

# Inhibition of glycogen synthase kinase-3 $\beta$ attenuates acute kidney injury in sodium taurocholate-induced severe acute pancreatitis in rats

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**Abstract.** The aim of the present study was to investigate the efficacy of 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8), the selective inhibitor of glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), on the development of acute kidney injury in an experimental model of sodium taurocholate-induced severe acute pancreatitis (SAP) in rats. The serum amylase, lipase, interleukin-1 $\beta$  and interleukin-6 levels, and the pancreatic pathological score were examined to determine the magnitude of pancreatitis injury. The serum creatinine and blood urea nitrogen levels, myeloperoxidase (MPO) activity and renal histological grading were measured to assess the magnitude of SAP-induced acute kidney injury. The activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) was examined using an immunohistochemistry assay. The expression of GSK-3 $\beta$ , phospho-GSK-3 $\beta$  (Ser9), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), intercellular adhesion molecule-1 (ICAM-1) and inducible nitric oxide synthase (iNOS) protein in the kidney was characterised using western blot analysis. TDZD-8 attenuated (i) serum amylase, lipase and renal dysfunction; (ii) the serum concentrations of proinflammatory cytokines; (iii) pancreatic and renal pathological injury;

(iv) renal MPO activity and (v) NF- $\kappa$ B activation and TNF- $\alpha$ , ICAM-1 and iNOS protein expression in the kidney. The results obtained in the present study suggest that the inhibition of GSK-3 $\beta$  attenuates renal disorders associated with SAP through the inhibition of NF- $\kappa$ B activation and the downregulation of the expression of proinflammatory cytokines, TNF- $\alpha$ , ICAM-1 and iNOS in rats. Blocking GSK-3 $\beta$  protein kinase activity may be a novel approach to the treatment of this inflammatory condition.

## Introduction

Severe acute pancreatitis (SAP) is an acute abdominal disease characterised by the development of systemic inflammatory and multiple organ failure syndromes (1,2). Acute renal failure (ARF) is a common severe complication of SAP. Once SAP occurs, ARF may induce the rapid progression of the disease and increases the risk of mortality in SAP patients (3,4). The exact mechanism of ARF that occurs in patients with SAP remains unclear and accumulating evidence indicates that the pathological sequelae of SAP and SAP-related organ failures are mediated by proinflammatory cytokines and adhesion molecules, including tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$ , IL-6, IL-10 and intercellular adhesion molecule-1 (ICAM-1) (5,6). The magnitude and duration of the systemic inflammatory response affects the development of tissue damage and multiple organ failure. Therefore, reducing the proinflammatory cytokines and adhesion molecule levels to inhibit the effect of inflammation may represent a valid strategy for the treatment of pancreatitis-associated kidney injury.

Glycogen synthase kinase (GSK)-3 is a serine-threonine protein kinase that participates in a multitude of cellular processes, including cell signalling, gene transcription, translation and cytoskeletal organisation, cell cycle progression and cell survival (7). Two isoforms have been isolated in mammals, GSK-3 $\alpha$  and GSK-3 $\beta$ . GSK-3 is constitutively active in cells and a wide variety of extracellular stimuli exert effects through the inhibition of GSK-3 activity (8,9). The phosphorylation of a specific serine residue (Ser21 in GSK-3 $\alpha$  and Ser9 in GSK-3 $\beta$ ) located in its N-terminal domain inhibits GSK-3 activity and therefore reduces the activity of this enzyme to alter cell function (7). GSK-3 $\beta$  has been implicated in the regulation of the

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**Abbreviations:** GSK-3 $\beta$ , glycogen synthase kinase-3 $\beta$ ; SAP, severe acute pancreatitis; STC, sodium taurocholate; TDZD-8, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione; IL, interleukin; MPO, myeloperoxidase; NF- $\kappa$ B, nuclear factor- $\kappa$ B; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; ICAM-1, intercellular adhesion molecule-1; iNOS, inducible nitric oxide synthase

**Key words:** severe acute pancreatitis, acute kidney injury, glycogen synthase kinase-3 $\beta$ , inflammation, cytokines

transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B). Hoefflich *et al* reported that GSK-3 $\beta$  knockout mice exhibited a similar phenotype to mice in which the gene for NF- $\kappa$ B subunit p65 and the inhibitor of I $\kappa$ B kinase- $\beta$  (IKK- $\beta$ , and hence NF- $\kappa$ B activation) has been deleted (10). Similar experimental models have confirmed that GSK-3 $\beta$  activity is closely correlated to the NF- $\kappa$ B activation pathway (11–13). Therefore, GSK-3 $\beta$  may have a key role in modulating the inflammatory response.

Accumulating studies have demonstrated that potent selective inhibitors of GSK-3 $\beta$  (TDZD-8, SB216763 and SB415286) alleviate the organ injury/dysfunction associated with endotoxemia and systemic inflammation (14–16). These findings provide support for the hypothesis that GSK-3 $\beta$  has an important role in the regulation of the inflammatory response. However, whether GSK-3 $\beta$  inhibition is effective in preventing acute kidney injury in SAP has not yet been elucidated. The present study hypothesised that GSK-3 $\beta$  inhibition suppresses the inflammation of acute kidney injury associated with SAP. Therefore, the aim of this study was to determine whether TDZD-8, a specific GSK-3 $\beta$  inhibitor, ameliorates the development of acute kidney injury in sodium taurocholate (STC)-induced SAP in rats.

