Protective effect of exogenous IGF-I on the intestinal mucosal barrier in rats with severe acute pancreatitis

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BACKGROUND: Severe acute pancreatitis (SAP) can result in intestinal mucosal barrier (IMB) dysfunction. This study was undertaken to demonstrate the effect of IGF-I on the intestinal mucosal barrier in rats with SAP and its possible mechanisms.

METHODS: Seventy-two male Wistar rats were randomly divided into three groups: a sham operation (SO group, n=24), a SAP group not treated with IGF-I (SAP group, n=24), and a SAP group treated with IGF-I (IGF-I group, n=24). SAP was induced in the rats by injecting 5.0% sodium taurocholate into the biliary-pancreatic duct. The SO rats were given an infusion of normal saline instead. The rats in the IGF-I group underwent the SAP procedure and were given a subcutaneous injection of IGF-I at 30 minutes before the operation and at 3 hours after the operation. Eight rats in each group were sacrificed at 6, 12 and 24 hours after operation. Apoptosis of mucosal cells in the small intestine was determined by TUNEL. The levels of endotoxin and DAO and serum amylase were also measured. Pathologic changes in the small intestine were monitored. Changes of bax and bcl-2 mRNA expression in the small intestine were determined by reverse transcription polymerase chain reaction (RT-PCR).

RESULTS: The levels of serum amylase were lower in the IGF-I group than in the SAP group at all three time points (P<0.05). The levels of endotoxin in the IGF-I group were higher than those in the SAP group at 6 hours, but lower in the IGF-I group than in the SAP group at 12 and 24 hours (P<0.05). The levels of diamine oxidase were higher in the IGF-I group at 6 hours but lower than those in the SAP group at 12 and 24 hours. The pathological score of the small intestine was lower in the IGF-I group than in the SAP group, and the difference was statistically significant at 12 and 24 hours. The pathologic changes observed under electron microscopy were better in the IGF-I group than those in the SAP group. The apoptosis index of intestinal epithelial cells was significantly decreased in the IGF-I group compared with the SAP group. Compared with the SO group, the mRNA expression levels of bax were increased at each time point in the SAP group, and were significantly decreased in the IGF-I group as compared with the SAP group at each time point (P<0.05). The expression levels of bcl-2 were weak and not different between the SO group and the SAP group (P>0.05). They were significantly increased in the IGF-I group versus the SO and SAP groups (P<0.05). The ratio of bax and bcl-2 mRNA expression levels at each time point in the SAP group were significantly higher than those in the SO group, but they were obviously decreased in the IGF-I group.

CONCLUSIONS: Exogenous IGF-I seems to protect mucosal cells in the small intestine against SAP-induced apoptosis and could alleviate SAP-induced injury of the intestinal mucosa. The underlying mechanisms include enhanced mRNA expression of bcl-2 and inhibition of bax mRNA expression.

KEY WORDS: Insulin-like growth factor I; Severe acute pancreatitis; Mucosal barrier; Apoptosis

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INTRODUCTION
Numerous studies show that the pathological nature of pancreatitis is a kind of aseptic inflammation combining pancreatic enzyme activation and autodigestion of the pancreas. The degree of pancreatic necrosis is not the only factor to determine disease prognosis, and secondary infection of the pancreas may play an important role in this course. This secondary infection is often caused by the bacterial translocation through the damaged gut barrier. The so-called "second-strike" theory describes that SAP could result in intestinal mucosal barrier (IMB) dysfunction\cite{3-5} and imbalance of the intestinal flora ecosystem and lead to the translocation of bacteria and endotoxins from the gut to the systemic circulation. Translocated bacteria/endotoxin will excessively activate mono-macrophages to release a series of inflammatory mediators and cytokines that induce a second peak of cytokines in the systemic circulation. This second peak can accelerate the septic process,\cite{3-5} promote the development of systemic inflammatory response syndrome (SIRS) and even multiple organ dysfunction syndrome (MODS), and at last may cause death. The gastrointestinal tract may be the origin of bacterial translocation during SAP, and SAP may influence the gut barrier function, leading to the occurrence of gut origin sepsis. It is important to explore effective methods to preserve the normal intestinal mucosal barrier and to prevent the gut-derived infection caused by SAP. IGF-I has an obvious anti-apoptotic role by regulating the gene expression of the bcl-2 family, therefore it protects the intestinal mucosal barrier function. It has been proved in some studies, for example, intestinal mucosa injury is induced by oxidative stress and ischemia-reperfusion,\cite{6-9} and so on. The present study was undertaken to investigate the effects of exogenous IGF-I on apoptosis and the bax and bcl-2 gene transcription in intestinal mucosal epithelial cells during SAP.

