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Protective Effects of Sivelestat in a Caerulein-Induced Rat Acute Pancreatitis Model

Jun Cao[1,2] and Quanyan Liu[1]

Abstract—In the present study, we investigated the protective effects of sivelestat on acute pancreatitis (AP) in a rat model. Sivelestat is a specific neutrophil elastase inhibitor, which has been developed in Japan in 1991. Varying doses of sivelestat in normal saline were infused continuously in sivelestat-treated groups through osmotic pumps. Blood and pancreas samples were collected for serological and histopathological studies, and ten rats in each group were taken for survival observation. Increasing doses of sivelestat inhibits the expression of lipase, amylase, corticosterone, IL-1β, TNF-α, and nuclear factor–κB. Furthermore, sivelestat reduces the inflammatory cells infiltration, histological damage, and mortality rate. Meanwhile, the total antioxidant power and serum level of IL-4 in high-dose sivelestat-treated groups were increased. Our findings suggest that the increasing doses of sivelestat protect against caerulein-induced AP in rats, and this protection is possibly associated with the anti-inflammatory ability of sivelestat.

KEY WORDS: sivelestat; acute pancreatitis; caerulein; anti-inflammation.

INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease of pancreas. The incidence of AP has increased in the past two decades [1, 2]. This disease is mild and resolves itself without serious complications in 80 % of patients, but it has some complications and substantial mortality in up to 20 % of patients [3]. AP involves a complex cascade of events, and its molecular mechanisms have not been completely elucidated yet. Most investigators believe that AP is caused by the unregulated activation of trypsin within pancreatic acinar cells, and enzyme activation within the pancreas leads to the auto-digestion and local inflammation [4]. The pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-8, and platelet activating factor) and the anti-inflammatory cytokines (IL-10, TNF-soluble receptors, and IL-1 receptor antagonist) have been shown to be intimately involved in the inflammatory response to AP [5]. Recent studies show that the severity of AP appears to be determined by the magnitude of the resultant systemic inflammatory response [6]. About half of deaths in AP occur within the first week; these patients develop an exaggerated systemic inflammatory response syndrome (SIRS) with the development of multiple organ dysfunction syndrome and death [7]. Thus, nowadays, the anti-inflammatory therapy has been considered as an important part in the clinical management of AP.

Sivelestat, a specific neutrophil elastase (NE) inhibitor, has been developed in Japan in 1991 and used clinically for the treatment of acute lung injury [8]. This agent is characterized as having no effects on proteases other than NE. The benefits of sivelestat on ischemia–reperfusion, endothelial cell injuries, and tissue injuries associated with systemic inflammatory response have been demonstrated in several experiments [9–11]. Pro-inflammatory cytokines play a central role in the pathogenesis of acute inflammatory response, and numerous studies suggest that sivelestat treatment could reduce inflammatory mediators [12, 13]. It is reported that sivelestat reduces the levels of pro-inflammatory cytokines by inhibiting nuclear factor–κB (NF–κB) activation [14]. Previous study also demonstrated a great therapeutic potential for sivelestat in managing AP-associated lung injury [15]. However, the authors did not systematically investigate the anti-inflammatory effect of sivelestat in that study. For this reason, our

[1] General Surgery Department of Zhongnan Hospital, Wuhan University, 169 Donghu Road, Wuchang, Wuhan, Hubei 430071, China
[2] To whom correspondence should be addressed at General Surgery Department of Zhongnan Hospital, Wuhan University, 169 Donghu Road, Wuchang, Wuhan, Hubei 430071, China. E-mail: juncaodoctor@yahoo.cn

study was aimed at assessing the effect of exogenous administration of sivelestat during the induction process of caerulein-induced AP in rats.

