

# Protective effects of carbon monoxide releasing molecule-2 on pancreatic function in septic mice

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**Abstract.** The present study aimed to investigate the effect of carbon monoxide (CO)-releasing molecule-2 (CORM-2) on pancreatic function in sepsis-model mice. To perform the present investigation, mice were rendered septic by cecal ligation and puncture (CLP). Then, mice were either treated with or without CORM-2 (8 mg/kg, intravenous) for different durations (6, 12 and 24 h) immediately following CLP. The levels of serum amylase and lipase, tumor necrosis factor  $\alpha$ , interleukin-1 $\beta$  and interleukin-6 in addition to myeloperoxidase (MPO) activity in pancreatic tissues were determined at 6, 12 and 24 h post-CLP. Histological scores and the expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), nuclear factor- $\kappa$ B (NF- $\kappa$ B) and phosphorylated inhibitor of  $\kappa$ B (p-I $\kappa$ B- $\alpha$ ) in the pancreas were also evaluated at 24 h post-CLP. The results of the present study revealed that compared with CLP-alone group, CORM-2 treatment significantly ( $P < 0.05$ ) reduced the levels of serum amylase, lipase and pro-inflammatory cytokines. In parallel, the severity of pancreatic histology, MPO activity and the expression levels of ICAM-1 and VCAM-1 in the pancreas of CORM-2 treated CLP mice were substantially decreased compared with the untreated group. Furthermore, CORM-2 treatment inhibited the expression levels of NF- $\kappa$ B and P-I $\kappa$ B- $\alpha$  in the pancreas of mice following CLP compared with the untreated group. CORM-2-liberated CO exerted protective effects on the pancreatic function of septic mice, and the beneficial effects may be due to the suppression of NF- $\kappa$ B activation and subsequent regulation of NF- $\kappa$ B-dependent expression of cytokines.

## Introduction

Sepsis is considered to be a harmful, non-resolving inflammatory response to infection with the presence of organ

dysfunction (1,2). In spite of the progress made in the development of medical technology, well-equipped intensive care units and improved practice treatments, the morbidity and mortality rates of sepsis remain high (3). Severe sepsis may cause multiple organ dysfunction (MODS) and the collapse of the circulatory system (septic shock), which mainly results from the extensive activation of inflammatory and coagulation pathways (3,4). Poor clinical outcomes are closely associated with the development of organ dysfunction. The greater the severity of the organ damage, the higher the risk of mortality (5). The dysfunction of organs including the lung, kidney, intestine, liver and brain, in addition to the disorder of including hematopoietic and cardiovascular systems, have been extensively studied (6). However, little attention has been focused on the function of the pancreas in sepsis. Previously, research had identified that the pancreas has a vulnerability to inflammation and injury in patients with sepsis and animal models of sepsis induced by cecal ligation and puncture (CLP) (7).

Carbon monoxide (CO) has long been known as a toxic gaseous molecule due to its ability to combine with hemoglobin. However, a small quantity of endogenous CO, a byproduct of inducible heme oxygenase-1, is continuously generated in mammals (8). Under stress condition, the intracellular levels of CO substantially increase, which has an effect on cyto-protective functions (9). Previously, transition metal carbonyls have been identified as potential CO-releasing molecules (CORMs) with the potential to facilitate the pharmaceutical use of CO by liberating it to the affected tissues and organs (8). Lipid-soluble metal carbonyl complex tricarbonyldichlororuthenium (II) dimer ( $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ ), known as CORM-2, is the first compound to corroborate the feasibility of this technology, in a controlled manner without substantially altering carboxy-hemoglobin (CO-Hb) levels (10-12). Studies have revealed that CORM-2 is able to modulate inflammation and inhibit the lipopolysaccharide (LPS)-induced production of cytokines *in vivo* and *in vitro* (13-15). Previous studies have revealed that CORM-2 attenuates leukocyte sequestration in organs including the lung, liver and small intestine in burned and CLP-induced mouse models of sepsis through interfering with nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation and inhibiting the expression of adhesion molecules (16-19). Nevertheless, to the best of our knowledge, no previous studies have determined the regulatory effects of exogenous CO on pancreatic function in a CLP-induced mouse model of sepsis.

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The present study used a CLP-induced septic mouse model, which was designed as a prospective experiment, to investigate the effects of exogenous CO on the regulation of pancreatic function and to investigate the molecular mechanisms of underlying the therapeutic effect of CO (20,21). The present study will provide further theoretical foundations and strategies for the treatment of sepsis.

## Materials and methods

**Ethics statement.** All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996; <https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf>). All experimental protocols were ethically authorized by the Council on Animal Care at Jiangsu University (Jiangsu, China) on the Protection and the Welfare of Animals and conducted in accordance with the National Institutes of Health of China guidelines for the care and use of experimental animals.

**Materials.** CORM-2, dimethyl sulfoxide (DMSO) and radioimmunoprecipitation assay buffer were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). CORM-2 was dissolved in DMSO to acquire a 40 mmol/l stock solution, as previously described (22). An inactive form of CORM-2 (iCORM-2, used as the negative control) was prepared as followed: The stock of CORM-2 was incubated at 37°C in a 5% CO<sub>2</sub> humidified atmosphere for 24 h to liberate CO. The iCORM-2 solution was finally bubbled with nitrogen to remove the residual CO present in the solution. The primary antibodies of NF-κB (sc-7386), phosphorylated inhibitor of κB (p-IκB-α; sc-52943), ICAM-1 (sc-1511) and VCAM-1 (sc1504) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The nuclear protein extraction buffer kit was obtained from Vazyme (Piscataway, NJ, USA). Other reagents and instruments included tumor necrosis factor-α (TNF-α; JER-06) interleukin-6 (IL-6; JEM-04) and IL-1β (JEM-01) enzyme-linked immunosorbent assay (ELISA) kits were all purchased from Joyee Biotechnics Co., Ltd. (Shanghai, China). All other chemicals were of reagent grade and obtained from Sigma-Aldrich (Merck KGaA) unless otherwise stated.