## Materials and methods

**Animals.** Male SPF Wistar rats, weighing 200–250 g, were obtained from the Experimental Animals Center of the Hubei Academy of Medical Sciences (Wuhan, Hubei, China). The study was approved by the Ethics Committee of Wuhan University (Wuhan, Hubei, China). The experimental animal procedures were conducted in compliance with the EEC regulations (Official Journal of European Community L358 12/18/1986) and NIH standards (Guide for the Care and Use of Laboratory Animal, National Institutes of Health's publication 85-23, revised 1996). The animals were housed in a controlled environment and provided with standard rodent chow and water.

**Experimental groups.** The rats were divided into four experimental groups: (i) the SAP-vehicle group, in which rats were subjected to STC (Sigma-Aldrich, St. Louis, MO, USA) induced SAP (as described below) and administered an equivalent volume of the vehicle for TDZD-8 (10% DMSO solution, i.v.) at 30 min prior to the STC infusion (n=20); (ii) the SAP-TDZD-8 group, in which rats were subjected to STC-induced SAP (as described below) and administered TDZD-8 (no. T8325; 1 mg/kg, i.v.; Sigma-Aldrich) at 30 min prior to STC infusion (n=20); (iii) the sham-vehicle group, in which rats were treated with sham surgery (saline instead of STC) in combination with the administration of an equivalent volume of the vehicle for TDZD-8 (10% DMSO solution, i.v.) and observed for 12 h (n=20); (iv) the sham-TDZD-8 group, in which rats were treated with sham surgery (saline instead of STC) in combination with the administration of TDZD-8 (1 mg/kg, i.v.) and observed for 12 h (n=20).

All of the rats were sacrificed at 12 h following the induction of pancreatitis. The TDZD-8 dose used to reduce acute kidney injury was selected based on a previous study in which similar doses of TDZD-8 exerted protective effects in experimental models of the inflammatory response (14,17).

**Induction of SAP and tissue procurement.** Prior to the study, the rats were deprived of food, but allowed access to water. The rats were anaesthetised through an intraperitoneal injection of chloral hydrate (10%, 3 ml/kg). SAP was induced through the retrograde infusion of 5% STC solution (1 ml/kg) into the bile-pancreatic duct transduodenally using an angiocath with a standardised pressure-controlled infusion rate under laparotomy. Following infusion, the section of bile-pancreatic duct entering the duodenum was clipped using a non-invasive angiocath for 5 min. After assessing the bile leakage, the hole in the duodenum lateral wall was sutured. Subsequently, the abdomen was closed, and the rats were left to recover from the anaesthesia and allowed access to water.

The rats from each group were sacrificed through exsanguination at 12 h following the induction of pancreatitis. The blood samples were obtained through direct intracardiac puncture and the serum was stored at -20°C. Subsequently, the pancreas and kidney were immediately removed, harvested and fixed in 4% phosphate-buffered formaldehyde for histopathological observation. The remaining pancreatic and nephridial tissues were frozen in liquid nitrogen and stored at -80°C until further use.

**Histological examination.** Continuous sections of the paraffin-embedded tissue were obtained for pathological examination with hematoxylin-eosin staining. Two independent pathologists performed a blinded morphometric assessment under a light microscope (Olympus, Tokyo, Japan). The results were recorded on an evaluation form for pancreatic and kidney injury. The pancreatic histological assessment was determined by oedema, necrosis, haemorrhage and inflammation according to a previously described scale (18).

The histological assessment of kidney injury was performed using the point-counting method of Paller *et al* (19). For each kidney, 100 cortical tubules from at least 10 different areas were scored, and care was taken to avoid the repeated scoring of different convolutions of the same tubule. Higher scores represented more severe damage (maximum score per tubule was 10) and points were provided for the presence and extent of tubular epithelial cell flattening (1 point), brush border loss (1 point), cell membrane bleb formation (1 or 2 points), interstitial oedema (1 point), cytoplasmic vacuolisation (1 point), cell necrosis (1 or 2 points) and tubular lumen obstruction (1 or 2 points).

**Serum amylase, lipase, creatinine (Cr) and blood urea nitrogen (BUN) assays.** The serum samples were separated, and used for biochemical and cytokine measurements. Plasma amylase, lipase, Cr and BUN levels were measured with an automatic biochemistry analyser using standard techniques (Olympus).

**Measurement of cytokines.** IL-1 $\beta$  and IL-6 levels were examined in the plasma from SAP and sham-treated rats as previously described (20). The assay was performed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, Vienna, Austria). The absorbance was measured using an automated microplate reader and the concentrations were calculated according to the standard curve run on each assay plate.