MATERIALS AND METHODS

Animal Model and Study Design

One hundred and forty, male, specific pathogen-free Sprague–Dawley rats weighing 180~200 g were obtained from the Center for Animal Experiment/Animal Biosafety Level III laboratory (ABSL-III lab) of Wuhan University in China. All the animal experiments were conducted in accordance with the guidelines of Animal Use and Care Committee of Wuhan University. Rats were housed individually in cages on a 12-h dark/light cycle and drinking water *ad libitum*. Experimental room conditions were controlled for temperature 26±2 °C and relative humidity 50±20 %.

Rats were randomly divided into control group (n=20), caerulein group (n=20), and caerulein + sivelestat group (five subgroups, n=20 in each subgroup). Rats were made to fast overnight before the induction of acute pancreatitis. As previously described [16], four subcutaneous injections of caerulein (Sigma-Aldrich, St. Louis, MO; 20 μg kg^{-1} body weight) or vehicles (normal saline) were applied consecutively at 2-h intervals. Varying doses of sivelestat (Ono Pharmaceutical Co. Ltd., Osaka, Japan) in normal saline were infused continuously at a rate of 0.5 μl/h for 24 h through osmotic pumps since the last injection of caerulein. The concentrations of sivelestat were 0.1, 1.0, 10, 100, and 1,000 μg/ml in the subgroups 1–5, respectively. Ten rats in each group were sacrificed with an overdose of pentobarbital 24 h after the last injection of caerulein for blood and pancreas samples collection. Another ten rats in each group were taken for a 14-day survival observation.

Measurement of Amylase, Lipase, Cytokines, Corticosterone and Total Antioxidant Power in Serum

Blood samples were centrifuged at 15,000 rpm under 4 °C and then stored in a −80 °C fridge for next analysis. Serum amylase and lipase were measured with a TBA-2000FR System (TOSIBA, Tokyo, Japan). The serum levels of cytokines and corticosterone were detected using a commercial enzyme-linked immunosorbent assay kits (Boster, Wuhan, China), according to the manufacturers' protocols. We also measured the serum level of total antioxidant power with a colorimetric assay kit (Genmed Scientifics Inc., USA).

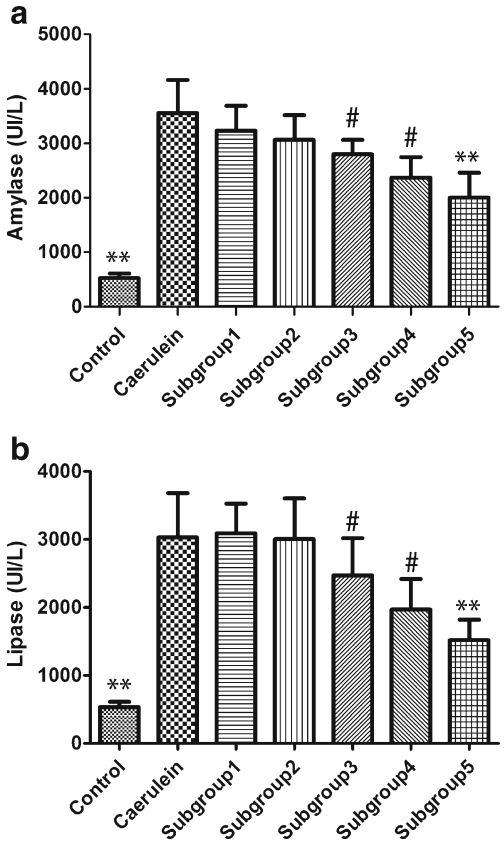

Fig. 1. Serum levels of amylase and lipase. *Subgroup* means sivelestat subgroup; compared with other groups, $^{**}p<0.01$; compared with caerulein group, $^{\#}p<0.05$, $^{\#\#}p<0.01$.

