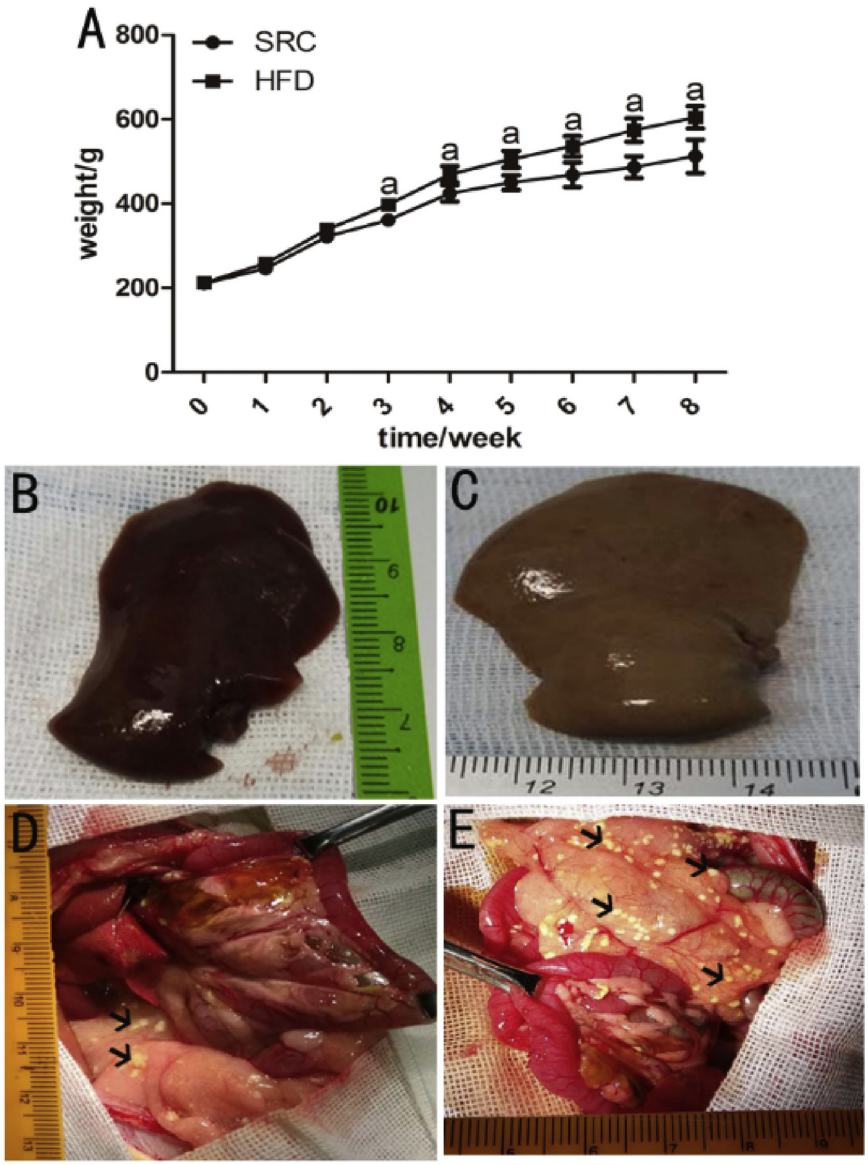


Fig. 1. General presentations of rats. A Weight of rats, B liver tissue of the S−N group, C liver tissue of the H−N group, D saponification spots of the S-ANP group, E saponification spots of the H-ANP group. $P < 0.05$ was considered statistically significant. [a]*P vs.* the same time point in the S−N and H−N groups.

Pro-Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) for quantitative analysis.

2.9. Statistical analysis

Experimental data were expressed with means ± standard deviation. Experimental data were analyzed using SPSS 22.0 software (IBM SPSS, Armonk, NY, USA). Data of all groups were analyzed by one-way ANOVA, excepting liver pathology which was analyzed by Kruskal-Wallis nonparametric test. $P < 0.05$ indicated a statistical difference.

