

Exogenous leptin protects rat models of sodium taurocholate-induced severe acute pancreatitis through endocrinal and immunological pathways

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Abstract. Acute pancreatitis (AP) is a common non-bacterial disease compromising pancreatic tissues. Adipocyte-derived leptin is closely associated with the severity and clinical outcome of pancreatitis. The potential protective effects of exogenous leptin administration on a rat model of severe AP (SAP) remain to be elucidated, and were examined in the present study. Male Wistar rats were divided into a sham operation group (SO), SAP model group (SAP) and leptin group (LEP). Each group was divided into three sub-groups by observation time (24, 48 and 72 h). The SAP models were prepared by retrograde injection of 6% sodium taurocholate into the pancreatic-bile duct. Following model establishment, exogenous leptin was intraperitoneally injected into mice at 50 mg/kg in the LEP group. Subsequently, serum amylase, lipase and glucose levels at particular time-points were analyzed using a fully-automatic biochemical analyzer, and serum levels of tumor necrosis factor (TNF)- α and interleukin (IL)-10 were detected using an enzyme-linked immunosorbent assay. The pathological changes in pancreatic tissues were observed using hematoxylin and eosin

staining, and the pancreatic expression of the long form of the leptin receptor (OB-Rb) was detected and evaluated using Nest-polymerase chain reaction analysis. The mortality rates of the model rats were compared between the groups. Following the administration of exogenous leptin, the serum level of amylase in the LEP group was significantly decreased at 48 h, compared with that in the SAP group, with serum lipase levels decreased at 48 and 72 h, and blood glucose levels decreased at 72 h. Regarding the serum inflammatory factors, the level of TNF- α in the LEP group was significantly lower, compared with that in the SAP group at 24 h; whereas no significant difference was observed in the serum level of IL-10 between the two groups. Regarding the pathological changes in the pancreas, the tissues in the LEP group showed significantly alleviated pancreatic inflammation. In addition, the pancreatic expression of OB-Rb in the LEP group was significantly higher, compared with that in the SAP group at 24 and 48 h. No significant difference in 3-day mortality rates were observed between the SAP group and the LEP group. Taken together, exogenous leptin administration regulated inflammatory factors and the expression of OB-Rb at the early stage of AP, which exerted protective effects by through the immunological and endocrinal pathways.

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Introduction

Acute pancreatitis (AP) is a common non-bacterial disease compromising the pancreatic tissues. In clinical practice, the disease has been further classified as mild, moderate and severe forms. The majority of mild cases of AP, the most common form, show local pancreatic inflammations and resolve within days, whereas moderate and severe AP (SAP) usually lead to life-threatening complications, including multiple organ failure, resulting in a high mortality rate (1). In previous decades, knowledge of AP has been improved through intensive investigations performed to reveal the pathogenic nature of the disease and to develop pragmatic therapies (2), however, the pathogenesis of AP remains to be elucidated,

and identification of an effective specific therapeutic modality remains a challenge in the treatment of patients with SAP.

In scenarios of AP, the most important determinant process is considered to be the intra-acinar activation of digestive enzymes, of which trypsin is the dominant component. The release of digestive enzymes subsequently leads to autodigestion of the pancreatic gland and initiates local (mild form of AP) or systemic (moderate and severe forms of AP) inflammatory responses (3). A number of cytokines, including interleukin (IL)-4 and tumor necrosis factor (TNF)- α have been reported to be upregulated in patients with SAP and in cerulein-induced pancreatitis (CIP) rat models (4), indicating that endocrinal and immunological factors contribute to the final pancreatic tissue damage and manifestation of overall symptoms. The innate defense system has been recognized as a key physiological mechanism in counteracting the effects of AP. Previous studies have shown that hormones, including ghrelin, leptin and melatonin, modulate immunological processes and protect the pancreatic tissues from inflammatory damage, shedding light on the potential use of leptin-based therapeutic strategies against AP (5,6).