Evaluation of Histological Damage and Inflammatory Cell Infiltration

Pancreas tissues were collected for morphological evaluation. The specimens were fixed in 10 % formalin, embedded in paraffin, cut into sections 4-microns in thickness, and stained with hematoxylin and eosin (H&E). Five observation fields under light microscope (Eclipse-600, Nikon) were randomly selected in each specimen and were blindly evaluated by two pathologists after randomization. All specimens were photographed using a digital camera (450D, Canon, Japan) and scored through the method of Schmidt *et al*. [17]. Additionally, inflammatory cells (PMN leukocytes, lymphocytes, and monocytes) were quantified by analyzing at least five random fields containing inter- or

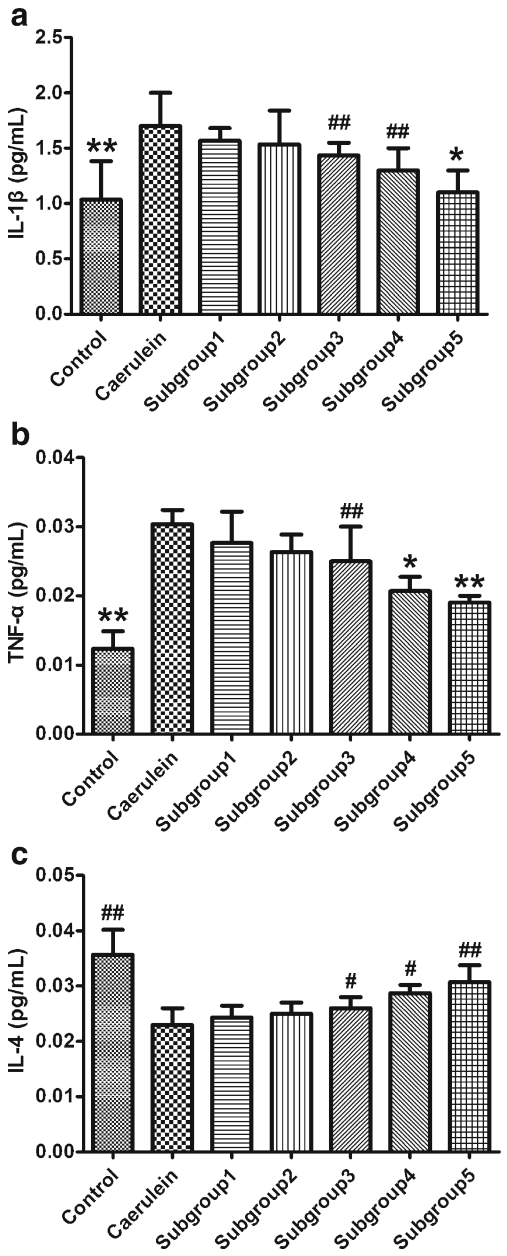

Fig. 2. Serum levels of cytokines. *Subgroup* means sivelestat subgroup; compared with other groups, $^{**}p<0.01$; compared with caerulein group, $^{\#}p<0.05$, $^{\#\#}p<0.01$.

intralobular blood vessels at a magnification of 400×. The numbers of inflammatory cells within the vascular lumen, marginated/adherent to the endothelium, and in the perivascular pancreatic tissue were counted. The inflammatory infiltrate is presented as the mean number of inflammatory cells per high power field. Myeloperoxidase (MPO) was measured as described by Bradley *et al.* [18].

WESTERN BLOT ASSAY

The NF–κB p65 expression in pancreatic tissue was determined using Western blot assay and carried out in duplicates. Proteins were extracted using protein extraction reagent (Sigma, USA), following a protocol provided by the manufacturer. Western blot assay was performed as described previously [19]. Briefly, protein samples (50 μ g) were resolved by 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis and membranes; primary antibody was added (Abcam, UK) followed by intubation for 2 h at room temperature (RT) and staying overnight at 4 °C; TBST was used to wash membrane four times with 10 min/time; secondary antibody was added (Boster, Wuhan, China) and combined with horse radish peroxidase (1:5,000) for incubation followed by jiggle at RT for 2 h, membrane washing, imaging, and exposure. The protein bands were normalized with GAPDH, and all blots were quantified with Software Quantity One (Bio Rad).

Survival Observation

In each group, ten rats were taken for survival observation, which lasted for 14 days. Rats were housed individually in cages in a same experimental room with adequate temperature and humidity. Experimenters checked and noted the physical conditions of rats every 6 h.