METHODS
Animal model and groups
Seventy-two healthy male Wistar rats, weighing 180–220 g, were obtained from the Experimental Animal Center of Lanzhou University (Lanzhou, China). All the rats were randomly divided into a sham operation group (SO group, \( n=24 \)), a SAP group not treated with IGF-I (SAP group, \( n=24 \)), and a SAP group treated with IGF-I (IGF-I group, \( n=24 \)). Each group was randomly divided into 3 time units (6, 12, and 24 hours) with 8 rats in each time unit. Before the experiment the rats were fed with standard rat chow and water. They were housed in metabolic cages at a controlled temperature and under a 12-hour light/dark cycle for at least a week to acclimate to the surroundings. Prior to experiment, the rats were fasted overnight with free access to water. Surgical anesthesia was induced by intraperitoneal injection with 2% pentobarbital sodium (2.5 mL/kg of body weight). After the conventional surgical degerm, laparotomy was performed through a linea alba abdominis under aseptic conditions. SAP was induced by retrograde infusion of 1 mL/kg of 5% sodium taurocholate into the pancreatoc duct via the common biliopancreatic duct. The infusion pressure (measured with a mercury manometer) was maintained below 25 mmHg. At the same time, one microaneurysm clip was placed on the bile duct below the liver and another around the common bilio-pancreatic duct at its entry into the duodenum to avoid the reflux of enteric contents into the duct. The common bilio-pancreatic duct of the SO group rats was infused with normal saline solution in the same way. When the infusion was completed, the microclips were left in place for 4 minutes. The clips were then removed and the abdomen was closed in two layers. The IGF-I group rats were subcutaneously injected with IGF-I (50 \( \mu g/kg, 2.5 \) mL/kg of body weight) on the posterior limb one half hour before operation and three hours after the SAP model was carried out. Rats in the SAP group and the SO group were given the same dose of normal saline solution (50 \( \mu g/kg, 2.5 \) mL/kg of body weight) at the same time point. Fasting with water restraint was imposed on all rats after operation. Eight rats of each group were killed at 6, 12, and 24 hours after laparotomy. All the experiments had been approved by the regional animal ethics committee.

Main reagents and equipments
Sodium taurocholate was purchased from Sigma Incorporation; IGF-I was purchased from Co. Biovision; Trizol reagent and RT-PCR-related reagents were purchased from TAKARA Biotechnology (Dalian) CO.LTD; DNA nick in situ endlabeling (TUNEL) kit was purchased from Roche Company(Germany). Bcl-2 and bax-primer were synthesized by TAKARA Biotechnology (Dalian) Co.Ltd. The serum amylase kit was purchased from Xiamen TAL Experimental Co. Ltd, plasma endotoxin tachypleus amebocyte lysate kit was purchased from Xiamen TAL Experimental Co. Ltd, with the calculation unit for content in EU/mL.
Specimen collection and pretreatment

Eight rats of each group were anesthetized at 6, 12, and 24 hours after laparotomy. Two mL of blood were taken with a 2 mL syringe from the portal vein. The specimen was centrifuged (2000 r/min) to obtain blood plasma, after which the plasma was conserved in fridge (−70 °C). The 5 cm of distal ileum from the ileocecal area was removed and placed in cold saline. The ileum was cut along the longitudinal axis and flushed clean, and then the water was drained using the filter. Part of the ileum was fixed in 10% phosphatebuffered formaldehyde for further studies. Another part was preserved in liquid nitrogen immediately. The whole pancreas was obtained and promptly fixed in 40 g/L phosphate buffered formaldehyde for further studies.

Detection of amylase

Plasma amylase was assayed with an amylase kit with a kinetic spectrophotometric method according to the manufacturer's instructions. Briefly, the method is based on the enzymatic degradation of ethylidene-p-nitrophenol-G7 by amylase coupled with glucosidase, thus producing p-nitrophenol that exhibits strong absorbance at 405 nm. The concentrations of amylase were expressed as units per liter.