Leptin is a 16-kDa protein encoded by the obese (OB) gene (7). The cytokine-like hormone is one of the adipokines produced in white adipose tissue by adipocytes, and in other organs, including the stomach, bones and muscles. Following secretion into blood, leptin combines its specific receptor (OB-R) on target cells. OB-R represents six isoforms of the OB-R gene product, among which, the full-length, long form of the leptin receptor (OB-Rb) is the only receptor molecule capable of transducing intracellular signals to activate downstream pathways. Studies have shown that plasma levels of leptin increase significantly in AP (8,9), and leptin is a valuable factor in predicting persistent organ failure in patients with acute pancreatitis (10). These studies indicate that leptin is closely associated with the severity and clinical outcome of AP. In addition, translational investigations have found that the administration of exogenous leptin can reduce AP severity and protect pancreatic tissues from damage in CIP rat models, through inhibiting the release of inflammatory factors, including TNF- α and IL-1 β (4). Further studies have shown that leptin exerts protective effects by activating nitric oxide pathways, which improves pancreatic blood flow and promotes the growth of pancreatic cells (8,11,12). These findings potentiate leptin as a promising agent for the treatment of SAP. However, the effects of exogenous leptin on SAP remain to be fully elucidated.

In the present study, SAP rat models were established via retrograde injection of sodium taurocholate into the pancreatic-bile duct, and the effects of exogenous leptin administration on the severity of SAP and 3-day short-term mortality rates of the rats in the SAP models were investigated. The results indicated that exogenous leptin protected the rat models against sodium taurocholate-induced SAP through endocrinal and immunological pathways.

Materials and methods

Reagents. The following items were used in the present study, sodium taurocholate was purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany); recombinant rat

leptin was purchased from Prospec-Tany TechnoGene, Ltd. (East Brunswick, NJ, USA), rat TNF- α and IL-10 enzyme-linked immunosorbent assay (ELISA) kits were purchased from RapidBio Laboratory (Calabasas, CA, USA), the total RNA extraction kit (TRIZol) was purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA); M-MLV reverse transcriptase, RNase inhibitor, Oligo(dT), dNTP, Taq enzyme and a 100 bp DNA ladder were purchased from Promega Corporation (Madison, WI, USA).

Rat models. The rat models were prepared as previously reported (13,14). In brief, eight-week old male Wistar rats weighing 260-320 g were purchased from the Laboratory Animal Center of Chinese Academy of Medical Sciences (Beijing, China). The experimental animals were housed in cages under standard conditions under a 12-h light/dark cycle (7:00 a.m.-7:00 p.m.) at room temperature and a relative humidity of ~40%. Following adaptive feeding for 3 days, the rats were randomly divided into three groups for experiments and were observed for 72 h. The groups included the sham operation (SO) group (n=30; 10 rats for 24, 48 and 72 h, respectively), the SAP model group (n=45; 15 rats for 24, 48 and 72 h, respectively) and the leptin (LEP) group (n=45; 15 rats for 24, 48 and 72 h, respectively). Prior to the start of experiments, the rats were deprived of food, with drinking water available. The rats were anesthetized through intraperitoneal (i.p.) injection of 1% pentobarbital sodium (30 mg/kg). The SAP model was generated by retrograde injection of 6% sodium taurocholate (0.1 ml/100 g) into the pancreatic-bile duct; an equal volume of normal saline was injected into the pancreatic-bile duct of rats in the SO group. The rats in the leptin group were administered with leptin by i.p. injection at 50 mg/kg, twice every 24 h; whereas the rats in the other groups were injected with an equal volume normal saline in parallel. The rats were sacrificed at 24, 48, and 72 h, respectively. Blood samples from the heart were obtained for the detection of serum concentrations of amylase, lipase, blood glucose, serum TNF- α and IL-10. Pancreatic tissues were obtained and washed with ice-cold saline solution and fixed with 10% formalin solution for pathological detection. Sections of the tissues were stored in liquid nitrogen for RNA detection using nested polymerase chain reaction (Nest-PCR) analysis. All animal experiments were performed with consent from the Animal Experimentation Committee and the Institutional Ethical Review Board of Beijing Friendship Hospital, Capital Medical University (Beijing, China).