Statistical Analysis

Data are expressed as mean±SD. The data were processed by the statistical analysis software SPSS version 16.0 (SPSS Inc., Chicago, IL). Comparison of several means was performed using one-way and repeated measure two-way analysis of variance followed by the Tukey–Kramer test to identify significant difference between groups. The Kaplan–Meier method was applied for survival analysis and comparison between groups was performed using the log-rank test. All *P* values were two-tailed, and a *P* value of less than 0.05 was considered significant.

RESULTS

Serum Cytokines and Enzymes Expression

The serum levels of the pancreatic enzymes amylase and lipase and cytokines are shown in Figs. 1 and 2. Compared with the control group, the expression levels of amylase and lipase significantly increased in caerulein

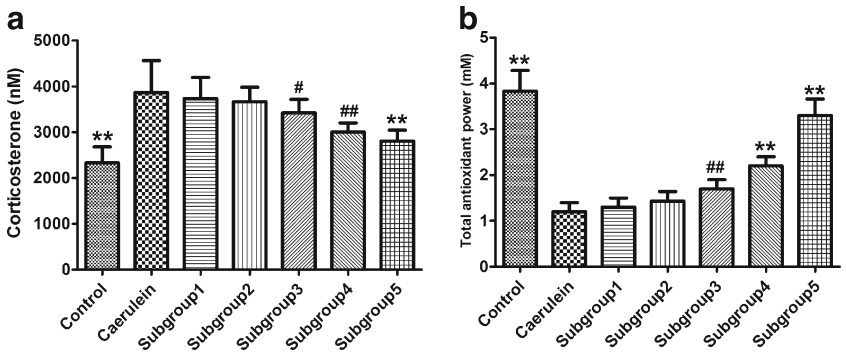

Fig. 3. Serum levels of corticosterone and total antioxidant power. *Subgroup* means sivelestat subgroup; compared with other groups, $^{**}p<0.01$; compared with caerulein group, $^{\#}p<0.05$, $^{\#\#}p<0.01$.

group ($P=0.001$). In the sivelestat subgroups 1 and 2, the serum enzymes levels were similar with that in the caerulein group. However, with increasing doses of sivelestat in different subgroups, the serum levels of amylase and lipase progressively decreased. The enzyme levels in the sivelestat subgroup 5 were significantly lower than that in other experimental groups ($P<0.05$, respectively). Serum levels of IL-1β and TNF-α significantly increased in the caerulein group compared with the control group ($P=0.004$), while the serum levels of anti-inflammatory cytokine IL-4 was significantly reduced due to the administration of caerulein. Compared with the caerulein group, the IL-1β and TNF-α levels in the sivelestat subgroups 1 and 2 insignificantly decreased. However, sivelestat significantly inhibited the secretion of pro-inflammatory cytokines IL-1β and TNF-α in the sivelestat subgroups 3, 4, and 5. In addition, this inhibitory effect was enhanced with the increasing doses of sivelestat. In contrast, the serum level of IL-4 in the sivelestat subgroups 3, 4, and 5 was significantly higher than that in the caerulein group ($P<0.05$, respectively).

Serum levels of Corticosterone and Total Antioxidant Power

Serum activities of total antioxidant power (TAP) and corticosterone present in rats with caerulein-induced acute pancreatitis are shown in Fig. 3. Compared with the control group, the concentration of corticosterone was significantly increased by the caerulein treatment. Likewise, TAP levels were significantly decreased due to the administration of the caerulein. Nevertheless, sivelestat treatment with higher doses (subgroups 3, 4, and 5) significantly reverted TAP and corticosterone expressions to basal levels.