**Myeloperoxidase (MPO) assay.** Neutrophil infiltration into the renal parenchyma was indirectly quantitated using an MPO

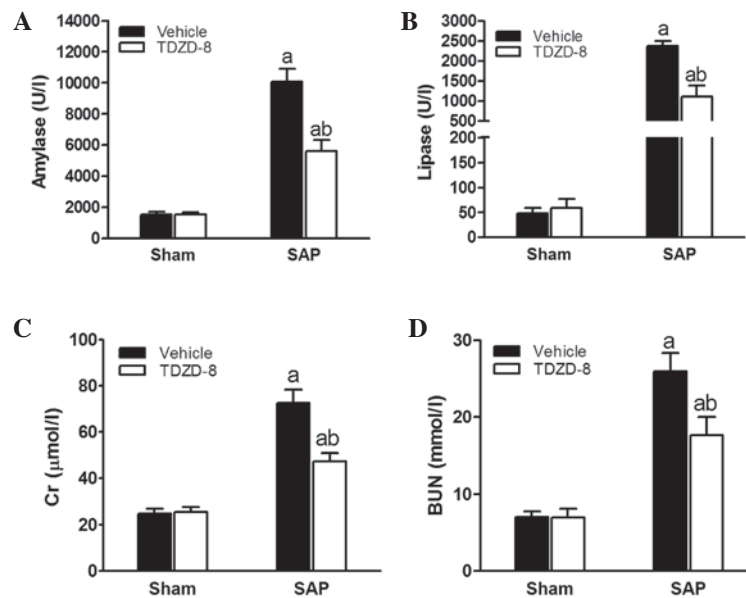


Figure 1. Effects of glycogen synthase kinase-3 $\beta$  inhibition on the amylase, lipase, Cr and BUN serum levels following SAP. TDZD-8 treatment significantly reduced the increase in (A) amylase, (B) lipase, (C) Cr and (D) BUN levels following SAP in rats. Each value represents the mean  $\pm$  standard deviation from 20 rats/group. <sup>a</sup>P<0.05 vs. the sham group; <sup>b</sup>P<0.05 vs. the SAP-vehicle group. TDZD-8, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione; Cr, creatinine; BUN, blood urea nitrogen; SAP, severe acute pancreatitis.

assay, as previously described for neutrophil elicitation (21). Renal cortical samples were weighed and homogenised in 1:19 (wt/vol) in ice-cold homogenate buffer. MPO activity was spectrophotometrically determined using a commercial assay kit according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The absorbance was measured at 460 nm.

**Immunohistochemical localisation of NF- $\kappa$ B p65.** Pancreatic and renal tissue was placed in a decalcifying solution for 24 h and 4  $\mu$ m sections were prepared from paraffin embedded tissues. Following deparaffinisation, 0.3% hydrogen peroxide was used to inactivate endogenous peroxidase activity. Non-specific adsorption was minimised following incubating the section in 5% normal goat serum in phosphate-buffered saline. Endogenous biotin and avidin binding sites were blocked using avidin and biotin, respectively. The sections were incubated overnight with a rabbit polyclonal anti-rat NF- $\kappa$ B p65 antibody (1:100 in PBS, v/v; Cell Signaling Technology, Inc., Danvers, MA, USA) in a moisture chamber. The sections were subsequently counterstained with hematoxylin. The negative control studies were performed in which PBS was used instead of the primary antibody.

**Western blot analysis.** Renal GSK-3 $\beta$ , phospho-GSK-3 $\beta$  (Ser9), TNF- $\alpha$ , ICAM-1 and iNOS levels at 12 h following STC injection were determined by western blot analysis. The proteins were extracted using the Nuclear-Cytosol Extraction kit (Appligen Technologies Inc., Beijing, China). The protein concentrations in the samples were determined using the Bradford method with bovine serum albumin as a standard. Briefly, equal amounts of protein samples were electrophoresed using 8 or 10% sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gels and subsequently transferred onto nitrocellulose membranes. The membrane was blocked with TBST buffer (TBS containing

5% non-fat dry milk and 0.1% Tween-20) at room temperature for 2 h and subsequently incubated with rabbit polyclonal anti-GSK-3 $\beta$  or anti-phospho-GSK-3 $\beta$  (Ser9) (Cell Signaling Technology, Inc.), anti-TNF- $\alpha$  (Abcam, Cambridge, MA, USA), anti-ICAM-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or anti-iNOS (Cell Signaling Technology, Inc.) antibodies overnight at 4°C. Following extensive rinsing with TBST, the blots were incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000; Pierce Biotechnology, Inc., Rockford, IL, USA) at room temperature for 1-2 h, developed using an ECL reagent (Millipore, Bedford, MA, USA) and captured on a light-sensitive imaging film (Kodak, Shanghai, China). The protein bands were quantified through densitometry (Quantity One 4.5.0 software; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

## Results

**Effects of GSK-3 $\beta$  inhibition on pancreatic injury and renal dysfunction in SAP.** As demonstrated in Fig. 1A and B, significant hyperamylasemia and hyperlipasemia developed following the induction of pancreatitis, suggesting that establishing the SAP model was successful. TDZD-8 reduced amylase and lipase at 12 h following pancreatitis compared with the SAP-vehicle group (P<0.05). The rats subjected to SAP were associated with an increase in Cr and BUN levels, indicating that the rats experienced aggravated renal dysfunction. The changes in renal function induced by SAP were significantly improved following TDZD-8 treatment at 12 h after the STC infusion (P<0.05; Fig. 1C and D). The sham-vehicle and sham-TDZD-8-treated rats demonstrated no elevation in the serum levels of amylase, lipase, Cr and BUN levels.