Serum amylase, lipase, blood glucose and TNF- α and IL-10. Rat serum amylase, lipase, and blood glucose levels were detected using a fully-automatic biochemical analyzer (Olympus, Tokyo, Japan). The serum concentrations of TNF- α and IL-10 were determined using commercial ELISA kits according to the manufacturer's instructions.

Histological examination. The dissected pancreatic tissue samples were fixed in 10% formalin and stained with hematoxylin and eosin. The pancreatic tissue samples were examined by an experienced pathologist in a blinded-manner. According to the Kusske criteria, the pancreatic histological scores were determined by grading of intralobular edema,

leukocytic infiltration, vacuolization of pancreatic acinar, necrosis and bleeding (15).

Determination of OB-Rb mRNA. The pancreas specimens were snap-frozen in liquid nitrogen for Nest-PCR detection. The total RNA was extracted from the pancreatic tissues using TRIzol in a single step method using the extraction kit. The RNA concentration was estimated by the absorbance at a wavelength of 260 nm. Single-strand cDNA was then generated from 5 μ g of total cellular RNA in a 50- μ l reverse transcription system (10 μ l 5X buffer, 0.5 μ l Oligo(dT) and 16 units M-MLV). The nucleotide sequence of OB-Rb was determined based on published cDNA sequences encoding rat OB-Rb (16). OB-Rb primers were designed Primer Express software version 3.0.1 (Thermo Fisher Scientific, Inc.). The outer sense primer was: 5'-TGGATGAAAGGGGACTTGAC-3' and the outer antisense primer was: 5'-TCCTGAGCCATCCAGTCTCT-3'. The inner sense primer was: 5'-CTGGTTTGGCGTATGGAAG-3' and the inner antisense primer was: 5'-CCAGTCTCTTGCTCCTCACC-3'. The expected length of this PCR product was 217 base pairs (bp). GAPDH was used as the internal control. The GAPDH primers were as follows: Sense, 5'-CCCTTCATTGACCTCAACTACATGG-3' and antisense, 5'-CATGGTGGTGAAGACGCCAG-3', with a GAPDH product length of 300 bp. A 25 μ l PCR reaction system was used for PCR detection (2.5 μ l 10X buffer, 0.4 mM dNTPs, 0.4 mM MgCl₂, 1 mM each primer, 1 unit Taq DNA polymerase and 2 μ l cDNA). The incubation and thermal cycling conditions were as follows: Denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min, running for 35 cycles. The template of the second amplification was from the first-round PCR product (2 μ l), and the PCR conditions were the same as the first round. The PCR products were detected by electrophoresis on a 1.5% agarose gel containing 0.5 μ g/ml ethidium bromide. To compare the mRNA expression of OB-Rb against the mRNA expression of the reference gene GAPDH, the gray scale values of the electrophoresis bands were estimated using Quantity one software version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Statistical analysis was performed using SPSS software version 21.0 (IBM Corp., Armonk, NY, USA). All data are expressed as the mean \pm standard error of the mean. Variations between two groups were compared using Student's t-test. Comparisons involving more than two groups were performed using two-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Exogenous leptin administration affects serum amylase, lipase and blood glucose levels in rat models of SAP. Following model preparation, the serum amylase levels in rats of the SAP model group and the leptin group gradually increased, peaked at 24 h and remained high at 48 h, compared with those in rats of the SO group. At 48 h, the serum level of amylase in rats of the leptin group was lower than that in the SAP group.

The serum levels of lipase in rats of the SAP group and LEP group increased significantly, and peaked at 24 h, compared