**Sepsis mouse model establishment.** C57BL/6 mice (n=60; male, 6-8 weeks, weight, 20±2 g) were obtained from the Experimental Animal Center of Jiangsu University, Zhenjiang, Jiangsu, China. Mice were housed in standard wire-topped cages and in temperature-controlled units (18-23°C with 40-60% humidity and 12-hour light/12-hour dark cycle). Food and water were supplied *ad libitum*. CLP was executed to induce polymicrobial sepsis as previously described (3,23). The mice were anesthetized with 2% isoflurane in oxygen through a face-mask. Under aseptic conditions, a midline incision (1-cm) was created through the abdominal wall. The cecum was identified then ligated with a 3-0 silk suture at a 1 cm position from the distal to the base of the cecum. Care was taken not to cause intestinal obstruction. Two punctures of the cecal wall were performed using a 22-gauge needle at the top and bottom. The cecum was lightly squeezed to extrude

about 1-mm<sup>3</sup> droplet of fecal material from the puncture sites to ensure a full-thickness perforation. The cecum was returned to the abdominal cavity, and the incision was closed with 5-0 surgical sutures in layers. Subsequent to the operation, the mice were immediately injected with 1 ml pre-warmed sterile saline subcutaneously to replace the fluid lost. For the sham group animals, mice underwent the same procedure, except with no CLP treatment.

**Experimental protocol.** A total of 3 time points were selected following a sham operation or CLP treatment in the experiments: 6, 12 and 24 h. At each point in time, a total of 60 mice were randomly divided into 4 groups: Sham group (n=15); CLP group (n=15); CLP+CORM-2 group (n=15); and CLP+iCORM-2 group (n=15). Mice in the CLP+CORM-2 and CLP+iCORM-2 groups received an injection of CORM-2 [8 mg/kg intravenously (i.v.)] or iCORM-2 (8 mg/kg, i.v.) immediately subsequent to CLP, respectively. The dosage of CORM-2 used in the present study was based on the results of previous studies (16,18). The negative control (iCORM-2) was used to examine whether the effects observed were due to CO liberated by CORM-2 or caused by other components of the molecules. Sham animals received normal saline intravenously following the sham operation in the same regimen.

**Tissue collection.** Mice were euthanized using excessive anesthesia administration and were sacrificed at 6, 12 and 24 h after CLP or sham surgery as previously described (23,24). Blood samples were collected using cardiac puncture, and the samples were stored in serum tubes and immediately centrifuged at 3,000 × g for 5 min at room temperature. The serum was isolated from these samples for subsequent lipase and amylase level determination. Pancreatic tissues were removed and immediately frozen in liquid nitrogen or fixed at room temperature overnight in 10% formalin for further studies.

**Biochemical measurement.** The activities of serum amylase and lipase were measured to evaluate the pancreatic injury by using a commercial kit (SNM144-BOU, Biolebo, Beijing, China). Levels of amylase and lipase were determined according to the manufacturers' protocol.

**Morphological examination.** Samples of 10% formalin-fixed pancreas tissue were embedded in paraffin and sectioned at a thickness of ~4 μm for routine histology for each group. Fixed tissues were then stained with hematoxylin and eosin (H&E) according to the manufacturer's protocol, and examined by two experienced morphologists who were blinded to the sample identity. For these studies, 10 randomly selected microscopic fields (at a magnification of 10x20) were examined for each tissue sample. Each section was scored for the severity of pancreatic injury on a scale of 0-4 (normal to severe) as previously described (25).

**Immunohistochemical staining.** Pancreatic tissues were fixed in 10% formalin and 4-μm thick sections were prepared from paraffin-embedded tissues. Following deparaffinization and rehydration, and sections were subjected to heat-mediated antigen retrieval in sodium citrate buffer (10 mM sodium citrate, pH 6.0) prior to blocking in 10% normal goat serum for

2 h at room temperature. The primary antibodies of ICAM-1 and VCAM-1 were diluted to 1/200 and the samples were incubated at 4°C overnight. Following three washes with phosphate buffered saline, a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (sc-2004, Santa Cruz Biotechnology, Inc.) was diluted 1:100 and incubated with the samples for 20 min at room temperature. The slides were then washed again three times with tris-buffered saline. Thereafter, the slides were filled with freshly prepared 3,3'-Diaminobenzidine chromogen (brown) and incubated at room temperature for 3 min, washed in distilled water and counter-stained with hematoxylin for 10 sec at room temperature. This was followed by dehydration with gradient ethanol (100, 95 and 80%) and clearing using xylene. The slides were then mounted with mounting medium, labelled and viewed under a light microscope. For these studies, 10 randomly selected microscopic fields (magnification, 10x20) were examined for each tissue sample. Mean optical density for ICAM-1 and VCAM-1 were evaluated using Image-Pro Plus version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

**Myeloperoxidase (MPO) activity.** MPO activity was determined in pancreatic tissues in a procedure similar to that documented in other studies (26-28). The tissue samples were homogenized in 50 mmol/l potassium phosphate buffer (PB; pH 6.0) and centrifuged at 10,000 x g for 10 min at room temperature. The pellets were suspended in 50 mmol/l PB including 0.5% hexadecyltrimethylammonium bromide. Subsequent to sonication, the samples were centrifuged at 10,000 x g for 10 min again at room temperature. Aliquots (0.3 ml) were added to 2.3 ml of the reaction mixture containing o-dianisidine, 50 mmol/l PB and 20 mmol/l H<sub>2</sub>O<sub>2</sub> solution. One unit of enzyme activity was regarded to indicate the quantity of MPO present that resulted in a change in the absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

**TNF- $\alpha$ , IL-6 and IL-1 $\beta$  level detection.** TNF- $\alpha$ , IL-6 and IL-1 $\beta$  levels in pancreas tissue homogenates were measured using ELISA kits according to the manufacturer's protocol of each kit.