**Effects of GSK-3 $\beta$  inhibition on the degree of pancreatic and renal histopathology.** Representative histological sections



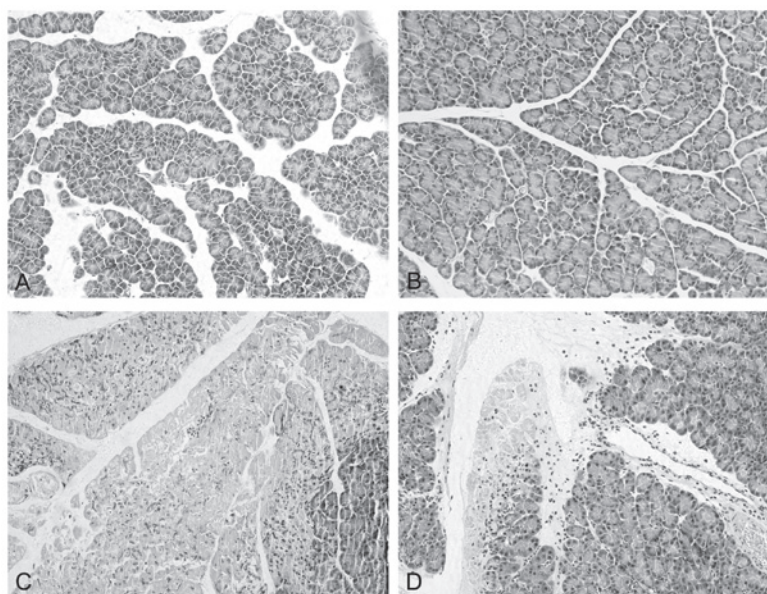


Figure 2. Morphological changes in the pancreas at 12 h following sodium taurocholate-induced severe acute pancreatitis. No histological alterations were observed in the pancreatic tissues obtained from (A) sham-vehicle and (B) sham-TDZD-8-treated rats. (D) TDZD-8 therapy significantly reduced the extent and severity of the histological signs of (C) pancreatic injury. This figure represents at least three experiments performed on different experimental days (original magnification, x200). TDZD-8, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione.

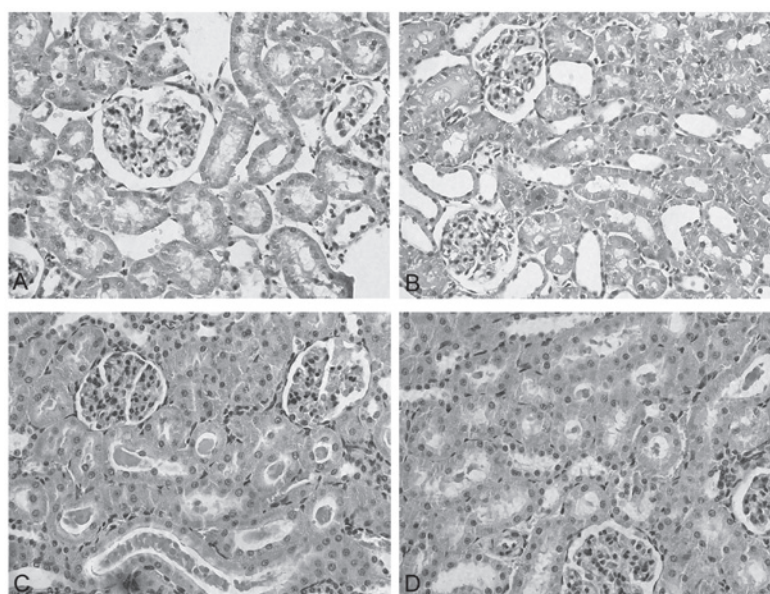


Figure 3. Morphological changes in the kidney at 12 h following SAP. (A and B) A normal renal tubule structure was observed in the sham-vehicle and sham-TDZD-8-treated rats; (C) SAP rats were treated with either vehicle or (D) TDZD-8. Hematoxylin-eosin staining of the renal tissue of SAP-vehicle rats demonstrated blurred cellular boundaries, tubular epithelial cell swelling and necrosis, tubular lumen stenosis and interstitial haemorrhage. The renal sections obtained from rats following TDZD-8 treatment demonstrated a significant reduction in the characteristic histological alterations. The figure represents at least three experiments performed on different experimental days (original magnification, x400). SAP, severe acute pancreatitis; TDZD-8, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione.

are demonstrated in Fig. 2A-D (pancreas) and Fig. 3A-D (kidney). STC-induced pancreatic damage was characterised by increased oedema, inflammatory cell infiltration, vacuolisation and necrosis. The sham rats demonstrated weak morphological evidence of pancreas injury, except for mild interstitial oedema. As revealed in Fig. 4A, there was a significant reduction in the pancreatic histological score in rats subjected to STC-induced pancreatitis and treated with TDZD-8 ( $P < 0.05$ ). In sham-vehicle and

sham-TDZD-8-treated rats, the histological features of the pancreas were typical of a normal architecture.