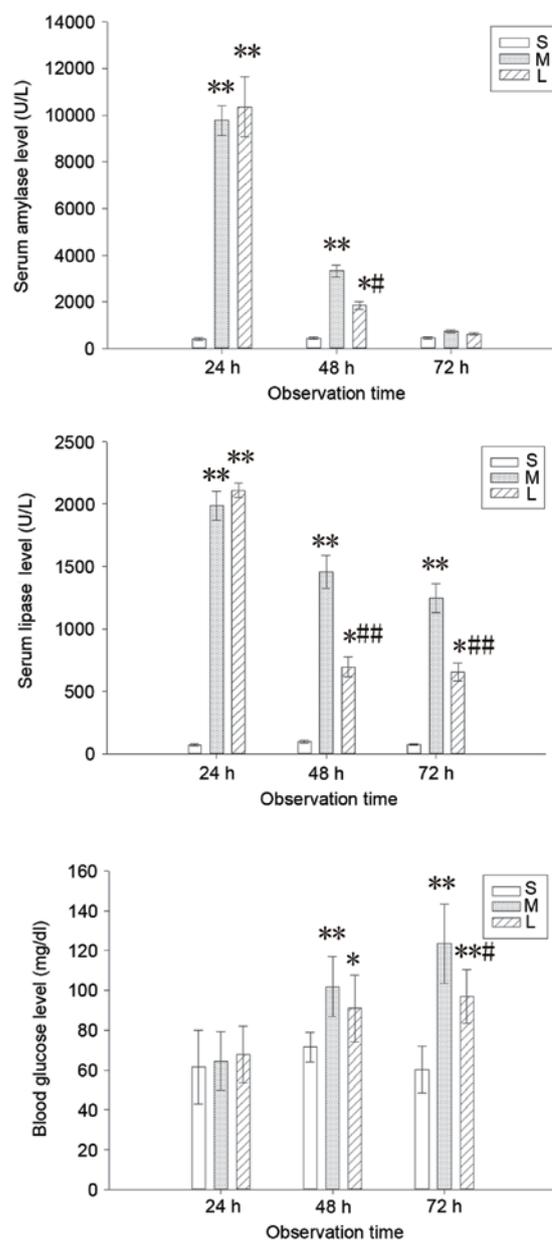


Figure 1. Serum amylase, lipase and blood glucose levels in the different groups. *P<0.05, compared with the S group; **P<0.01, compared with the S group; #P<0.05, compared with the M group; ##P<0.01, compared with the M group. S, sham operation; M, severe acute pancreatitis model; L, leptin.

with the rats in the SO group. These higher levels were maintained at 48 and 72 h, despite a decrease from 24 h. At 48 and 72 h, the lipase level in the LEP group was significantly lower compared with that in the SAP group.

In terms of serum glucose levels, the average levels in the SAP group and LEP group did not differ significantly from that in the SO group at 24 h, but were significantly higher at 48 and 72 h. The serum glucose level in the LEP group was significantly lower than that in the SAP group at 72 h (Fig. 1).

Serum levels of TNF- α and IL-10 are reduced in the leptin-treated model rats. Following model preparation, the serum levels of TNF- α in the SAP group were significantly higher than those in the SO group at 24 and 48 h. The level of TNF- α in the LEP group was significantly lower than that