3. Results

3.1. General presentations of rats

After 8 weeks of SRC or HFD feeding, the HFD-fed rats had a higher body weight than the SRC-fed rats ($P < 0.05$; Fig. 1A). The edge of the liver was sharp, and the liver colour was dark red in the S−N group. The edge of the liver was relatively dull, and liver colour was relatively yellow in the H−N group (Fig. 1B, C). The amount of intra-abdominal fat and saponification spots of intra-abdominal fat was higher in the H−ANP group than in the S−ANP group (Fig. 1D, E).

3.2. Serum levels of AMY, LIP, ALT, AST, TG and TC

Serum AMY, LIP, ALT and AST levels were not significantly different among the N, S−N and H−N groups ($P > 0.05$). Serum TG and TC levels in the H–N group were significantly higher than those in the N and S−N groups ($P < 0.05$). Serum AMY, LIP, ALT, AST, TG and TC levels in the S-ANP group were markedly higher than those in the S−N group ($P < 0.05$). Serum AMY and TG levels were markedly decreased and serum LIP, ALT and AST levels were markedly increased in the H-ANP group compared with those in the S-ANP group ($P < 0.05$). Serum AMY, LIP, ALT and AST levels were markedly decreased and TG was markedly increased in the H-ANPT group compared with those in the H-ANP group ($P < 0.05$), (Fig. 2).

3.3. Histopathological analysis of pancreatic and liver tissue

Oedema, vacuolization, haemorrhage, inflammation and necrosis

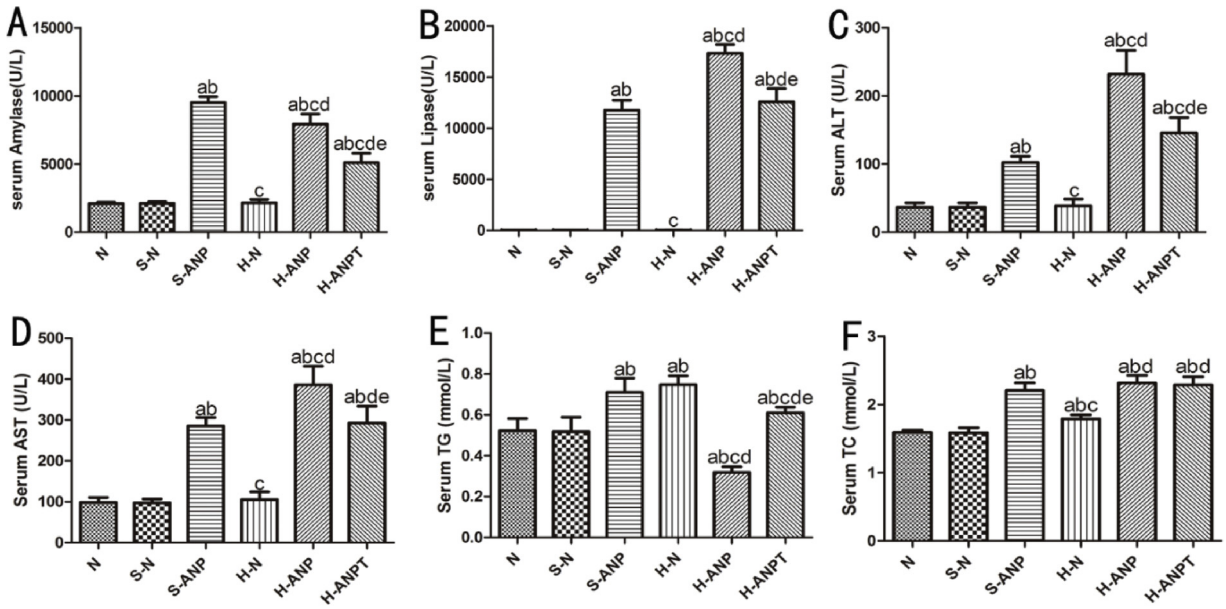


Fig. 2. Serum levels of AMY, LIP, ALT, AST, TG and TC. The levels of (A) serum AMY, (B) serum LIP, (C) serum ALT, (D) serum AST, (E) serum TG, (F) serum TC. $P < 0.05$ was considered to be statistically significant. [a]*P vs.* the N group, [b]*P vs.* the S−N group, [c]*P vs.* the S−ANP group, [d]*P vs.* the H−N group, [e]*P vs.* the H−ANP group.

were observed in the S-ANP group, and the pancreatic damage scores in the S−ANP group were markedly higher than those in the S−N group ($P < 0.05$). The severity of the pancreatic injury and the pancreatic damage scores were markedly increased in the H−ANP group compared with those in the S−ANP group ($P < 0.05$) but were significantly decreased in the H-ANPT group compared with those in the H−ANP group ($P < 0.05$). (Fig. 3A, C).