Compared with sham-vehicle and sham-TDZD-8-treated rats (Fig. 3A and B), rats subjected to pancreatitis for 12 h exhibited the recognised features of kidney injury, including characteristic histological signs of tubular damage. Tubular epithelial cell flattening, brush border loss, cell membrane bleb formation, interstitial oedema, cytoplasmic vacuolisation, cell necrosis and tubular lumen obstruction were observed

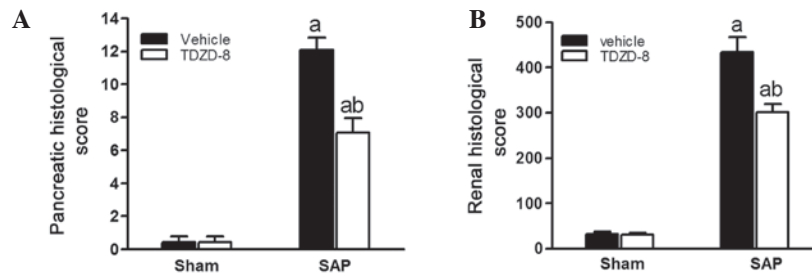


Figure 4. Comparison of the total pathological score in the (A) pancreas and (B) kidney. <sup>a</sup>P<0.05 vs. the sham group; <sup>b</sup>P<0.05 vs. the vehicle-treated SAP group. SAP, severe acute pancreatitis; TDZD-8, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione.

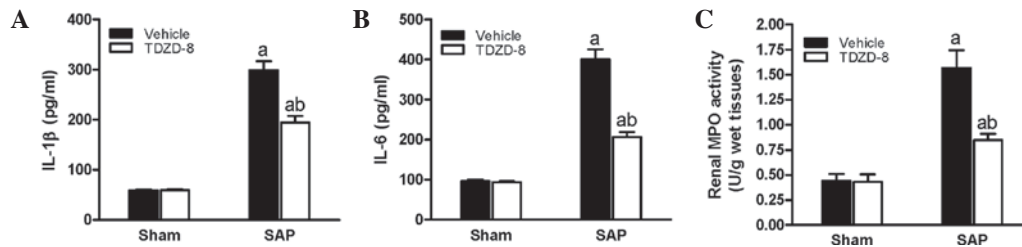


Figure 5. Effects of GSK-3β inhibition on proinflammatory cytokine production and renal neutrophil infiltration following SAP. (A and B) The rats demonstrated a significant increase in the plasma levels of IL-1β and IL-6 at 12 h following SAP. (A and B) IL-1β and IL-6 plasma levels were significantly reduced following TDZD-8 treatment. (C) Treatment with TDZD-8 significantly reduced the STC-induced increase of MPO activity in the kidney. The data represent the mean ± standard deviation from 20 rats/group. <sup>a</sup>P<0.05 vs. the sham group; <sup>b</sup>P<0.05 vs. the vehicle-treated SAP group. SAP, severe acute pancreatitis; TDZD-8, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione; IL-1β, interleukin-1β; IL-6, interleukin-6; MPO, myeloperoxidase.

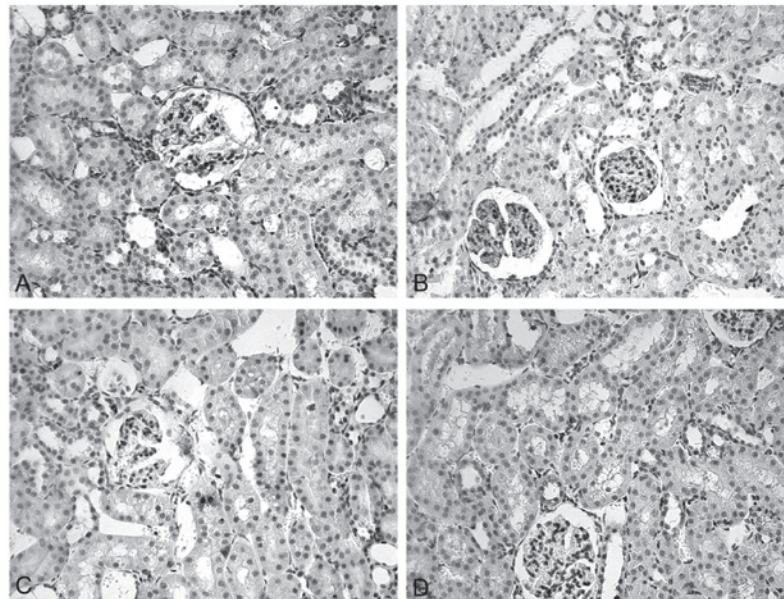


Figure 6. Immunohistochemical localisation of NF-κB p65 in the kidney. Weak immunoreactivity in the cytoplasm was observed in renal sections obtained from (A) sham-vehicle and (B) sham-TDZD-8-treated rats. (C) By contrast, intense positive staining in the nucleus was observed in the renal sections obtained from vehicle-treated severe acute pancreatitis rats. (D) The intensity of the positive staining in the nucleus for NF-κB p65 was markedly reduced after the administration of TDZD-8. The figures represent at least three experiments performed on different experimental days (original magnification, x400). NF-κB, nuclear factor-κB; TDZD-8, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione.