**Western blot analysis.** Nucleic proteins were extracted using a nuclear protein extraction buffer kit (Vazyme) according to the manufacturer's protocol. A BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to evaluate the protein concentrations. Then, samples (10  $\mu$ g protein) were subjected to electrophoresis on 10% SDS-PAGE gels using a discontinuous system and transferred onto polyvinylidene fluoride membranes, which were then incubated with an anti-mouse NF- $\kappa$ B-specific polyclonal antibody (1:1,000; cat no. Sc-514451; Santa Cruz Biotechnology, Inc.), anti-mouse P-I $\kappa$ B- $\alpha$ -specific polyclonal antibody (1:1,000; cat no. Sc-7977; Santa Cruz Biotechnology, Inc.) or  $\beta$ -actin (1:1,000; cat no. Sc-81178; Santa Cruz Biotechnology, Inc.) at 4°C overnight and a HRP-conjugated goat anti-mouse immunoglobulin G antibody (1:10,000; cat no. Sc-2031; Santa Cruz Biotechnology, Inc.; incubated at room temperature for 1 h) was used as the secondary antibody. Electrochemiluminescence reagent was used to visualize the bands using FluorChem FC3 (ProteinSimple, San Jose, CA, USA), and AlphaView version

3.4.0 software (ProteinSimple, Santa Clara, CA, USA) was used for quantification analysis.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Data were presented as the mean  $\pm$  the standard deviation. One-way factorial analysis of variance followed by a Tukey's post-hoc test were performed for comparisons between the groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Effect of CORM-2 on the function of the pancreas in septic mice.** Sepsis results in MODS, which includes injury of the pancreas (29,30). To demonstrate the protective effects of CORM-2 on the function of pancreas in a CLP-induced mouse sepsis model, the levels of serum amylase and lipase (commonly used as bio-markers to detect pancreatic injury) (30,31) were examined. The level of serum amylase was significantly increased at 6, 12 and 24 h post-CLP compared with the sham mice ( $P < 0.05$ ) (Fig. 1A). Administration of CORM-2 in septic mice significantly decreased the level of serum amylase at 6, 12 and 24 h post-CLP compared with the CLP-alone group ( $P < 0.05$ ; Fig. 1A). The septic mice treated with iCORM-2 did not present altered serum amylase activity when compared with the CLP group (Fig. 1A). Similar results were obtained when assessing the serum lipase levels (Fig. 1B).

The damage to the pancreas was also examined by histological evaluation. The pancreatic tissue from the sham group mice exhibited normal architecture, while the pancreatic tissues in the septic mice exhibited severe pathological injury at 24 h post-CLP (Fig. 2A). The pancreatic tissues in the septic mice exhibited characteristic edema, inflammatory cell infiltration and necrosis of the acinar cells (Fig. 2A). Treatment of the CLP mice with CORM-2 for 24 h significantly reduced the extent and severity of the histological signs of pancreatic injury compared with the CLP-alone group, including edema, inflammatory cell infiltration and necrosis ( $P < 0.05$ ; Fig. 2A and B), whereas alleviation in the injury of pancreas was not observed in the iCORM-2 treatment group compared with the CLP-alone group (Fig. 2A and B). The present study revealed that CORM-2 treatment exerted protective effects in the CLP-induced pancreatic damage.

**Effect of CORM-2 on MPO activity in the pancreas of septic mice.** Inflammatory cell infiltration is a notable pathogenesis of sepsis-induced acute pancreatic injury (32). To evaluate pancreatic neutrophil infiltration, MPO activity, an index of neutrophil accumulation, was measured in the pancreatic tissues obtained from the sham group, the CLP group, the CLP+CORM-2 group and the CLP+iCORM-2 group post-operation at 6, 12 and 24 h. The results revealed that MPO activity was significantly increased in septic mice at 6, 12 and 24 h post-CLP when compared with the sham group ( $P < 0.05$ ; Fig. 3). Treatment with CORM-2 subsequent to CLP treatment significantly attenuated MPO activity at 6, 12 and 24 h compared with the CLP-alone group ( $P < 0.05$ ; Fig. 3), indicating that treatment with CORM-2 decreased the infiltration of neutrophils in the pancreatic tissues. No significant

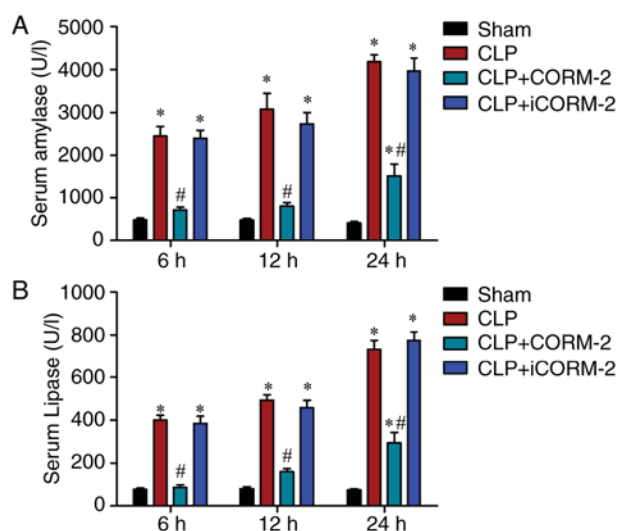


Figure 1. Effect of CORM-2 on serum amylase and lipase levels in CLP-induced septic mice. Serum (A) amylase and (B) lipase levels were determined at 6, 12 and 24 h after the induction of CLP treatment or a sham operation in four groups: A sham group; CLP, mice subjected to CLP to induce polymicrobial sepsis; CLP+CORM-2, mice subjected to CLP and treated with CORM-2 (8 mg/kg, i.v.); and CLP+iCORM-2, mice subjected to CLP and treated with iCORM-2 (8 mg/kg, i.v.). \* $P < 0.05$  vs. the sham group; # $P < 0.05$  vs. the CLP group. These data are expressed as the mean  $\pm$  standard deviation,  $n = 5$  for each group. CLP, cecal ligation and puncture; CORM-2, carbon monoxide releasing molecule-2; iCORM2, inactive CORM-2; i.v., intravenously.

difference was identified between the CLP group and the CLP+iCORM-2 group (Fig. 3).