Conspicuous hepatocyte oedema, loss of intercellular borders, inflammatory cells and necrosis were observed in the S−ANP group, and the hepatic injury grades in the S-ANP group were markedly higher than those in the S−N group ($P < 0.05$). The severity of the hepatic injury and the hepatic injury grades were significantly higher in the H−ANP group than in the S-ANP group ($P < 0.05$) but were significantly lower in the H−ANPT group than in the H-ANP group ($P < 0.05$). (Fig. 3B, D).

3.4. The levels of MDA and SOD in liver tissue

The hepatic MDA level was markedly higher and the SOD level was significantly lower in the H−N group than in the N and S−N groups ($P < 0.05$). The hepatic MDA level was markedly higher and the SOD level was significantly lower in the S−ANP group than in the S−N group ($P < 0.05$). The hepatic MDA level was markedly higher and the SOD level was significantly lower in the H−ANP group than in the S-ANP group ($P < 0.05$). The hepatic MDA level was markedly lower and the SOD level was significantly higher in the H−ANPT group than in the H-ANP group ($P < 0.05$), (Fig. 4).

3.5. Macrophages and neutrophils in liver tissue

The number of M1 macrophages ($CD68^+CD11C^+$), M2 macrophages ($CD68^+CD206^+$) and neutrophils (MPO^+) in the N, S−N and H–N groups were not significantly different ($P > 0.05$). The number of M1 macrophages, M2 macrophages and neutrophils was markedly higher in the S-ANP group than in the S−N group ($P < 0.05$). The number of M1 macrophages and neutrophils was markedly higher in the H-ANP group than in the S-ANP group ($P < 0.05$). The number of M1 macrophages and neutrophils were markedly lower in the H-ANPT group than in the H-ANP group ($P < 0.05$), (Fig. 5).

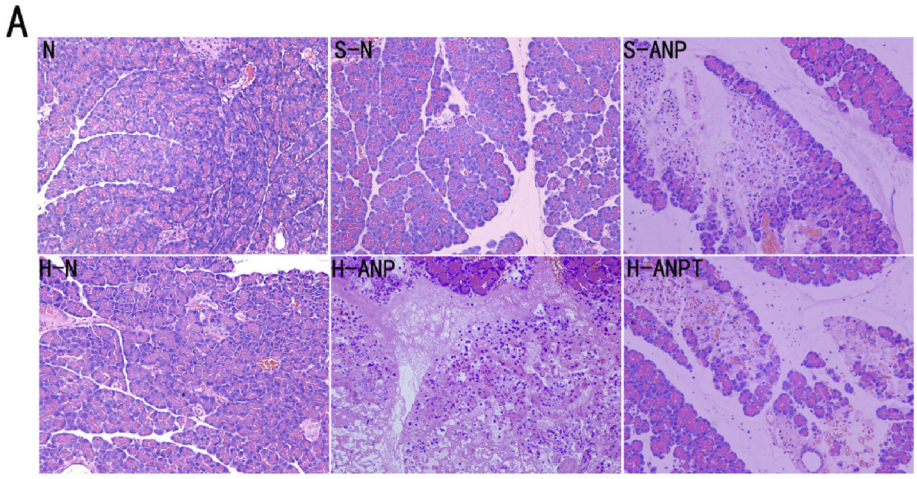
3.6. The expression of NF-κB, IL-1β and caspase-3 in liver tissue