(P<0.05; Fig. 3C). The kidneys obtained from rats treated with TDZD-8 (Fig. 3D) demonstrated reduced histological features and pathological grading of kidney injury compared with the SAP-vehicle rats (P<0.05; Fig. 4B)

*Effects of GSK-3β inhibition on IL-1β and IL-6 production, and renal neutrophil infiltration following SAP.* To determine

whether GSK-3β inhibition modulates the inflammatory process through the regulation of IL-1β and IL-6, the plasma levels of IL-1β and IL-6 were analysed. A substantial increase in IL-1β and IL-6 formation was observed (Fig. 5A and B). By contrast, the plasma and pancreas levels of IL-1β and IL-6 were significantly reduced in the animals treated with TDZD-8 (P<0.05). No elevation in IL-1β and IL-6 plasma



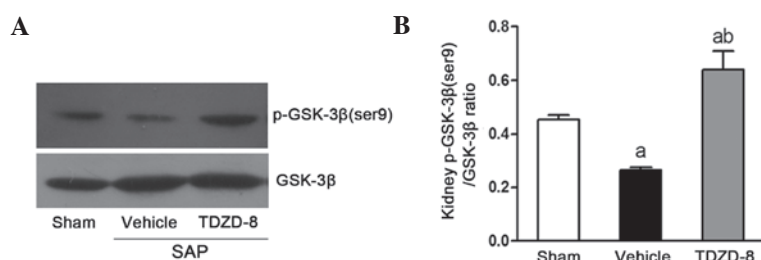


Figure 7. (A) Western blotting to measure the phospho-GSK-3 $\beta$  (Ser9) in renal tissue at 12 h following SAP; (B) densitometric analysis of the bands for the phospho-GSK-3 $\beta$  (Ser9) ratio. TDZD-8 treatment induced a significant increase in phospho-GSK-3 $\beta$  (Ser9). The results are expressed as a fold-increase over sham in at least three independent experiments. <sup>a</sup>P<0.05 vs. the sham group; <sup>b</sup>P<0.05 vs. the vehicle-treated SAP group. SAP, severe acute pancreatitis; TDZD-8, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione; GSK-3 $\beta$ , glycogen synthase kinase-3 $\beta$ .

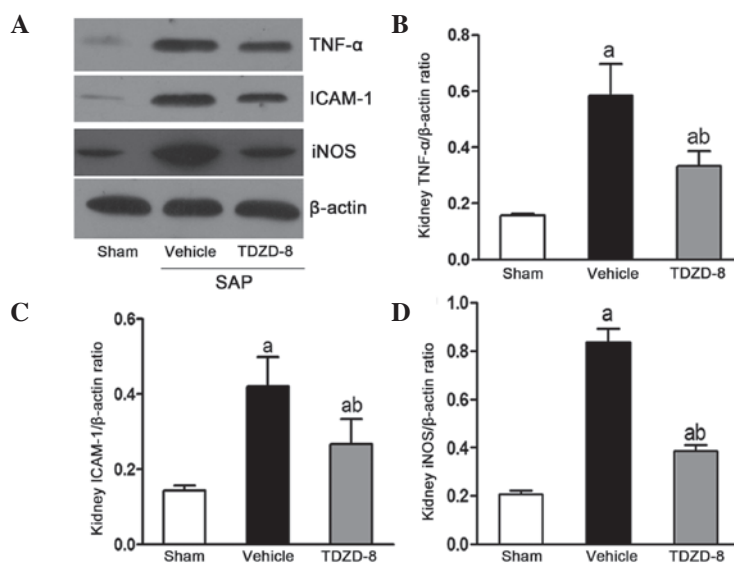


Figure 8. Western blotting to measure the activation of TNF- $\alpha$ , ICAM-1 and iNOS in renal tissue at 12 h following SAP. SAP markedly increased the tissue concentration of TNF- $\alpha$ , ICAM-1 and iNOS compared with the sham rats. TDZD-8 treatment effectively inhibited TNF- $\alpha$ , ICAM-1 and iNOS activity in the kidney following SAP. (A) A representative image and densitometric analysis of the bands for the (B) TNF- $\alpha$ , (C) ICAM-1 and (D) iNOS ratios. The results are expressed as a fold-increase over sham in at least three independent experiments. <sup>a</sup>P<0.05 vs. the sham group; <sup>b</sup>P<0.05 vs. the vehicle-treated SAP group. SAP, severe acute pancreatitis; TDZD-8, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione; TNF- $\alpha$ , tumour necrosis factor- $\alpha$ ; ICAM-1, intercellular adhesion molecule-1; iNOS, inducible nitric oxide synthase.