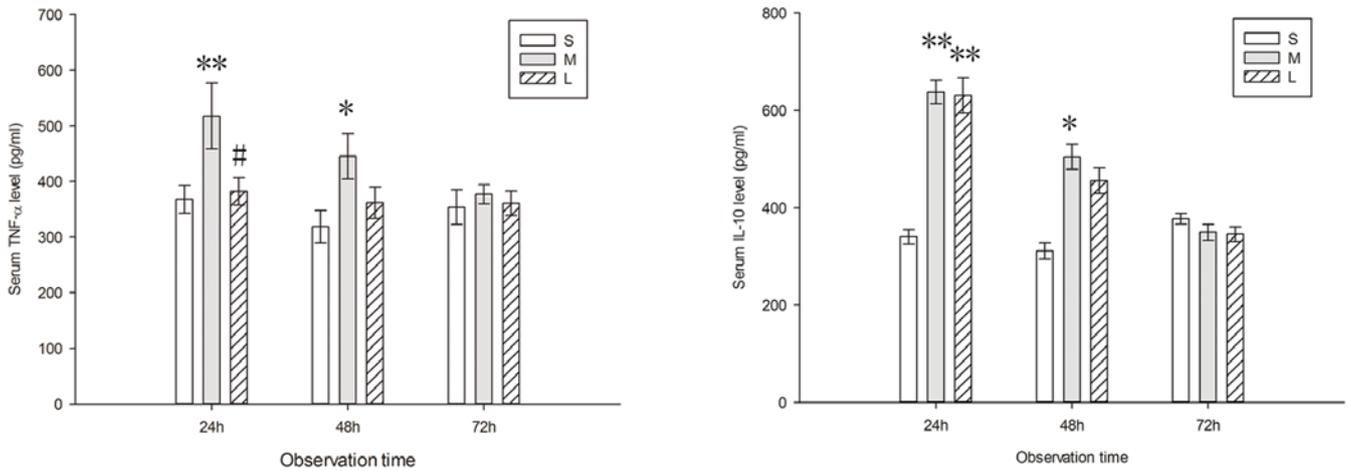


Figure 2. Concentrations of serum TNF- α and IL-10 (pg/ml) in the SO, SAP and LEP groups. *P<0.05, compared with the SO group; **P<0.01, compared with the S group; #P<0.05, compared with the M group. TNF- α , tumor necrosis factor- α ; IL, interleukin; S, sham operation; M, severe acute pancreatitis; L, leptin.

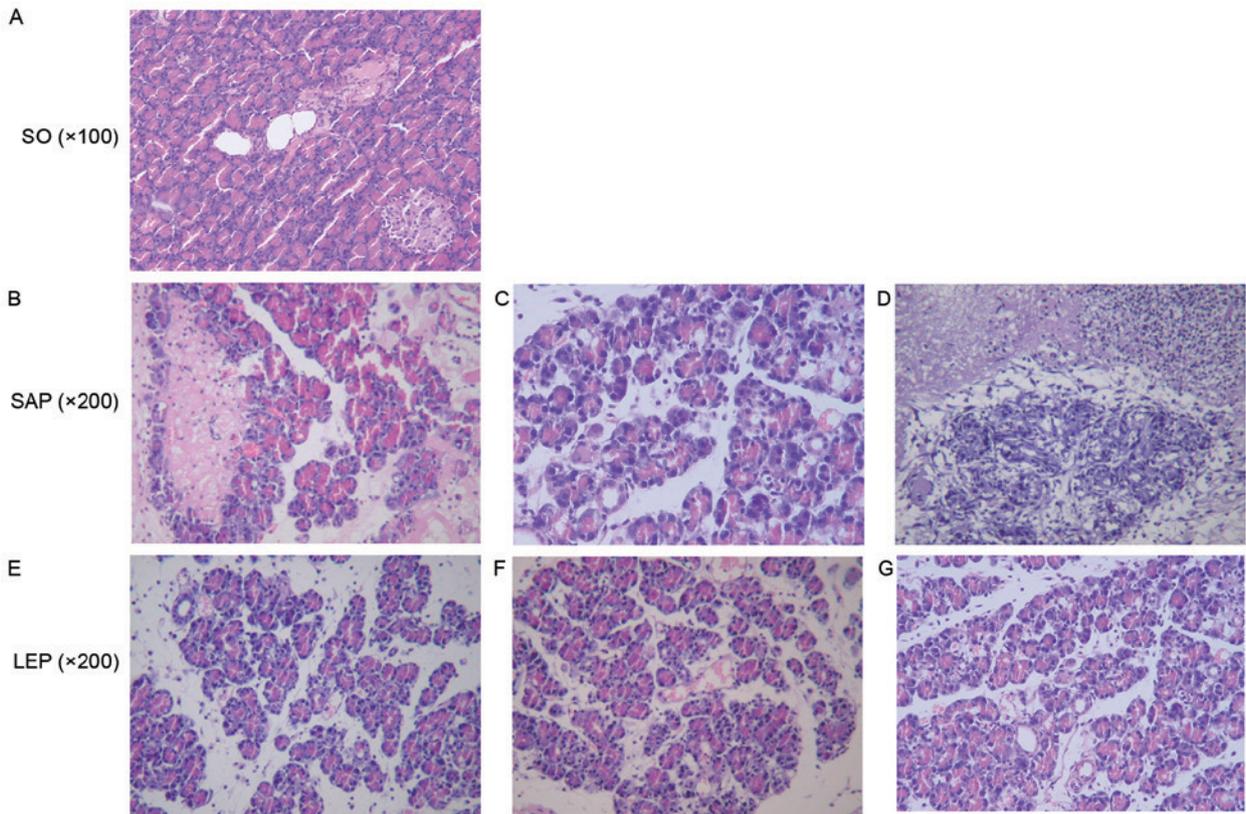


Figure 3. Hematoxylin and eosin staining of pancreatic tissues from rats of the SO, SAP and LEP groups. (A) Representative staining of samples from the SO group (magnification, x100). Representative staining of samples from the SAP group at (B) 24 h, (C) 48 h and (D) 72 h. Representative staining of samples from the LEP group at (E) 24 h, (F) 48 h and (G) 72 h. SO, sham operation; SAP, severe acute pancreatitis; LEP, leptin.