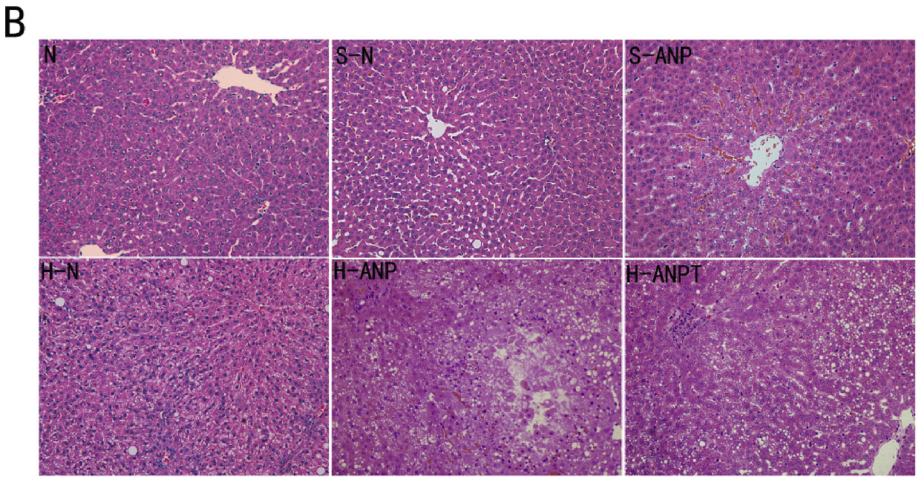
The expression of NF-κB, IL-1β and caspase-3 was not significantly different among the N, S−N and H−N groups ($P > 0.05$). The expression of NF-κB, IL-1β and caspase-3 was markedly increased in the S-ANP group compared with the S−N group ($P < 0.05$). The expression of NF-κB, IL-1β and caspase-3 were markedly increased in the H-ANP group compared with S-ANP group ($P < 0.05$). The expression of NF-κB, IL-1β and caspase-3 was markedly decreased in the H-ANPT group compared with the H-ANP group ($P < 0.05$), (Fig. 6).

4. Disscussion

AP is a common, life-threatening inflammatory disorder that can cause local injury, SIRS, and multiple organ failure. The global incidence of AP is 30–50 per 100 000 people annually, and its incidence is increasing globally, leading to a significant burden on health care systems [17]. The increasing incidence of AP is linked to increased alcohol use, while increasing obesity may also play an important role [18]. A study reported that a BMI higher than 30 kg/m^2 raises the risk of the acute severe pancreatitis three times and doubles mortality rates [6]. In the present study, we establish a diet-induced obesity rat model and found that the amount of intra-abdominal fat and the pathological pancreas scores in the H-ANP group were higher than those in the S-ANP group. It has been reported that obesity increases the severity of ANP due to the release of excess unsaturated free fatty acids from fat, which worsens ANP [19]. Furthermore, fat tissue is an important source of pro-inflammatory cytokines and releases a large number of inflammatory factors during the ANP that accelerate the pancreatic and extra-pancreatic organ injuries [20].

The liver is a frequently major site of extra-pancreatic organ damaged during AP, and liver injury can worsen AP [9]. Several studies suggest that oxidative impairment and numerous inflammatory mediators play critical roles in liver injury during AP [8,21,22]. A study has shown that obesity alters the release of inflammatory cytokines and increases the vulnerability of the liver during the AP [11]. In this study, serum ALT, serum AST and pathological changes in the liver in the H-ANP group were markedly higher than those in the S−ANP group, implying that obesity can aggravate liver injury during ANP.





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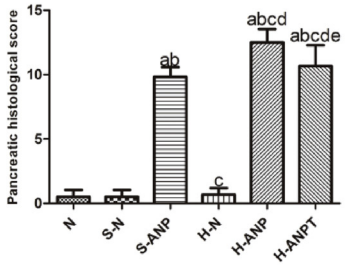
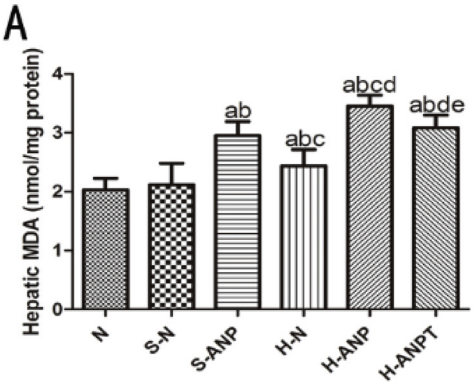