**Effect of CORM-2 on the expression of ICAM-1 and VCAM-1 in the pancreas of septic mice.** The accumulation of neutrophils in the tissue results from the increased adherence of neutrophils to endothelial cells, which is mediated by adhesion molecules (26). Infiltration of neutrophils in the pancreas augments the injury of tissues. Therefore, the present study evaluated the expression levels of ICAM-1 and VCAM-1 adhesion molecules, which serve critical roles in the firm attachment of neutrophils to the endothelium. No positive staining for ICAM-1 was observed in the pancreatic tissue sections obtained from the sham group mice (Fig. 4A). Sections obtained from CLP-induced septic mice 24 h after CLP exhibited significant positive staining for ICAM-1 and VCAM-1 compared with the sham group ( $P < 0.05$ ; Fig. 4A-C). The degree of pancreatic staining for ICAM-1 and VCAM-1 were significantly reduced in tissue sections obtained from 24 h post-CLP mice that had received CORM-2 treatment compared with the CLP-alone group ( $P < 0.05$ ; Fig. 4A-C). No significant changes in staining were observed in CLP-treated mice that had additionally been treated with iCORM-2 when compared with CLP-alone mice (Fig. 4A-C).

**Effect of CORM-2 on the production of pro-inflammatory cytokines in the pancreas of septic mice.** In order to investigate the regulation of inflammation in the pancreas by CORM-2 treatment, the expression levels of pro-inflammatory cytokines IL-6, IL-1 $\beta$  and TNF- $\alpha$  were determined using ELISA. The levels of IL-6, IL-1 $\beta$  and TNF- $\alpha$  significantly increased at 6, 12 and 24 h after CLP when compared with the sham controls

( $P < 0.05$ ; Fig. 5A-C). Treatment with CORM-2 significantly decreased the CLP-induced increase in the expression of IL-6, IL-1 $\beta$  and TNF- $\alpha$  compared with the CLP-alone group ( $P < 0.05$ ; Fig. 5A-C). Additionally, iCORM-2 treatment did not exert any effect in the expression of these cytokines (Fig. 5A-C).

**Effect of CORM-2 on NF- $\kappa$ B activation in the pancreas of septic mice.** To determine whether NF- $\kappa$ B was involved in the observed anti-inflammatory effects of CORM-2-derived CO, the levels of NF- $\kappa$ B p65 in the pancreas were measured using western blot analysis. The results demonstrated that 24 h post-CLP mice exhibited a significantly increased protein level of NF- $\kappa$ B compared with the sham group mice ( $P < 0.05$ ; Fig. 6A). This increase was significantly inhibited by treatment with CORM-2 compared with the CLP-alone group ( $P < 0.05$ ), whereas iCORM-2 treatment had no effect on the levels of NF- $\kappa$ B (Fig. 6A). Furthermore, p-I $\kappa$ B- $\alpha$  in the pancreas was also examined using western blot analysis, which is required for the initiation of NF- $\kappa$ B activation. There was significant increase of the levels of p-I $\kappa$ B- $\alpha$  in the pancreas of septic mice 24 h after CLP compared with the sham group ( $P < 0.05$ ), which was significantly suppressed by the administration of CORM-2 compared with the CLP-alone group ( $P < 0.05$ ; Fig. 6B). No significant change in the p-I $\kappa$ B- $\alpha$  levels in the pancreas was observed by treatment with iCORM-2 when compared with the CLP-alone mice (Fig. 6B).

## Discussion

Sepsis is a common phenomenon amongst critically ill patients, and is associated with the progressive failure of multiple organs (1). Although the most common dysfunctions of organs in sepsis are in the lung and kidney, the pancreas is also susceptible to inflammation and injury in patients with sepsis (3,6). Research suggests that sepsis has a notable impact on pancreatic secretory function, which augments the severity of sepsis and is worse in septic shock compared with sepsis without shock (6). Impaired exocrine function in the pancreas is associated with Acute Physiology and Chronic Health Evaluation III and Sequential Organ Failure Assessment scores (6).

Previous studies have demonstrated the specific and independent function of exogenous CO in the modulation of inflammation (14,19,33-36). As novel metal carbonyl-based compounds, CORMs have the ability to deliver CO in biological systems in a controlled manner (11). CORM-derived CO has been demonstrated to have various benefits, including vasoactive, antihypertensive, cardio-protective and anti-rejection effects (37-40). CORM-2, one of the novel group of CORMs, has been determined to possess anti-inflammatory effects and to ameliorate the function of the pancreas in acute pancreatitis (20,21). In previous studies, the administration of CORM-2 improved the function of numerous organs in CLP and thermal injury animal models of sepsis (11,18). However, it remains unclear whether CORM-2 serves a protective effect on the function of the pancreas.

To determine the therapeutic function of CO, the present study initially established CLP septic mice and sham mice. The animals were separated into four groups: Sham group; CLP group; CLP+CORM-2 group; and CLP+iCORM-2