Evaluation of Histological Damage and Inflammatory Cell Infiltration

Histological evaluation was used to investigate if sivelestat attenuated caerulein-induced pancreas damage. Light microscope observations of H&E-stained sections revealed the presence of edema, acinar necrosis, hemorrhage, fat necrosis, inflammation, and perivascular infiltrate (Fig. 4). Caerulein group had significantly higher mean scores than did other groups, except the sivelestat subgroup 1 ($P=0.001$ to 0.10, respectively). Sivelestat subgroups 2 and 3 demonstrated moderate edema and occasional single-cell necrosis, frequently with moderate necrosis, but no hemorrhage. With increasing doses of sivelestat, the tissue damage has been significantly attenuated according to the variable scores. Sivelestat subgroups 4 and 5 present only a mild edema, little acinar necrosis, and inflammation. Total histological scores in the subgroup 5 were significantly lower than that in the caerulein group ($P<0.01$). Determination of the number of infiltrating cells revealed that the number of PMN leukocytes was significantly reduced when the animals were treated with high-dose sivelestat. This was also confirmed by the evaluation of MPO concentrations of the pancreatic tissues, which have been shown to be an accurate marker for the infiltration of PMN leukocytes (Fig. 5).

NF–KB EXPRESSION IN PANCREATIC TISSUES

Compared with the control group, the expression of NF–κB p65 in the caerulein group was significantly higher ($P<0.01$). Compared with the caerulein group, treatment with low dose of sivelestat in subgroups 1 and 2 has no significant impact on the expression of NF–κB