D

Group	n	Hepatic pathological grades				Mean rank
		0	I	II	III	
N	6	6	0	0	0	8.0
S-N	6	5	1	0	0	9.67
S-ANP	6	4	2	0	0	25.67[ab]
H-N	6	0	1	3	2	11.33[c]
H-ANP	6	0	0	2	4	29.33[abcd]
H-ANPT	6	0	1	2	3	27.0 [abcde]

Fig. 3. Histopathological analysis of pancrtic and liver tissue. A Morphological study of the pancreas. B Morphological study of the liver. C Pathological scores of the pancreas (original magnification, × 200). D Hepatic injury grades. N N group, S—N S—N group, S—ANP S—ANP group, H—N H—N group, H—ANP H—ANP group, H—ANPT H—ANPT group. $P < 0.05$ was considered to be statistically significant. [a]*P vs.* the N group, [b]*P vs.* the S—N group, [c]*P vs.* the S-ANP group, [d]*P vs.* the H—N group, [e]*P vs.* the H-ANP group.



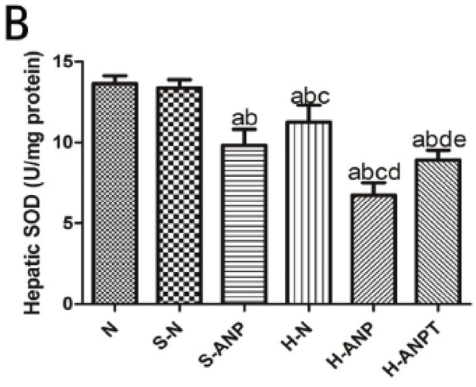


Fig. 4. The levels of MDA and SOD in liver tissue. The levels of (A) hepatic MDA, and (B) hepatic SOD. $P < 0.05$ was considered to be statistically significant. [a]*P vs.* the N group, [b]*P vs.* the S—N group, [c]*P vs.* the S-ANP group, [d]*P vs.* the H—N group, [e]*P vs.* the H—ANP group.