levels was observed in sham-vehicle and sham-TDZD-8 treated rats. The renal MPO activity following SAP was measured to examine the infiltration of neutrophils into the kidney. MPO activity was significantly elevated at 12 h following STC infusion in the vehicle-treated rats compared with the sham group (P<0.05). The MPO activity in the renal tissue from SAP rats treated with TDZD-8 was significantly reduced (P<0.05; Fig. 5C)

**Effects of GSK-3 $\beta$  inhibition on NF- $\kappa$ B p65 subunit expression in the kidney following SAP.** An immunohistochemical assay was used to assess the localisation of NF- $\kappa$ B p65 subunit expression. NF- $\kappa$ B p65 expression was primarily detected in the cytoplasm of cells from renal tissue. Immunostaining for NF- $\kappa$ B p65 (Fig. 6C) was markedly expressed in the nucleus following the administration of STC compared with that in the sham-control rats (Fig. 6A). Positive immunostaining for NF- $\kappa$ B p65 was significantly reduced in rats treated with TDZD-8 (Fig. 6D). As demonstrated in Fig. 6B, weak immunoreactivity was primarily observed in the cytoplasm in sham-TDZD-8-treated rats.

**Effects of inhibition of GSK-3 $\beta$  on the phosphorylation of GSK-3 $\beta$  (Ser9) in the kidney following SAP.** To obtain a mechanistic insight into the effects of the GSK-3 $\beta$  inhibitor used in the present study, a western blot analysis of the phosphorylation of GSK-3 $\beta$  (Ser9) was performed (Fig. 7A). As demonstrated in Fig. 7A, the phosphorylation of GSK-3 $\beta$  (Ser9) in renal tissue was significantly increased in the TDZD-8-treated SAP group than in the vehicle-treated SAP group, consistent with the inactivation of the kinase (P<0.05; Fig. 7B).

**Inhibition of GSK-3 $\beta$  reduced activation of TNF- $\alpha$ , ICAM-1 and iNOS in the kidney following SAP.** Taken together, these observations indicated that the degree of pancreatitis and associated kidney injury was gradually aggravated. Therefore, the effects of TDZD-8 treatment on TNF- $\alpha$ , ICAM-1 and iNOS expression in the kidney following 12 h were then examined. As demonstrated in Fig. 8, TNF- $\alpha$ , ICAM-1 and iNOS expression in renal tissue was significantly increased in the vehicle-treated SAP group (P<0.05). In addition, the inhibition of GSK-3 $\beta$  significantly decreased the activation of TNF- $\alpha$ , ICAM-1 and iNOS expression in the kidney following SAP (P<0.05).

## Discussion

The inhibition of GSK-3 $\beta$  has been successfully used in basic studies and animal models of a number of different diseases, including non-septic shock, organ injury/dysfunction through endotoxemia, inflammation and ischemia-reperfusion injury (12,14,16,22,23). However, the mechanism through which GSK-3 $\beta$  inhibition provides renal protection in SAP remains unclear. Therefore, it was hypothesized that GSK-3 $\beta$  may contribute, in part, to acute kidney injury following SAP.

These data demonstrated that TDZD-8 treatment exerts an important protective effect against acute kidney injury in SAP. Therefore, the present study provides evidence that TDZD-8 attenuates (i) serum amylase and lipase levels; (ii) serum Cr and BUN levels; (iii) renal neutrophil infiltration; (iv) NF- $\kappa$ B activation; (v) proinflammatory cytokine IL-1 $\beta$  and IL-6 production; (vi) TNF- $\alpha$ , ICAM-1 and iNOS upregulation in the renal cortex and (vii) the degree of pancreatic and kidney injury. These findings are consistent with the hypothesis that TDZD-8 ameliorates the degree of SAP and associated kidney injury in rats.

Inhibiting GSK-3 $\beta$  has demonstrated promising efficacy in animal models of several inflammatory diseases, including AP (11,22). However, the mechanism by which TDZD-8 protects the kidney against the inflammatory injury following SAP remains unknown. The transcription factor NF- $\kappa$ B has a critical role in the regulation of numerous genes responsible for the generation of mediators or proteins, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, ICAM-1 and iNOS (17,20,24,25), in several inflammatory diseases, including AP. Various therapeutic strategies, including antioxidants (26,27), anti-inflammatory agents (28,29) or pharmacological inhibition (30), that target the NF- $\kappa$ B signalling pathway, have demonstrated beneficial effects in experimental AP models. Accumulative evidence has revealed that the NF- $\kappa$ B p65 subunit, particularly the COOH-terminus of p65 at Ser536, is phosphorylated through GSK-3 $\beta$  to enhance the transcriptional responses of NF- $\kappa$ B (31,32). Therefore, the pharmacological inhibition of GSK-3 $\beta$  may inhibit NF- $\kappa$ B transcriptional activity and have anti-inflammatory effects. Immunohistochemical analysis revealed that NF- $\kappa$ B translocates from the cytoplasm to the nucleus upon activation in renal tissues, and the significant inhibition of NF- $\kappa$ B p65 was observed in the renal cortex of rats following TDZD-8 treatment, confirming that the inhibition of GSK-3 $\beta$  suppressed NF- $\kappa$ B signalling in the inflamed kidney during SAP. These results also directly demonstrate that the dosing regimen of the TDZD-8 is sufficient to suppress GSK-3 $\beta$  in the kidney.