Endotoxin detection

The levels of endotoxin in the portal vein were determined by turbidimetry after all the specimens were obtained. A 0.05 mL sample of the portal vein plasma was added to 0.45 mL of processing solution B and mixed for 10 minutes at 70 °C. This was then transferred immediately to an ice water bath and constituted the serum sample to be measured. Then, 0.2 mL of the mixed sample from the first step was mixed into 0.45 mL of the main enzyme reaction agent (agent A) and then transferred to a standard 10×75 mm glass reaction tube with the trace sample. The glass tube was inserted into the MB-80 Rapid Microbial Detection System, and the computer system linked with the MB-80 automatically calculated the endotoxin level.

Diamine oxidase detection

Diamine oxidase activity was assayed according to the modified method of Nobumichi et al.[10] In the final volume of 3.8 mL, the assay mixture contained 3 mL of phosphate buffer (0.2 mol/L, pH 7.2), 0.1 mL (4 kg) of horseradish peroxidase solution, 0.1 mL of o-dianisidine methanol solution, 0.5 mL of plasma, and 0.1 mL of substrate solution (175 kg of cadaverine dihydrochloride).

This sample was incubated for 30 minutes at 37 °C, and DAO activity was measured as the absorbance at 436 nm.

Pathological examination for intestinal mucosa and pancreas

Paraffin-embedded tissue sections (2–3 mm thick) were stained with hematoxylin & eosin. Intestinal mucosal damage was evaluated blindly under a microscope by two pathologists. Histological evaluation was performed blindly under a light microscope by two pathologists on two separate occasions. Mucosal damage was assessed according to the standard scale of Chiu et al.[11] The pancreas specimens and the histological characters were judged in terms of the Schmidt criteria.[12] Mucosal grading was performed and classified as: 0=normal mucosa; 1=development of subepithelial space at the tip of the villus; 2=extension of the space with epithelial lifting; 3=massive epithelial lifting; 4=denuded villi; 5=disintegration of the lamina propria.

Apoptosis detection of intestinal mucosal cells

TUNEL was performed according to the method proposed by Gavrelle et al.[13] with some modification. The sections were deparaffinized by heating for 20 minutes at 60 °C, rehydrated in descending concentrations of ethanol (100, 95, 90, and 80), and then immersed in double-distilled water (DDW). After rehydration, the sections were incubated with 20 μg/mL proteinase K for 15 minutes at 37 °C. The slides were washed four times in DDW for 2 minutes, then they were incubated with a 50 μL TUNEL reaction mixture for 60 minutes at 37 °C. After washing in PBS, the slides were incubated with 50 μL Converter-POD for 30 minutes at 37 °C. The reaction products were visualized by staining them with diaminobenzidine solution for 7 minutes. Then the sections were stained with hematoxylin for 15 seconds in ascending concentrations of ethanol, permeabilized in xylene and mounted in gum. For positive controls, TUNEL was performed after deoxyribonuclease treatment; for negative controls, TUNEL was performed with PBS (0.01 mol/L) instead of the TUNEL reaction mixture. TUNEL-positive nuclei were counted in fields of×40 magnification chosen randomly, and the number of labeled nuclei per 100 nuclei in those fields was expressed as the apoptotic index (AI).[14]

Reverse transcriptase polymerase chain reaction of Bcl-2 and Bax mRNA reagents and primers

The sequences of bax, bcl-2 and β-actin primers
(designed by Primer 3 software, synthesized by TaKaRa Biotechnology (Dalian) Co. Ltd) were as follows: Forward primers and reverse primers, respectively: 5′-GAAGCTGAGCGAGTGTCTCCG-3′ and 5′-TGGTCACTGTCTGCCATGTGG-3′ for bax (524 bp of amplification products); 5′-CGGGCTGGGGATGACTTCTCT-3′ and 5′-GCATCCCAGCCTCCGTATCC-3′ for bcl-2 (296 bp of amplification products); 5′-ATGGATGACGTATCGCTG-3′ and 5′-ATGAGGTAGTTGTCAGGT-3′ for β-actin (445 bp of amplification products).