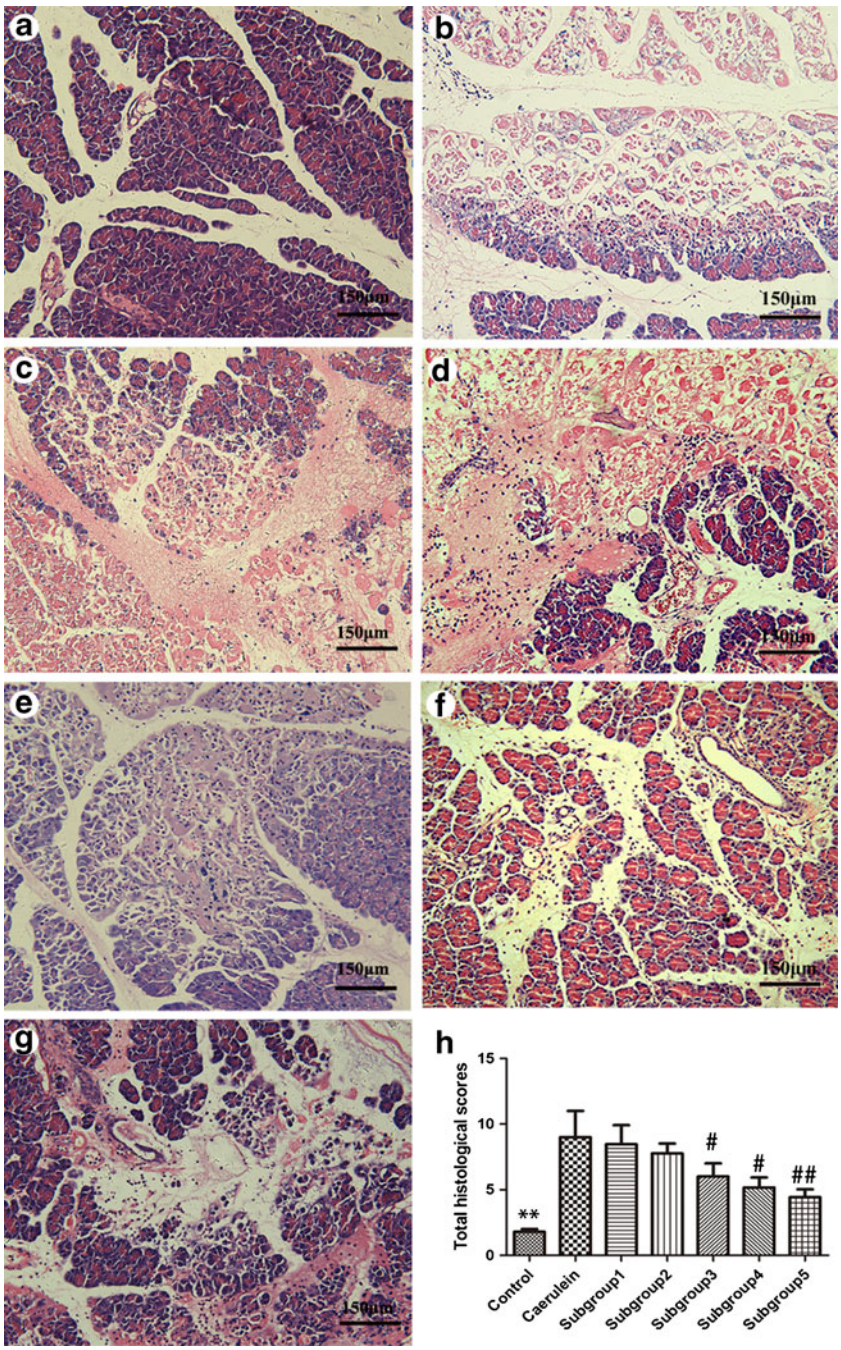

Fig. 4. Light microscope observations of H&E-stained sections (×200). **a** Control group; **b** caerulein group; **c**-**g** sivelestat subgroups 1–5, respectively; **h** total histological scores; sections present varying degrees of edema, acinar necrosis, hemorrhage and fat necrosis, inflammation and perivascular infiltrate. Compared with other groups, $^{**}p<0.01$; compared with caerulein group, $^{\#}p<0.05$, $^{\#\#}p<0.01$.

p65. With increasing dose of sivelestat, the expression of NF−κB p65 has been inhibited. The level of NF−κB p65 in the subgroups 3, 4, and 5 was significantly lower than that in the caerulein group (respectively, $P<0.01$) (Fig. 6).

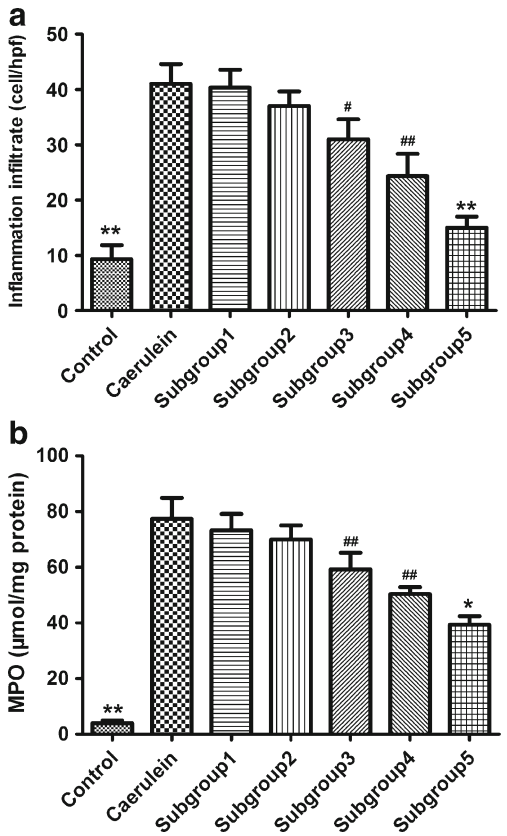

Fig. 5. Evaluation of inflammatory cells infiltration. **a** Amount of inflammatory infiltrating cells per high power field (hpf); **b** Concentration of MPO at 24 h after the last injection of caerulein. *Subgroup* means sivelestat subgroup; compared with other groups, $^{*}p<0.05$, $^{**}p<0.01$; compared with caerulein group, $^{\#}p<0.05$, $^{\#\#}p<0.01$.