in the SAP group at 24 h. No significant differences were observed between the levels of TNF- α in the LEP group and the SAP group at 48 and 72 h.

The serum levels of IL-10 in the SAP group and the LEP group were significantly higher compared with that in the SO group at 24 h, whereas the SAP group exhibited a higher level of IL-10 compared with that in the SO group at 48 h. No significant difference was found in the levels of IL-10 between the LEP and SAP groups (Fig. 2).

Histological examination. Tissue samples from the three groups were examined macroscopically and microscopically. The pancreatic tissues from the SO groups showed a normal abdominal cavity without ascites. The tissue structure exhibited maintained contact and normal compartments with marginal inflammatory cell infiltration under microscopic observation (Fig. 3A).

As expected, the pancreatic tissues in the SAP group showed signs of edema with scattered necrosis and saponated patches.

Table I. Pancreatic pathological grading status.

Group (h)	SO (n)	SAP (n)	LEP (n)
24	0.30±0.08 (10)	7.70±0.11 (11) ^a	7.38±0.24 (12) ^a
48	0.20±0.02 (10)	8.63±0.13 (9) ^a	6.00±0.16 (11) ^{a,b}
72	0.20±0.02 (10)	9.00±0.60 (6) ^a	6.60±0.14 (8) ^{a,b}

^aP<0.05, compared with the SO group; ^bP<0.05, compared with the SAP group. SO, sham operation. SAP, severe acute pancreatitis model; LEP, leptin-treated SAP rats.

Table II. Pancreatic mRNA expression of OB-Rb in SAP rats.

Group (h)	SO (n)	SAP (n)	LEP (n)
24	1.05±0.18 (10)	1.88±0.32 (11) ^a	2.40±0.06 (12) ^{a,b}
48	1.05±0.18 (10)	1.38±0.21 (9)	1.77±0.14 (11) ^{a,b}
72	1.07±0.15 (10)	1.16±0.15 (6)	1.25±0.16 (8)

^aP<0.05, compared with the SO group; ^bP<0.05, compared with the SAP group. SO, sham operation; SAP, severe acute pancreatitis model; LEP, leptin-treated SAP rats.

Tissue adhesions and hemorrhagic ascites were predominant in the abdominal cavity. At 48 and 72 h, following a longer duration of SAP conditions, the pancreatic inflammation was aggravated with severe interlobular and intralobular edema, leukocyte infiltration and pancreatic acinar vacuolization. Severe pancreatic necrosis and bleeding were also observed. The pancreas islets exhibited signs of inflammatory cell infiltration and local islet necrotization (Fig. 3B-D). In the LEP group, the pathological changes were similar to those in the SAP group at 24 h. The pancreatic inflammation in rats of the LEP group was markedly attenuated compared with that in the rats of the SAP group at 48 and 72 h (Fig. 3E-G). The pancreatic histologic scores in the SAP group and the LEP group were significantly higher than those in the SO group at 24, 48 and 72 h. However, the pathological grading of rats in the LEP group was significantly lower compared with that of the SAP rats at 48 and 72 h (Table I).

mRNA expression of pancreatic OB-Rb. The mRNA expression levels of pancreatic OB-Rb in the rats of the SAP and LEP groups were increased significantly and peaked at 24 h compared with the expression in rats of the SO group (P<0.05). The mRNA expression of OB-Rb in the LEP group was significantly higher than that in the SAP group at 24 and 48 h (P<0.05), with no difference between the two groups at 72 h (Table II; Fig. 4A-C).