**Total intestine RNA extraction**

Total RNAs were extracted from approximately 100 mg of intestine tissue using TRIZOL reagent according to the manufacturer's protocol. One hundred mg of intestine tissue was homogenized in 1 mL of TRIZOL reagent. Following homogenization, 0.2 mL of chloroform was added and the homogenized tissue was incubated for 5 to 10 minutes at a room temperature. The sample was centrifuged at 11 000 r/min for 15 minutes at 4 °C and the upper aqueous phase was transferred to another centrifuge tube. After that 0.5 mL of isopropyl alcohol was added, and stored at -20 °C. The next day, the sample was centrifuged at 12 000 r/min for 5 minutes at 4 °C. The supernatant was discarded and the RNA pellets were washed with 0.8 mL of 75% ethanol. The samples were centrifuged at 11 000 r/min for 5 minutes at 4 °C, and the supernatant was discarded again. At the end of the procedure, the RNA pellets were air-dried for 10 minutes, dissolved in 50 μL of DEPC water, and stored at -80 °C.

**Procedure of RT-PCR**

RT-PCR was carried out using the One Step method. The total RT-PCR volume of each eppendorf tube was 50 μL, including 5 μL of 10×One Step RNA PCR buffer, 10 μL of MgCl₂ (25 mol/L), 5 μL of dNTPs (10 mol/L), 1 μL of RNase inhibitor (40 U/μL), 1 μL of AMV RTase XL (5 U/μL), 1 μL of AMV-Optimized Taq (5 U/μL), 1 μL of upstream specific primer, 1 μL of downstream specific primer, 1 μL of experimental sample (≤1 μg total RNA), and 24 μL of RNase Free dH₂O. Each RT-PCR conditions were as follows: 30 minutes at 50 °C for RT reactions, 2 minutes at 94 °C for RTase inactivation, 30 seconds at 94 °C, 30 seconds at 58 °C, 90 seconds at 72 °C for 34 cycles (bax); 30 seconds at 94 °C, 30 seconds at 57 °C, and 90 seconds at 72 °C for 34 cycles (bcl-2); 30 seconds at 94 °C, 30 seconds at 60 °C, and 90 seconds at 72 °C for 35 cycles (β-actin). RT-PCR was terminated with an elongation step at 72 °C for 5 minutes, and PCR products were stored at 4 °C. Ten μL of PCR products were separated by electrophoresis through a 1 % agarose gel at 45 V for 120 minutes. The cDNA bands were visualized by ultraviolet illumination after the gels were stained with 0.5 g/L ethidium bromide dissolved in Tris-borate-EDTA buffer. The gels were photographed, and the films were scanned and analyzed with a computerized densitometer (Labworks4.0). Band intensity was determined by optical density with individual PCR product of β-actin cDNA ratios. All experiments were repeated 3 times.

**Statistical analysis**

Values of the results were expressed as mean±SD. Normally distributed data were assessed with a one-way analysis of variance (ANOVA). All statistical measurements were carried out using the SPSS PC version 11.5 (SPSS, USA). Differences were considered statistically significant if \( P<0.05 \).

**RESULTS**

**Serum amylase examination**

The levels of serum amylase were significantly higher in the SAP group than in the SO group at corresponding time points \( (P<0.05) \). But the levels of amylase were lower in the IGF-I group than in the SAP group at 12 and 24 hours \( (P<0.05) \) (Figure 1).

**Serum endotoxin**

The levels of serum endotoxin were significantly higher in the SAP group than in the SO group at 12 and 24 hours \( (P<0.05) \). The levels of endotoxin were higher in the IGF-I group than in the SAP group at 6 hours, but the difference was not statistically significant \( (P>0.05) \). The levels of endotoxin were lower in the IGF-I group than in the SAP group at 12 and 24 hours, but the difference was statistically significant at 24 hours \( (P<0.05) \) (Figure 2).

**Serum diamine oxidase**

The levels of serum diamine oxidase were higher in the SAP group than in the SO group at 12 and 24 hours, with a statistically significant difference \( (P<0.05) \). The levels of diamine oxidase were higher in the IGF-I group than in the SAP group at 6 hours, but the difference was not statistically significant \( (P>0.05) \). The levels of diamine oxidase were lower in the IGF-I group than in...
the SAP group at 12 and 24 hours, but the difference was statistically significant only at 24 hours (P<0.05) (Figure 3).