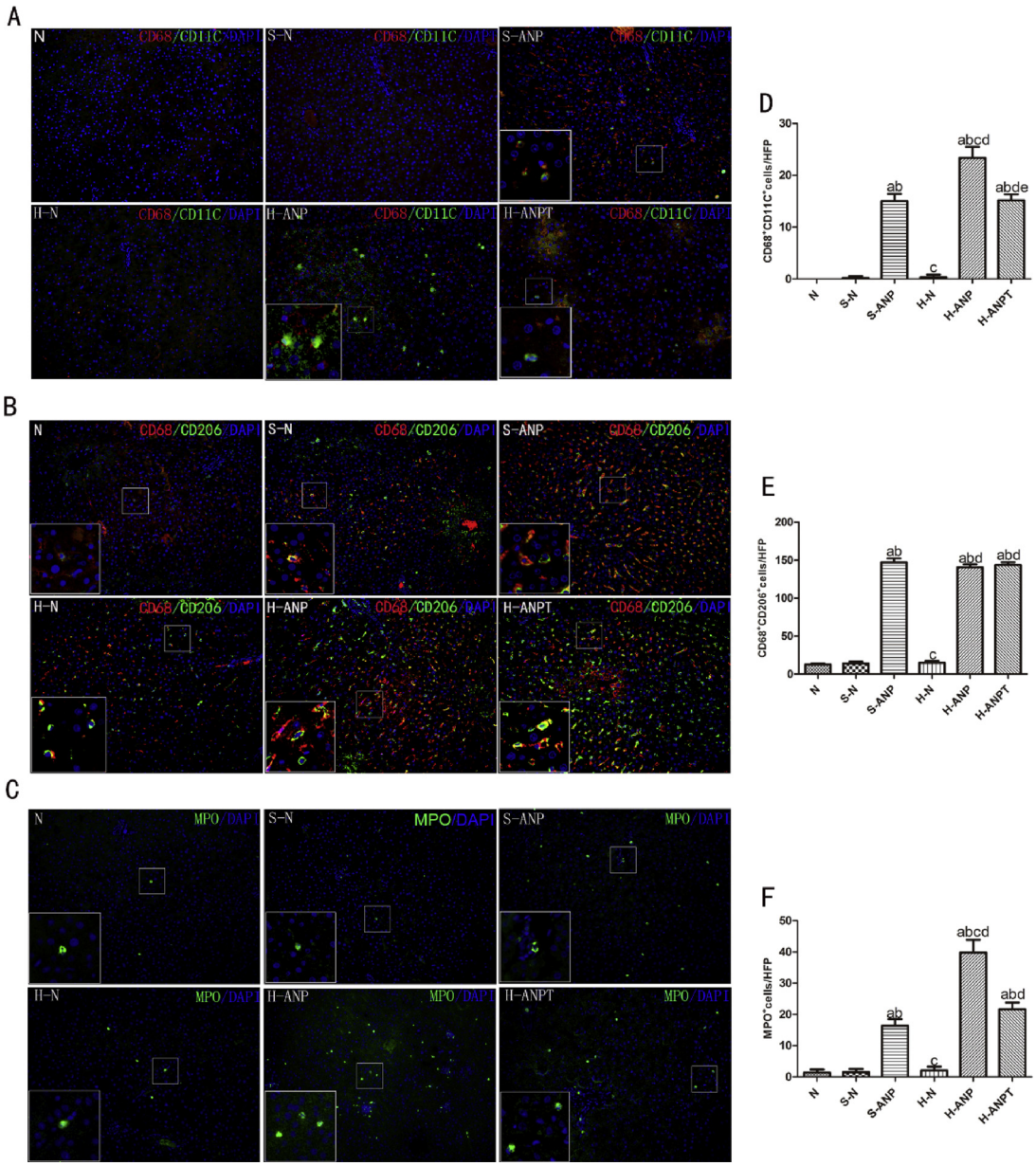


Fig. 5. Representative M1 macrophages ($CD68^+CD11C^+$), M2 macrophages ($CD68^+CD206^+$) and neutrophils (MPO^+) in liver tissue (original magnification, × 200). Representative images of (A) M1 macrophages, (B) M2 macrophages, and (C) neutrophils (white line square). DAPI(blue) was used to counter stain nuclei. The number of (D) M1 macrophages, (E) M2 macrophages, (F) neutrophils were counted. $P < 0.05$ was considered to be statistically significant. [a]*P vs.* the N group, [b]*P vs.* the S−N group, [c]*P vs.* the S-ANP group, [d]*P vs.* the H−N group, [e]*P vs.* the H−ANP group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Additionally, we found that the inflammatory cell infiltration of M1 macrophages and neutrophil was higher, the SOD activity was lower, and the MDA content was higher in the livers of the H-ANP group than in the S-ANP group. The mechanism underlying the more serious liver injury during ANP with obesity is complicated and not well-understood. Obesity aggravates liver oxidative stress and the inflammatory response, which may be associated with a more serious liver injury during the ANP.

Oxidative stress, including an imbalance between the oxidant and antioxidant activities, is involved in the pathogenesis of liver injury *via* different pathways [23]. Other research showed that improvement of liver oxidative stress status could attenuate the progression of AP-associated liver injury in rats [22]. Excessive production of reactive oxygen species (ROS) can cause degradation of polyunsaturated fatty acids in cell membranes, leading to membrane damage and MDA formation with a decrease in antioxidant defence enzymes, such as SOD [21]. Mitochondrial are the main site of ROS production and the area that is most sensitive to the effects of ROS. A study reported that obesity can repress the expression of mitofusin2 (Mfn2), which influences mitochondrial energy metabolism and thermogenesis [24]. Additionally, nuclear factor erythroid 2-related factor 2 (Nrf2) is involved in the defence against oxidative stress; which is lower in the liver of obese rats [25]. ROS generation increases and ROS clearance decreases, resulting in a disruption of the balance between the oxidant and antioxidant systems in the liver in obesity. Moreover, obesity can increase the severity of pancreatic damage through oxidative stress during AP [26]. The results of the current study show that hepatic MDA levels increased and SOD levels decreased in the H−ANP group compared with the S−ANP group, suggesting a more severe oxidative stress in the liver of obese rats during the ANP.