Exogenous leptin reduces the mortality rates of rats in the SAP model. The 3-day mortality rates of rats in the SAP group and the LEP group were 45.46 and 33.3%, respectively. Despite a lack of a significant difference between the two groups, the mortality rate in the LEP group was generally decreased compared with that in the SAP group.

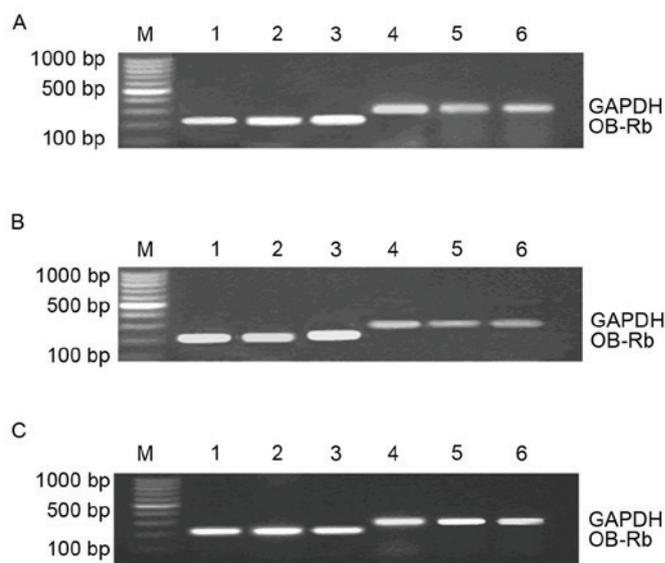


Figure 4. mRNA expression levels of OB-Rb in pancreatic tissues. Representative gels are included at (A) 24, (B) 48 and (C) 72 h. M, marker; lanes 1 and 4, samples from rats in the sham operation group; lanes 2 and 5, samples from rats in the severe acute pancreatitis group; lanes 3 and 6, samples from rats in the leptin group. OB-Rb, long form of the leptin receptor.

Discussion

Comprehensive therapeutic strategies for SAP have been widely investigated. In the present study, the protective effects of exogenous leptin on rat models with sodium taurocholate-induced SAP were investigated. The results demonstrated a profile of classical markers involved in the endocrinal and immunological pathways associated with the pancreas, supporting the hypothesis that exogenous leptin administration protects the pancreas against inflammatory damage in AP.

In the present study, the effects of exogenous leptin on the secretion of serum amylase and lipase activity in SAP rats were examined. The serum levels of amylase and lipase activity were significantly increased 24 h following model preparation. In previous investigations, leptin was found to inhibit the secretion of amylase induced by cerulein and the vagus nerve (17). The results of the present study showed that leptin decreased the serum level of amylase at 48 h, and the serum levels of lipase at 48 and 72 h, providing evidence that leptin alleviated pancreatic inflammation in the SAP rats by inhibiting the auto-digestion chain reaction of pancreatic enzymes.

According to the results of the present study, the blood glucose levels in the SAP rats increased gradually with progression of the disease at 24 h. By contrast, the blood glucose level in the leptin-treated rats decreased sharply at 48 h, and was significantly lower at 72 h compared with that in the rats of the SAP group. These results indicated a protective profile of leptin in SAP, which was in accordance with previous evidence that hyperglycemia affects the prognosis of AP (18). In the case of SAP, the endocrine dysfunction induced by pancreatic necrosis results in hyperglycemia, and the uncontrolled pancreatic inflammation induced by hyperglycemia leads to an acute diabetes syndrome. These pathogenic changes are life-threatening due to circulatory failure,

renal/cerebral dysfunction and acidosis (3). The possible mechanisms through which leptin decreases blood glucose levels are as follows: i) Leptin protects pancreatic islet structure from SAP damage to induce islet β cells to continuously secrete insulin; ii) leptin inhibits adipogenesis by regulating sterol regulatory element binding protein-1 (19), namely, to protect islet β cell function by promoting oxidization of free fatty acids in islet β cells, reducing further esterification into triglyceride circles, and maintaining normal insulin secretion; iii) leptin regulates the production and activity of various key enzymes, including glucokinase and phosphorylase, which increase glucose oxidization by promoting glucose transfer in myotubules and decreases blood glucose levels; and iv) leptin interacts with OB-R in the hypothalamus to indirectly affect glucose metabolism and decrease blood glucose levels (20). Notably, the results of the present study indicated that the regulation of blood glucose by leptin attenuated the severity of SAP, which may reduce the risks of infectious complications and aggravation of the disease.