Pathological examination of the small intestine

It was found that the entire mucous membrane of the small intestine was almost preserved in the SO group. In the SAP group, the pathological changes of the intestine demonstrated that edema, degradation of villi, degeneration of mucosal cells, mucosal cell necrosis, bleeding, and leukocytic infiltration were observed at 6 hours. A longer duration resulted in increased damage. However, these histological changes in the IGF-I group were obviously alleviated compared with those in the SAP group. The pathology score of the small intestine was significantly increased in the SAP group compared with the SO group at each time point (Table 1, and Figures 7, 8). There was no marked difference between the SO group and the IGF-I group (P>0.05).

Expressions of Bax and Bcl-2 mRNA in the small intestine

Bax mRNA expression was increased significantly in the SAP group at each time point (P<0.01) and tended to peak at 6 hours post-injection. It was noticeably reduced in three time units of the IGF-I group, and nearly reached the level of the SO group expression at 24 hours post-injection. Bcl-2 mRNA expression was weak and had no difference between the SO group and SAP group, but increased significantly in the IGF-I group at each time point (P<0.05). The ratio of bax and bcl-2 mRNA expression at each time point in the SAP group was significantly higher than that in the SO group, and lower in the IGF-I group. Compared with the SAP group, the difference was statistically significant at each corresponding time point (Table 1, and Figures 7, 8).

Apoptosis of intestinal mucosal cells

The apoptotic cell index of mucosal cells in the SAP group was significantly higher than that in the SO group at different time points (P<0.05). The apoptotic cell index was decreased in the IGF-I group compared with the SAP group at corresponding time points (P<0.05) (Figures 5, 6). There was no marked difference between the SO group and the IGF-I group (P>0.05).

Figure 1. Changes of the serum amylase level in three groups. Compared with the SO group, *P<0.05, †P<0.01; compared with the SAP group,  #P<0.05,  P<0.01.

Figure 2. Changes of the serum endotoxin level in the three groups. Compared with the SO group, *P<0.05, †P<0.01; compared with the SAP group,  #P<0.05,  P<0.01.

Figure 3. Changes of the serum diamine oxidase level in the three groups. Compared with the SO group, *P<0.05 and †P<0.01; compared with the SAP group,  #P<0.05,  P<0.01.

Figure 4. Changes of score after small intestine injury in the three groups. Compared with the SO group, *P<0.05, †P<0.01; compared with the SAP group,  #P<0.05,  P<0.01.
DISSCUSSION

About 80% of patients with SAP died from secondary infection of the pancreas or pancreatic area. Secondary infection comes from the bacterial translocation that was caused by intestinal barrier dysfunction. It has been demonstrated that IMB dysfunction and imbalance of the intestinal flora ecosystem is the precondition of the translocation of bacteria and endotoxins. Endotoxin is a by-product produced by gram-negative bacteria primarily in the bowel. Under normal circumstances, gram-negative bacteria are limited to the enteric cavity. However, owing to the IMB dysfunction with SPA, they may move to the systemic circulation from the gut. Therefore, plasma endotoxin can be considered as a sensitive indicator of IMB dysfunction. DAO exists in high concentrations in the intestinal mucosa. DAO in the blood comes mostly from the intestine. The serum DAO is reported to be proportional to the amount of intestinal DAO, and it is a reliable marker of intestinal mucosal integrity.

Our study confirmed that the incidence of epithelial apoptosis of the intestinal mucosa increased significantly at the early stage of SAP. The increase of apoptotic cells can decrease the total number of cells, and break the tight junction between epithelial cells, and this allows biomacromolecules to permeate, leading to the translocation of bacteria and endotoxins from the gut to the systemic circulation. Therefore excessive apoptosis is a cause of IMB dysfunction and endotoxemia in SPA. While the apoptosis is regulated by many genes, the bcl-2 genetic family is the primary gene which controls the apoptosis. The bcl-2 genetic family involves two kinds: the gene of inhibiting the apoptosis, such as bcl-2, bcl-x, bcl-w, etc., and the gene of promoting the apoptosis, such as bax, bad, bak, etc. Bcl-2 and bax are the important members in the bcl-2 genetic family which has the inhibiting effect. When the bcl-2 genetic expression increases, more and more bax with bcl-2 form bax-bcl-2 heterodimers.