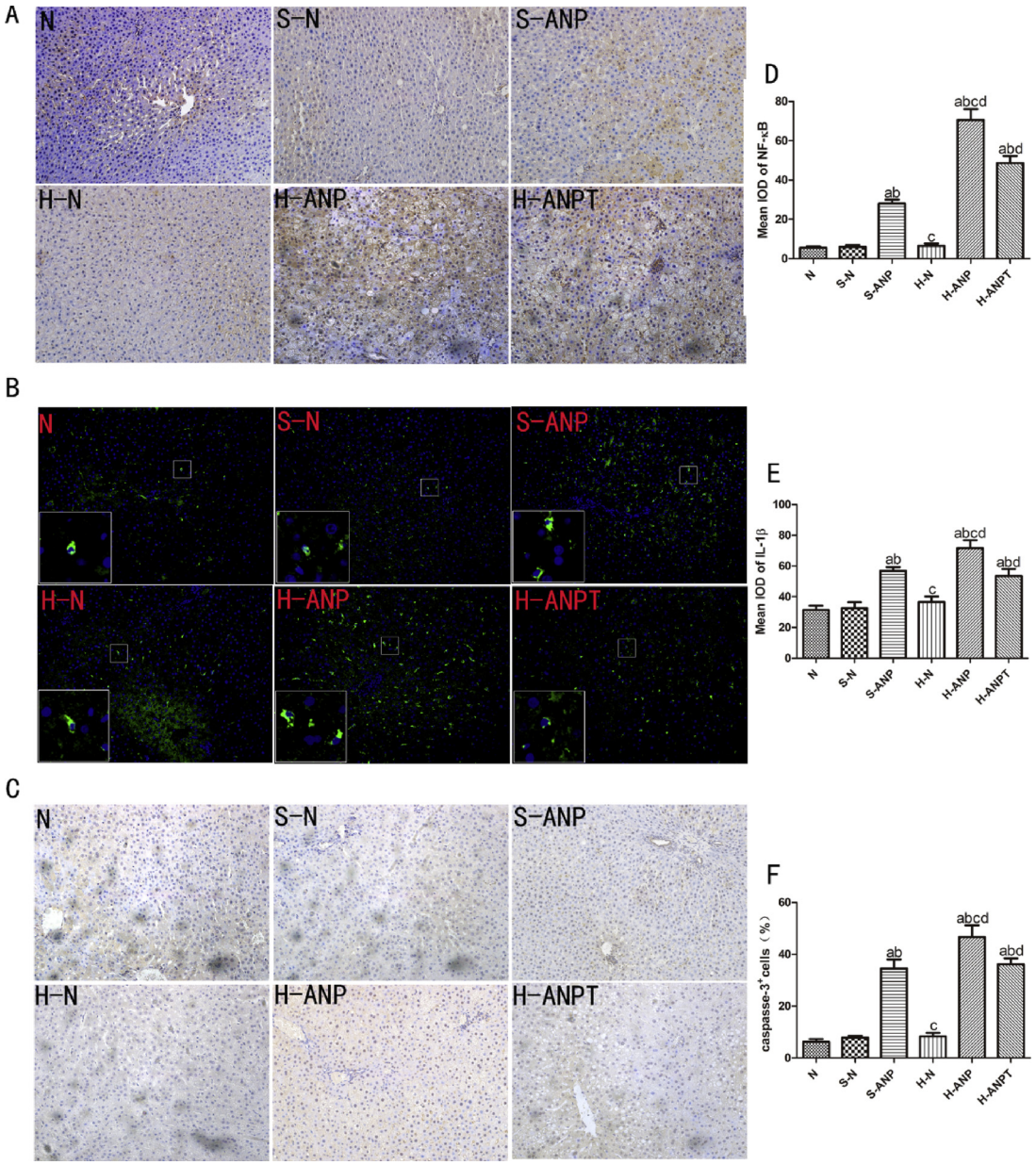


Fig. 6. Representative of NF-κB, IL-1β and caspase-3 expression in liver tissue (original magnification, × 200). The representative images of (A) NF-κB, (B) IL-1β, and (C) caspase-3. The level of (D) NF-κB, (E) IL-1β, (F) caspase-3 were measured. $P < 0.05$ was considered to be statistically significant. [a]*P vs.* the N group, [b]*P vs.* the S—N group, [c]*P vs.* the S-ANP group, [d]*P vs.* the H—N group, [e]*P vs.* the H—ANP group.

Pro-inflammatory cell infiltration and inflammatory cytokines release are associated with the pathogenesis of liver damage in AP, and inhibiting inflammation can alleviate the progression of AP-induced liver injury in rats [27,28]. As is well known, macrophages are divided into two classes: M1 macrophages with high expression of pro-inflammatory cytokines, such IL-1β and TNF-α, and M2 macrophages with overexpression of anti-inflammatory factors, such as IL-10. M1 macrophages [29] and neutrophils [30] can produce inflammatory cytokines and play a key role against intracellular bacteria and inflammatory diseases. In obese people, both the saturated fatty acids arising from within triglyceride stores of adipose tissue and the lipopolysaccharide (LPS) arising from increased intestinal permeability which can increase the inflammatory response though the TLR4 signalling pathway [31]. One study reported that the expression levels of HMGB1 and CCL2 were greater and the expression levels of IL-10 and IL-17 were decreased in the lung tissue of obese mice compared to those of control mice during the lung mechanical ventilation [32].Another study showed the occurrence of pancreatic tissue injury and inflammatory responses, accompanied by increased macrophage/neutrophil infiltration and elevated levels of pro-inflammatory mediators in high-fat diet mice compared with normal chow mice [33]. Additionally, it has been reported that the expression of chemokines, such as CXCL-1 and CCL3, was significantly higher in livers of obese mice than in control mice during the ischemia damage model [34]. In the present study, the number of $CD68^+CD11C^+$(M1 macrophages) and MPO^+ (Neutrophils) cells, as well as the expression of NF-κB and IL-1β, in the liver were higher in the H-ANP group than in the S-ANP group. Synthesizing the results of this study and related research, we hypothesized that obesity aggravates the inflammatory response in liver tissue and that changes is associated with the greater severity of liver injury in ANP.