Survival Observation Results

Compared with the control group, the mortality rate at 14 days in the caerulein group (mortality rate of 80 %, eight of ten rats), subgroup 1 (mortality rate of 70 %, seven of ten rats) and subgroup 2 (mortality rate of 70 %, seven of ten rats) was significantly higher (respectively, $P<0.05$). With increasing doses of sivelestat, more rats survived in the sivelestat-treated groups. The mortality rate of the subgroup 3 (40 %, four of ten rats), subgroup 4 (20 %, two of ten rats), and subgroup 5 (20 %, two of ten rats) was significantly lower than that in the caerulein group and the subgroups 1 and 2(respectively, $P<0.05$). In each group, most of the rats died within the first week after the induction of AP. In the control group, all rats survived (Fig. 7).

DISCUSSION

In this study, we used a caerulein-induced rat acute pancreatitis model to investigate the protective effects of sivelestat. Our results demonstrated that sivelestat inhibited the production of pro-inflammatory cytokines and serum levels of enzymes (lipase and amylase) and corticosterone in caerulein-induced rat acute pancreatitis. The treatment with sivelestat also caused the serum level of TAP significantly revert. In addition, rats received sivelestat treatment showed a slighter pancreas histological damage and higher survival rate in 14 days.

AP is an inflammatory disease with wide clinical variations, which may present sepsis, multiple organ failure, and even death [20]. Currently, extensive research efforts are focusing on investigating the pathophysiological mechanism of this disease. Many theories have been proposed attempting to explain the mechanisms underlying AP [21]. However, no ideal theories on the pathogenesis of AP are available at present. Although controversial, most investigators believe that AP is caused by the unregulated activation of trypsin within pancreatic acinar cells [4]. During AP, amylase and lipase are released from acinar cells, and their concentration in the serum is used to confirm diagnosis [22]. In this study, these two enzymes significantly increased in the caerulein-treated rats, and their concentrations exceeding three times the normal upper limit support the successful establishment of AP. Importantly, our results showed that sivelestat was able to cause lipase and amylase serum concentrations to revert to basal levels, that is, to reduce uncontrolled liberation of pancreatic enzymes to bloodstream, which suggests that sivelestat has the potential to inhibit pancreatic injury. In mild form of AP, besides the aetiological treatment, therapy is supportive. By contrast, severe episodes need management by a multidisciplinary team. However, despite efforts to start an appropriate treatment, mortality rate of severe acute pancreatitis has not substantially changed during the past decades. A previous study demonstrated that the duration of organ failure is a powerful marker of subsequent poor outcome in AP [23]. Sivelestat could prevent organ failure by inhibiting an increase in vascular permeability, as well as reducing production of cytokines and reactive oxygen species [24]. Our study showed a lower mortality and slighter histological damage in sivelestat-treated groups, which was possibly associated with these characteristic of sivelestat. Although the organ functions have not been detected in

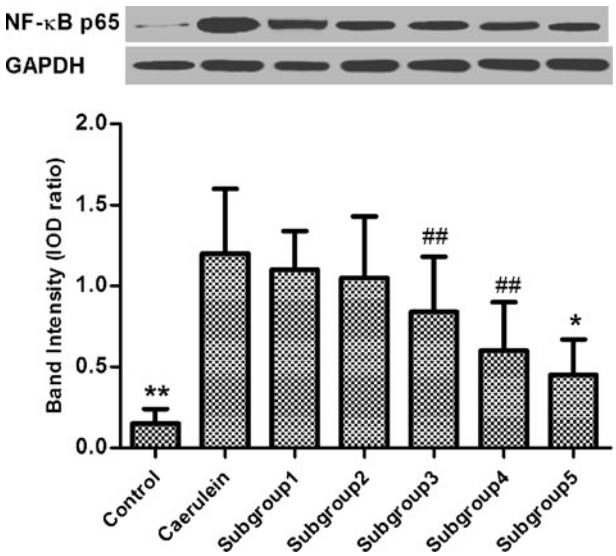

Fig. 6. Expression of NF–κB in pancreas. *Subgroup* means sivelestat subgroup; compared with other groups, $^{*}p<0.05$, $^{**}p<0.01$; compared with caerulein group, $^{\#\#}p<0.01$.

this study, the clinical efficacy of sivelestat for organ injuries has been established in previous studies [25].