Table 1. Changes of the expression of bax, bcl-2 mRNA and bax / bcl-2 in the three groups (mean ±SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>bax / bcl-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO</td>
<td>0.85±0.04</td>
</tr>
<tr>
<td>SAP</td>
<td>1.19±0.04</td>
</tr>
<tr>
<td>IGF-I</td>
<td>1.10±0.02</td>
</tr>
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</table>

Compared with the SO group, *P<0.05, #P<0.01; compared with the SAP group, ΔP<0.05, ȚP<0.01.
which is more stable than bax-bax heterodimers, and inhibits promoting apoptosis effect of the bax.\[^{[21-26]}\] Thus the change of these two reflects cellular apoptosis in some degree.

Insulin-like growth factor-1 (IGF-I), also called somatomedin C, is a single chain polypeptide that plays an important role in regulating the growth and development of body.\[^{[27]}\] Previous study has shown that IGF-I mRNA expression in the ileum was decreased significantly in rats with SAP. These findings suggest that the changes of IGF-I mRNA expression may be related to IMB.\[^{[28]}\] Experimental studies in radiation enterocolitis, small bowel resection and severe burns also have shown that administration of IGF-I can promote the synthesis of DNA in enterocyte, and the construction and function of the intestinal mucosa recovered to some degree. In addition, a series of experiments have demonstrated that IGF-I can inhibit cell apoptosis.\[^{[29-31]}\]

Our study has shown that the plasma amylase levels, endotoxin levels and DAO levels were significantly higher in the SAP group than in the SO group. The apoptosis of intestinal mucosa cells was visible, and AI and endotoxin levels and DAO levels were all positively correlated with the pathology scores of the small intestine. These results indicate that excessive apoptosis of the intestinal mucosa by SAP induced injury to the intestinal mucosa would lead to translocation of intestinal bacteria and endotoxin. The longer this process continues, the more severe the injury to the intestinal mucosa. In the present study, the plasma amylase levels were decreased significantly in the IGF-I group than in the SAP group, and the levels of endotoxin and DAO changed similarly as the levels of plasma amylase. The apoptosis index of intestinal epithelial cells decreased significantly in the IGF-I group as compared with the SAP group at corresponding time points. Pathological changes of the small intestine were significantly less in the IGF-I group as compared with the SAP group.

Bax mRNA expression was increased significantly in the SAP group at each time point. While bcl-2 mRNA expression was weak in the SO group, its expression was increased slightly in the SAP group, but not significantly. This suggests that the body may have a kind of regulatory mechanism. As some factors cause excessive apoptosis of intestinal epithelial cells, there is a self-regulation mechanism, in which the apoptosis suppressor gene expression is increased, thereby inhibiting apoptosis of the intestinal mucosa. In our study, Bax mRNA expression was noticeably reduced at three time points in the IGF-I group. Bcl-2 mRNA expression was increased significantly in the IGF-I group at each time point ($P<0.05$). This difference was statistically significant as compared with that in the SO group and the IGF-I group. The ratio of bax and bcl-2 mRNA expression at each time point in the SAP group was significantly higher than that in the SO group, and showed a steady decrease in the IGF-I group, reaching the level of the SO group. This finding suggests that exogenous IGF-I which is related to the regulation to Bcl-2 mRNA and Bax mRNA expression could inhibit excessive apoptosis of small intestinal epithelial cell that induced by SAP. This effect may be related to the mechanisms of IGF-I that could promote Bcl-2 mRNA transcription and inhibit the mRNA expression of bax mRNA.

In summary, IGF-I could decrease endotoxin translocation and alleviate intestinal epithelial cell apoptosis induced by SAP and protect the function of the intestinal mucosal barrier. It may be related to the mechanisms of IGF-I that may promote bcl-2 mRNA transcription and inhibit bax mRNA expression. Further studies are necessary to define the role of IGF-I including its effects on pancreatic microcirculation and a variety of inflammatory mediators, cytokines, and so on.

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Ethical approval: The study was approved by the Local Animal Ethical Committee.

Conflicts of interest: None.

Contributors: Wang YZ proposed the study, analyzed the data and wrote the first drafts. All authors contributed to the design and interpretation of the study and to further drafts.

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