There is evidence that inhibitors of GSK-3 $\beta$  reduce the activation of NF- $\kappa$ B by preventing the association of the transcriptional co-activator CBP with p65, which potentially reduces the formation of pro-inflammatory cytokines (e.g., IL-1 $\beta$  and IL-6) and enhances the formation of anti-inflammatory cytokines (e.g., IL-10) (33). Cytokines, including IL-1 $\beta$  and IL-6, propagate the extension of local or systemic inflammation, including SAP. In the present study, these data also demonstrated an increase in the expression of these cytokines following SAP. However, their serum levels were reduced in rats treated with TDZD-8. These findings are consistent with those obtained from previous studies revealing that IL-1 $\beta$  and IL-6 serum levels in cerulein-induced AP mice were significantly reduced following

treatment with chemically distinct GSK-3 $\beta$  inhibitors (11). Therefore, it is hypothesized that the inhibition of IL-1 $\beta$  and IL-6 formation observed in the TDZD-8-treated rats in the present study may reflect the GSK-3 $\beta$ -mediated inhibition of NF- $\kappa$ B activation.

Various studies have evidently demonstrated that the inhibition of TNF- $\alpha$  formation significantly prevents the development of inflammation in SAP (34). In addition, Takada *et al* revealed a fundamental role for GSK-3 $\beta$  in the TNF-mediated activation of NF- $\kappa$ B and NF- $\kappa$ B-regulated gene products (35). It was observed that TNF- $\alpha$  expression was markedly increased in the kidney following SAP. Treatment with TDZD-8 eliminated the expression of TNF- $\alpha$ , suggesting that the inhibition of GSK-3 $\beta$  downregulates NF- $\kappa$ B activation, followed by TNF- $\alpha$  downregulation.

A number of studies have revealed that neutrophil sequestration has a critical role in the development and full manifestation of AP (36,37). Neutrophils infiltrate injured tissues through the production of reactive oxygen metabolites, granule enzymes and cytokines that further amplify the inflammatory response (37,38). Adhesion molecules, including ICAM-1, have a deleterious role in the course of the disease through neutrophil recruitment to the pancreas and distant organs (20,37,39). In the present study, it was observed that ICAM-1 expression is markedly increased in the kidney following SAP. Treatment with TDZD-8 eliminated the expression of ICAM-1, and this effect was associated with the reduction of leukocyte infiltration (detected using the specific granulocyte enzyme MPO) and the moderation of the tissue damage (examined by histological examination). These results demonstrate that the inhibition of the GSK-3 $\beta$  pathway suppresses neutrophil recruitment and supports the concept of a therapeutic strategy directed against leukocyte infiltration in multiple organs and kidney injury.

Enhanced formation of NO by iNOS may contribute to the inflammatory process associated with acute pancreatitis (11,39,40). The results obtained in the present study demonstrated that iNOS expression in renal tissues was increased following SAP, which markedly indicates that iNOS is involved in the development of SAP-associated acute kidney injury. However, TDZD-8 attenuates iNOS expression in the kidney in STC-induced SAP rats. The mononuclear phagocyte system is the main source of NO in SAP (41). Therefore, low-level iNOS expression produces adequate NO levels under normal physiological conditions. However, when SAP is induced, the excessive NO may cause microvascular endothelium cell injury and dysfunction, thereby increasing vascular permeability. However, the overproduction of NO may generate oxygen-free radicals, which increase cytotoxic effects and exacerbate kidney injury. The reduced iNOS expression following TDZD-8 treatment may contribute to the attenuation of nitrotyrosine formation and lipid peroxidation in the kidney in STC-induced SAP animals. These results suggest that the inhibition of the GSK-3 $\beta$  pathway attenuates iNOS expression.

In the present study, a mechanism for the renal-protective effects of GSK-3 $\beta$  inhibition was proposed. The results revealed that the inhibition of GSK-3 $\beta$  reduced the renal inflammatory changes associated with the SAP. The mechanisms for the beneficial effects observed following GSK-3 $\beta$  inhibition were partially dependent on the reduction of (i) NF- $\kappa$ B activation; (ii) the formation of proinflammatory cytokines (IL-1 $\beta$  and

IL-6); (iii) the expression of TNF- $\alpha$ , ICAM-1 and iNOS; and (iv) neutrophil infiltration. Therefore, the findings presented in this study may stimulate interest in the development of more potent and specific GSK-3 $\beta$  inhibitors for the prevention and treatment of SAP, and the associated kidney injury.

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