Inflammatory cytokines are important in the pathogenesis of SAP. Previous studies have revealed that cytokine networks and immunological dysfunction have a marked affect on SAP prognosis (21). In the present study, the levels of TNF- α and IL-10 were evaluated in SAP models. TNF- α is produced at the early stage of AP, and initiates AP pathogenesis. The activation of IL-10, an anti-inflammatory cytokine, can alleviate the severity of SAP and improve prognosis of SAP (22-25). Konturek *et al* (9) and Jaworek *et al* (4) found that leptin protects the pancreas against the development of acute CIP in rats by decreasing plasma levels of TNF- α . The results of the present study showed that leptin significantly decreased serum levels of TNF- α at 24 h, which decreased further at 48 h. Leptin did not significantly affect the serum levels of IL-10, similar to results reported by Warzecha *et al* (11). The results of the present study indicated that leptin alleviated the severity of pancreatitis by decreasing the levels of TNF- α in SAP, and exerted a protective effect by inhibiting the cascade release of cytokines.

The histological experiments in the present study showed that the administration of leptin markedly alleviated pancreatic inflammation in the SAP rats at 48 and 72 h. It has been reported that leptin suppresses pancreatic inflammation induced by cerulein, decreases pancreatic weight, increases pancreatic blood flow and improves pancreatic DNA synthesis (9). Jaworek *et al* (26) reported that pretreatment with leptin in CIP rats led to only mild pancreatic damage. Consistent with these studies, the results of the present study confirmed the protective effects of leptin on pancreatic tissues in SAP.

In conditions similar to AP, the pancreatic expression of OB-R increases, and activated OB-R may be involved in the protective effect of leptin in AP (27,28). In the present study, the expression of OB-Rb in the SAP model group peaked at 24 h, and decreased gradually at 48 and 72 h; the expression levels of OB-R in the leptin group were higher than those in the SAP model group at 24 and 48 h. The enhanced expression of OB-R in the SAP model group at 24 h may have resulted from local inflammatory responses, stress-induced insulin secretion and increased leptin induction at inflammation. At 48 and 72 h, predominantly due to aggravated pancreatic inflammation in the SAP group, pancreatic islet damage

was observed and the expression of OB-Rb was decreased. Insulin secretion has been shown to decrease sharply in serious metabolic disorders, and the insulin-stimulated expression of OB-Rb is synchronously decreased (29). By contrast, the exogenous leptin and increased insulin secretion in the rats of the LEP group enhances the pancreatic expression of OB-Rb.

The present study examined the effect of leptin on the 3-day mortality rate of rats in the SAP model. SAP is recognized as a serious and rapidly progressive disorder with a high mortality rate, primarily due to its life-threatening status accompanied with unbalanced metabolism and energy supply. Previous investigations have shown that leptin may have a physiological effect at the acute stage of inflammation by regulating the inflammatory response and energy metabolism, and decreasing the mortality rates induced by acute inflammation (30). In the present study, despite the insignificant difference between the SAP model rats and leptin-treated rats, the mortality rates of the leptin-treated rats showed a decreasing trend, compared with that of rats in the SAP model group, indicating that leptin exerted a protective effect against SAP in these rat models.

Taken together, the results of the present study confirmed that leptin regulated inflammatory factors at the early stage of AP by affecting immunological and endocrinal pathways. Leptin inhibited the production and release of pro-inflammatory factors through regulating immune cells and fat cells to prevent inflammatory cascade responses. In addition, leptin administration may improve the energy and nutrient metabolic balance in SAP. Whether leptin exerts anti-inflammatory effects through the regulation of stress-induced glucocorticoids requires further confirmation, however, the results of the present study suggested that exogenous leptin administration may be a promising therapeutic strategy for the treatment of SAP.

Acknowledgements

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