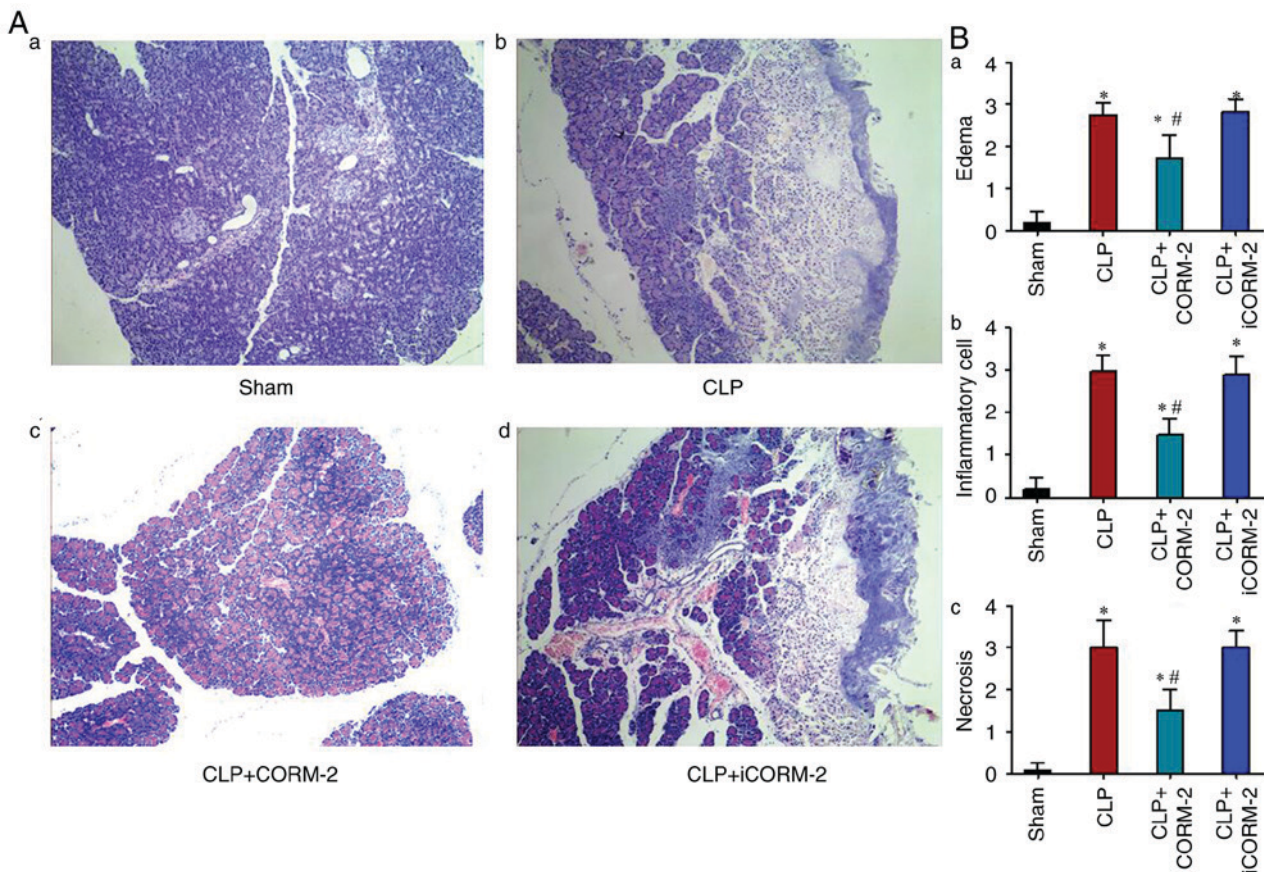


Figure 2. Histological evaluation of the effect of CLP and CORM-2 on the pancreas. (A) Effect of CORM-2 on pancreatic injury at 24 h after the induction of CLP (H&E staining, magnification x200), in the pancreatic tissue obtained from: (Aa) The sham group, (Ab) the CLP group (mice subjected to CLP to induce polymicrobial sepsis), (Ac) the CLP+CORM-2 group (mice subjected to CLP and treated with CORM-2, 8 mg/kg, i.v.) and (Ad) the CLP+iCORM-2 group (mice subjected to CLP and treated with iCORM-2, 8 mg/kg, i.v.). (B) Quantified effect of CORM-2 on histopathological scores of pancreatic injury. H&E-stained sections were evaluated for (Ba) edema, (Bb) inflammatory cell infiltration and (Bc) acinar necrosis. These data are expressed as the mean  $\pm$  standard deviation, n=10 for each group. \*P<0.05 vs. the sham group; #P<0.05 vs. the CLP group. CLP, cecal ligation and puncture; CORM-2, carbon monoxide releasing molecule-2; iCORM2, inactive CORM-2; i.v., intravenously; H&E, hematoxylin and eosin.

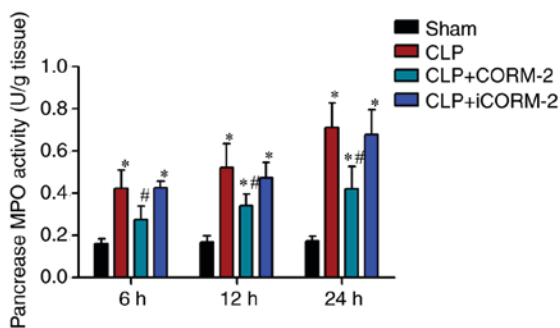


Figure 3. Effect of CORM-2 on the MPO activity in the pancreatic tissue of CLP-induced septic mice. MPO activity in the pancreas was measured at 6, 12 and 24 h after the induction of CLP in four groups: Sham, sham group; CLP, mice subjected to CLP to induce polymicrobial sepsis; CLP+CORM-2, mice subjected to CLP and treated with CORM-2 (8 mg/kg, i.v.); and CLP+iCORM-2, mice subjected to CLP and treated with iCORM-2 (8 mg/kg, i.v.). These data are expressed as the mean  $\pm$  standard deviation, n=5 for each group. \*P<0.05 vs. the sham group; #P<0.05 vs. the CLP group. CLP, cecal ligation and puncture; CORM-2, carbon monoxide releasing molecule-2; iCORM2, inactive CORM-2; i.v., intravenously; MPO, myeloperoxidase.

group. Each group was divided again at postoperative 6, 12 and 24 h time points. CORM-2 or iCORM-2 were injected immediately following CLP. The levels of serum amylase

and lipase (which are used to detect pancreatic injury) were examined in the present study. The results indicated that treatment with CORM-2 in CLP mice suppressed the levels of serum amylase and lipase compared with the sham group mice at all three time points. The septic mice treated with iCORM-2 did not exhibit altered serum amylase and lipase activity when compared with the CLP group mice at all three postoperative time points. Furthermore, the damage to the pancreas was also examined by histological evaluation. At all three time points, CORM-2 exerted a protective effect on the function of pancreas, and 24 h post-CLP exhibited the most substantial effect. Therefore, the present study selected the representative H&E stained pancreatic sections in the four groups at the 24 h time point (Fig. 1). The pancreas from the mice of the sham group exhibited normal architecture, while the pancreatic tissues in the septic mice exhibited characteristic edema, inflammatory cell infiltration and necrosis of the acinar cells. Treatment with CORM-2 substantially reduced the extent and severity of the histological signs of pancreatic injury compared with the CLP-alone group, whereas alleviation in the injury of the pancreas was not observed in the iCORM-2 treatment group. The results were consistent with the experimental data of serum amylase and lipase levels. As the specific mechanisms for the therapeutic effects of