Pro-inflammatory cytokines play a pivotal role in the early pathophysiological events of AP. Cytokines such as IL-1β and TNF-α initiate and propagate almost all consequences of the SIRS [26]. The inflammatory response is similar in AP and sepsis. Pro-inflammatory cytokines IL-1β and TNF-α are released *via* portal vein and lymph fluid drainage to the circulation [27]. These cytokines activate the vascular endothelium, which leads to enhanced leakage of the capillary veins, triggers migration of leukocytes into tissues, and promotes activation of coagulation cascades [28]. When neutrophils and monocytes become activated, the enzymes and oxygen radicals would be released, which damage vascular endothelial cells and organ parenchymal cells [29]. Increased vascular permeability with impaired microcirculation lead to lack of oxygen, which results in dysfunction of vital organs or organ failure [30]. This study and other previous reports have detected that these two cytokines increased serum levels, in both human and laboratory animal AP [31, 32]. In the current study, the serum IL-1β and TNF-α levels in sivelestat subgroups 3, 4, and 5 were significantly decreased by administration of high-dose sivelestat. It has been reported that NF–κB activity in pancreatic acinar cells plays a role in the inflammatory response that occurs during acute pancreatitis [33] and selective NF–κB inhibition resulted in decreased inflammation in the pancreas [19]. Additionally, sivelestat may reduce the levels of inflammatory mediators by inhibiting NF–κB [14]. In our study, treatment with high dose of sivelestat resulted in decreased inflammation, which might be associated with its ability to inhibit the expression of NF–κB.

Basic and clinical evidence suggests that the pathogenesis of AP can be associated with oxidative stress, because oxidative stress is observed in different experimental pancreatitis models [34, 35]. Oxidative stress can oxidize lipids in the cell membrane, depolarize the mitochondrial membrane, and induce DNA fragmentation during AP. To the best of our knowledge, this is the first study to demonstrate that sivelestat causes serum antioxidant biomarkers to revert to control levels. This study and

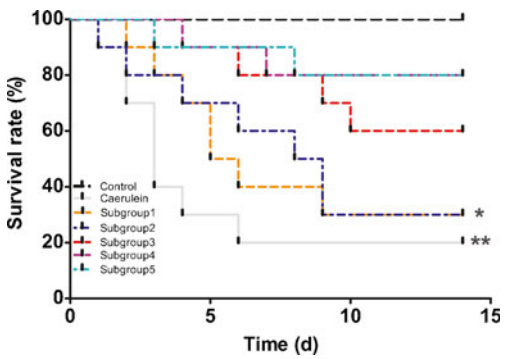

Fig. 7. Survival analysis. Survival observation lasted for 14 days. *Subgroup* means sivelestat subgroup; compared with control group, $^{*}p<0.05$, $^{**}p<0.01$.

other previous reports demonstrated that serum corticosterone levels increased in animal AP models [36]. Sivelestat treatment in this study inhibited the increase of corticosterone. It is reported that stress aggravates pancreatic pathology in AP [37], and the secretion of corticosterone is possibly a response to stress. In addition, secretion of endogenous glucocorticoids such as corticosterone may play an important role in mitigating the progress of AP, probably by inhibiting cytokine production [36]. Thus, in this study, whether the effect of sivelestat on corticosterone in AP is beneficial remains unknown.

CONCLUSION

Our results suggest that sivelestat may exert an anti-inflammatory effect and bring survival benefits in rat acute pancreatitis due to its ability to inhibit the inflammatory cells infiltration and the production of cytokines and to cause serum antioxidant biomarkers to revert to control levels. The anti-inflammatory ability of sivelestat in acute pancreatitis might be related to the inhibition of NF−κB. Thus, sivelestat may have a protective effect against acute pancreatitis.

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Conflicts of interests. The authors have declared that there is no conflict of interests.

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