MgIg can easily bind to receptors on target cell or steroid hormones receptors to suppress inflammatory reactions, and maintain the integrity of hepatocyte membranes [35]. One study showed that MgIg

exerted the protective effects against triptolide-induced hepatotoxicity by decreasing the expression of Nrf2 [36]. Another recent study found that MgIg could play an essential role in protecting hepatocytes from ethanol feeding-induced injury by suppressing the ROS generation by neutrophils and suppressing hepatocellular oxidative stress [37]. Additionally, it has been reported that MgIg can improve fructose-induced liver injury by blocking of NF-κB/NLRP3 inflammasome activation [38]. Furthermore, MgIg can inhibit the TLR4/NF-κB signalling pathway and alleviate the myocardial fibrosis induced by isoproterenol [39]. In this study, the levels of MDA, SOD, NF-κB and IL-1β decreased and the number of M1 macrophages and neutrophils was reduced in the liver tissue of the H-ANPT group compared with the H-ANP group, suggesting that MgIg may alleviate oxidative stress and inflammatory reactions to protect against hepatic injury during H-ANP. The specific mechanisms underlying the alleviation of hepatic oxidative stress and inflammatory reactions by MgIg during ANP with obesity require further study.

In summary, this study demonstrated that obesity could markedly aggravate liver oxidative stress and inflammatory response and lead to a more severe liver injury during the ANP. MgIg reduced hepatic oxidative stress and pro-inflammatory cytokine production to protect against the liver damage induced by ANP with obesity. The results of this study may provide a good option for alleviating liver injury in cases of ANP with obesity.

Conflict of interest

The authors statement that they have no conflicts of interest associated with the contents of this article.

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