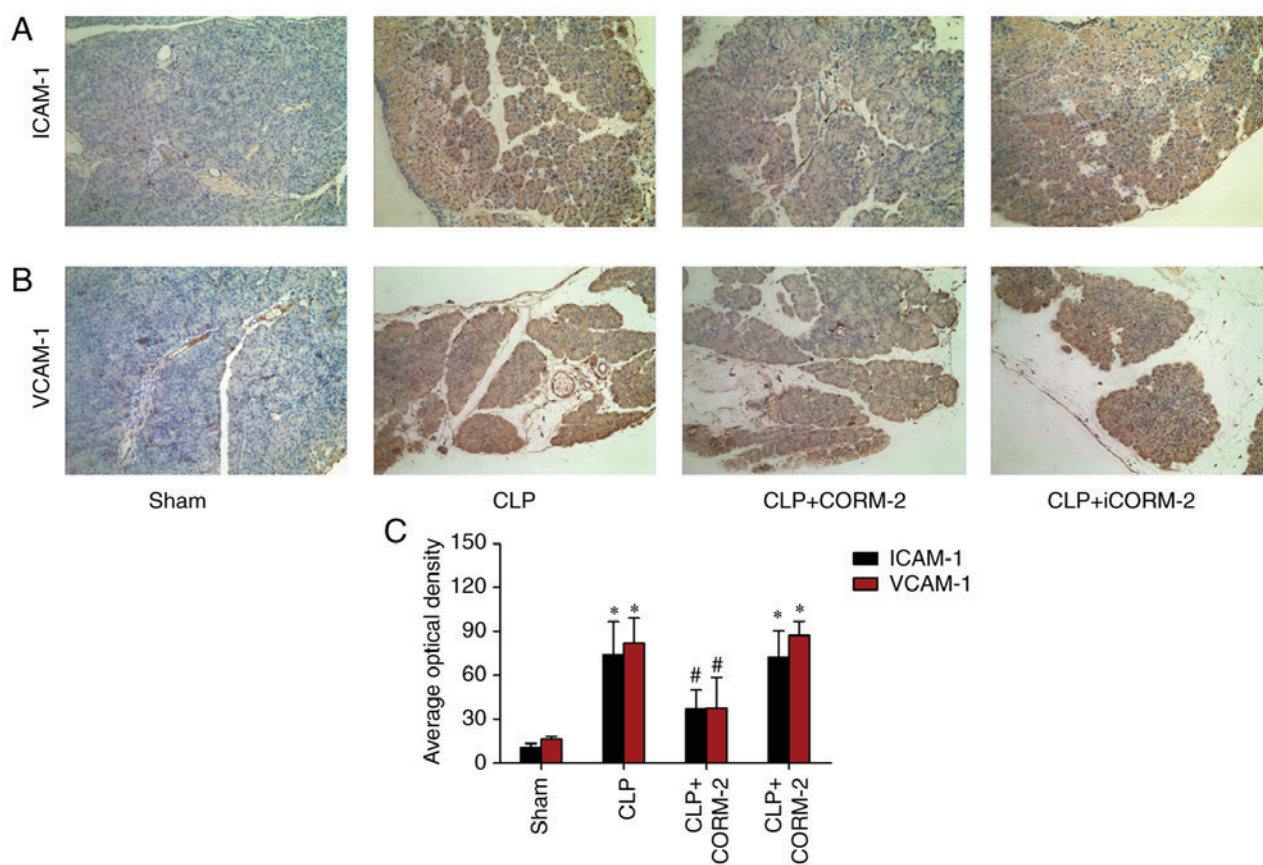


Figure 4. Effect of CORM-2 on ICAM-1 and VCAM-1 levels in the pancreatic tissue of CLP-induced septic mice (magnification, x200). Expression of (A) ICAM-1 and (B) VCAM-1 levels in the pancreas were determined at 24 h post-CLP by immunohistochemical staining, in the following four groups: Sham, sham group; CLP, mice subjected to CLP to induce polymicrobial sepsis; CLP+CORM-2, mice subjected to CLP and treated with CORM-2 (8 mg/kg, i.v.); and CLP+iCORM-2, mice subjected to CLP and treated with iCORM-2 (8 mg/kg, i.v.). (C) Quantitative analysis of the mean optical density for ICAM-1 and VCAM-1 in the pancreatic tissues. These data are expressed as the mean  $\pm$  standard deviation,  $n=10$  for each group. \* $P<0.05$  vs. the sham group; # $P<0.05$  vs. the CLP group. CLP, cecal ligation and puncture; CORM-2, carbon monoxide releasing molecule-2; iCORM2, inactive CORM-2; i.v., intravenously; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1.

CORM-2 in CLP are unknown, the aim of the present study was to investigate the potential mechanisms and identify CORM-2 mediated cellular targets.

MPO is an enzyme that is present predominantly in the azurophilic granules of polymorphonuclears (PMNs), and is frequently used to estimate tissue PMN accumulation (26,27). Neutrophil infiltration that aggravates tissue inflammation and damage is considered a principal contributor to sepsis mortality (41,42). In the present study, it was revealed that MPO activity in the pancreas was substantially elevated at 6, 12 and 24 h after CLP compared with the sham group. These effects were notably attenuated following the administration of CORM-2 *in vivo*, but not iCORM-2, suggesting that CORM-2 effectively inhibited neutrophil chemotaxis and infiltration in the pancreas subsequent to CLP, eventually decreased the production of oxidants and reduced oxidant-mediated injury and the inflammatory response in the pancreas.

The direct cause of neutrophil infiltration into tissues following CLP is considered to be the overexpression of adhesion molecules (for example, ICAM-1 and VCAM-1) (36). Adhesion molecules activate neutrophils and endothelial cells, which in turn accelerate the release of various inflammatory mediators (43,44). In the present study, immunohistochemical

staining was used to detect the expression of ICAM-1 and VCAM-1 in pancreas tissue. The present results demonstrated that the expression levels of ICAM-1 and VCAM-1 in the pancreas were increased at 24 h post-CLP compared with the sham group. Administration of CORM-2 inhibited the upregulation of ICAM-1 and VCAM-1 induced by CLP. No significant change in the expression levels were observed in iCORM-2 treated CLP mice when compared with the CLP-alone treated mice.

Research has revealed that sepsis induces a widespread inflammatory response with a substantial increase of pro-inflammatory cytokines in the serum, which are important mediators in the pathogenesis of acute pancreatic injury (45). TNF- $\alpha$ , IL-1 $\beta$  and IL-6 are induced in exaggerated production in the early stages of infectious diseases (46,47). Studies have demonstrated that CORM-2 treatment abolishes the elevation in the levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in the serum in CLP-induced peritonitis sepsis and LPS-induced sterile sepsis (3,20). Therefore, the levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in the pancreas were assessed in the present study. Similarly, the levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 increased at 6, 12 and 24 h post-CLP compared with the sham group, which were attenuated by CORM-2 treatment. These data indicated that the cytoprotective effects of CORM-2 were,

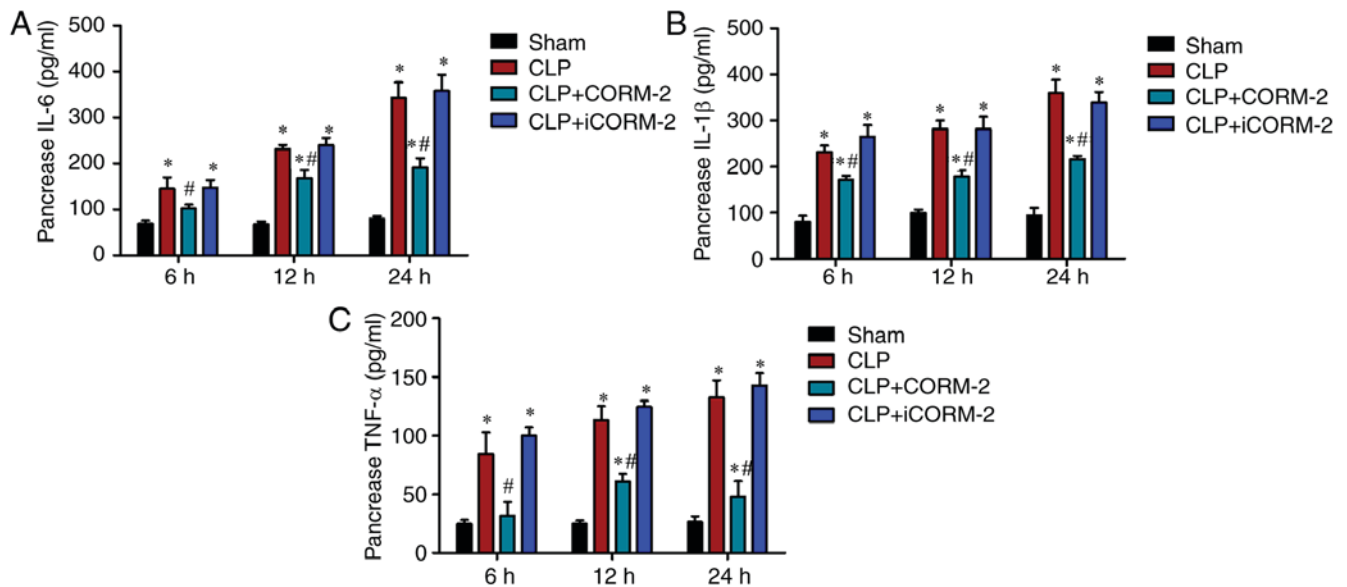


Figure 5. Effects of CORM-2 on the levels of pro-inflammatory cytokine in the pancreas of CLP-induced septic mice. The expression levels of (A) IL-6, (B) IL-1 $\beta$  and (C) TNF- $\alpha$  in the pancreas were assessed at 6, 12 and 24 h after the induction of CLP or sham operation in the following four groups: Sham, sham group; CLP, mice subjected to CLP to induce polymicrobial sepsis; CLP+CORM-2, mice subjected to CLP and treated with CORM-2 (8 mg/kg, i.v.); CLP+iCORM-2, mice subjected to CLP and treated with iCORM-2 (8 mg/kg, i.v.). These data are expressed as the mean  $\pm$  standard deviation, n=5 for each group. \*P<0.05 vs. the sham group; #P<0.05 vs. the CLP group. CLP, cecal ligation and puncture; CORM-2, carbon monoxide releasing molecule-2; iCORM2, inactive CORM-2; i.v., intravenously; IL-6, interleukin 6; IL-1 $\beta$ , interleukin 1 $\beta$ ; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ .

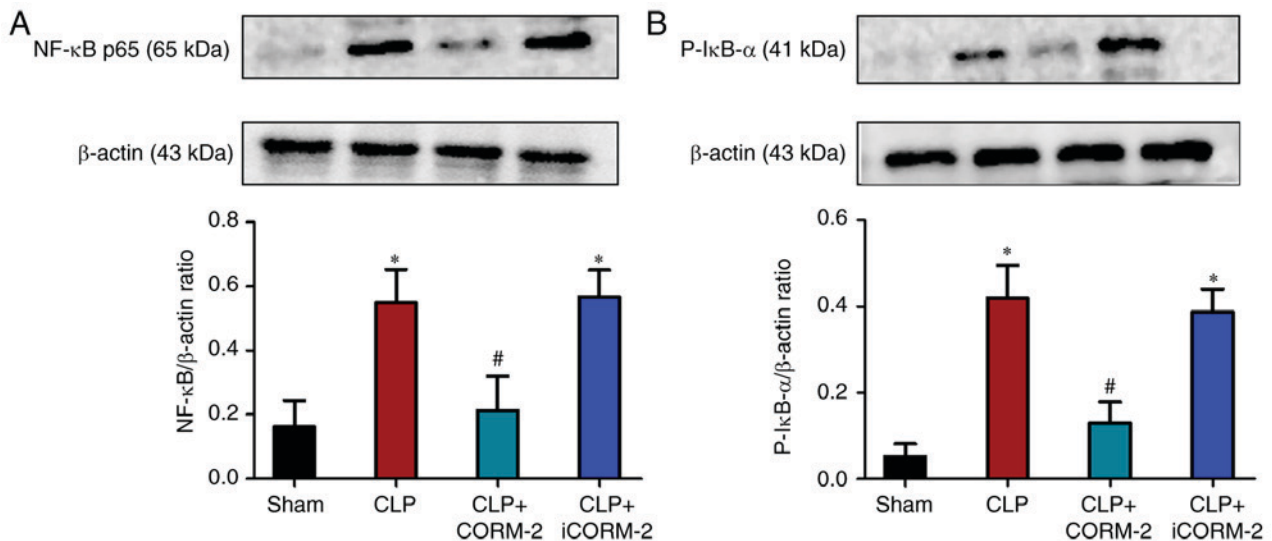


Figure 6. Effect of CORM-2 on the levels of NF- $\kappa$ B p65 and p-I $\kappa$ B- $\alpha$  in the pancreas of CLP-induced septic mice. The levels of (A) NF- $\kappa$ B and (B) p-I $\kappa$ B- $\alpha$  were analyzed using western blot analysis at 24 h after the induction of CLP or sham operation in the following four groups: Sham, sham group; CLP, mice subjected to CLP to induce polymicrobial sepsis; CLP+CORM-2, mice subjected to CLP and treated with CORM-2 (8 mg/kg, i.v.); and CLP+iCORM-2, mice subjected to CLP and treated with iCORM-2 (8 mg/kg, i.v.). These data are expressed as the mean  $\pm$  standard deviation, n=5 for each group. \*P<0.05 vs. the sham group; #P<0.05 vs. the CLP group. CLP, cecal ligation and puncture; CORM-2, carbon monoxide releasing molecule-2; iCORM2, inactive CORM-2; i.v., intravenously; NF- $\kappa$ B, nuclear factor- $\kappa$ B; p-I $\kappa$ B- $\alpha$ , phosphorylated inhibitor of  $\kappa$ B.

at least partly, due to the inhibition of the production and secretion of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 during the early period of sepsis.

The NF- $\kappa$ B family members are ubiquitous, which rapidly trigger transcription factors that mediate immune and inflammatory reactions by regulating the expression of certain chemokines and cytokines (48,49). Previously, it was reported that CORM-2 inhibited the activity of NF- $\kappa$ B in the tissues of the liver and lung in thermal injury mice

models and E coli-induced murine sepsis models (11,26). Therefore, the present study evaluated the involvement of NF- $\kappa$ B in the observed anti-inflammatory effects of CORM-2-derived CO on the pancreatic damage induced by CLP. For this purpose, the nuclear translocation and DNA binding of p65, the key component of NF- $\kappa$ B (50-52), was measured in the pancreas using western blot analysis. Additionally, p-I $\kappa$ B- $\alpha$  in the pancreas was also examined using western blot analysis, which is required for the

initiation of NF- $\kappa$ B activation. The results indicated that NF- $\kappa$ B p65 and p-I $\kappa$ B- $\alpha$  increased 24 h after CLP treatment compared with the sham group, and this increase was suppressed by treatment with CORM-2. The levels of NF- $\kappa$ B p65 and p-I $\kappa$ B- $\alpha$  at 24 h post-CLP were evaluated as CORM-2 exerted a notable protective effect on the pancreatic function of septic mice 24 h post-CLP (as presented in Fig. 1). In fact, at post-operative 6 and 12 h time points, similar results of NF- $\kappa$ B p65 and p-I $\kappa$ B- $\alpha$  were observed (data not shown). Inhibition of CORM-2 on the activation of NF- $\kappa$ B p65 and p-I $\kappa$ B- $\alpha$  may explain the decrease in the expression of inflammatory factors including TNF- $\alpha$ , IL-1 $\beta$  and IL-6 observed in the pancreas. However, despite p-I $\kappa$ B- $\alpha$  being required for the initiation of NF- $\kappa$ B activation, and numerous other studies have only assessed the expression of p-I $\kappa$ B- $\alpha$  to demonstrate the activation of NF- $\kappa$ B (53-55), in order for the experimental results to be more convincing, total I $\kappa$ B- $\alpha$  also needs to be tested. This is a limitation of the present study.

Despite the therapeutic and anti-inflammatory effects of exogenous CO in various disease and injury models, there has been little progress with regard to its use in clinical illness. The main problem is the method of administration of CO. Although low dose inhalational CO has been revealed to have anti-inflammatory effects against ventilatory-induced lung injury and has been associated with a decreased level of TNF- $\alpha$  (21,56), it is difficult to ensure a certain dose range of therapeutic CO without increasing the level of CO-hemoglobin (57). CORM-2 or iCORM-2, at the concentrations used in the present study, have previously been demonstrated not to be toxic to mammalian cells *in vitro* and to mice *in vivo* (21,58). However, the safety of intravenous injection with CORM-2 *in vivo* has not been demonstrated satisfactorily. Therefore, a novel method of administration of CO requires further investigation.

In summary, the present study demonstrated that the application of CORM-2 attenuated the severity of sepsis and improved the function of the pancreas. The mechanism by which CORM-2-derived CO inhibited pro-inflammatory cytokines of the pancreas may be via inhibiting NF- $\kappa$ B activation. However, this was a preliminary study on the function of CORM-2 in pancreatic function in sepsis model mice and its mechanism of action. There remain numerous issues that require further investigation, for example, whether the effect of CORM-2 on NF- $\kappa$ B activation is direct or indirect; what will happen if NF- $\kappa$ B and/or p-I $\kappa$ B- $\alpha$  are blocked; and if NF- $\kappa$ B and/or p-I $\kappa$ B- $\alpha$  are blocked, will any reverse effect occur or not. Future studies will conduct in-depth research on these issues.

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## Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

BS designed the study. YL was responsible for data access and analysis and manuscript preparation. XW, XX and WQ collaborated to perform data analysis and interpret the results.

## Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Affiliated Hospital of Jiangsu University (Jiangsu, China).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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