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Original Article

Protective effect of nimesulide on acute lung injury in mice with severe acute pancreatitis

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Abstract: The study was designed to investigate the effect of Nimesulide (NIM) on acute lung injury (ALI) in mice with severe acute pancreatitis (SAP). In our study, caerulein and LPS were employed to establish the ALI mice model induced by SAP. All animals were divided into four groups randomly: control, model (SAP), NIM low and high dosages groups. Following treatment with NIM, histopathology observation of pancreatic tissues and lung tissues were detected by hematoxylin and eosin (H&E) staining. The levels of serum amylase, lipase, tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and IL-6 were measured by ELISA. The ratio of wet lung to dry lung (W/D) was calculated. In addition, the expression levels of TNF-α, IL-1β and IL-6 were measured by Western blotting. Moreover, the expression of cyclooxygenase-2 (COX-2) was detected using Immunohistochemistry analysis. The results revealed that NIM markedly improved pancreatic histological injury and decreased the levels of serum amylase, lipase, TNF-α, IL-1β and IL-6 in a dose-dependent after NIM treatment. For ALI induced by SAP, pulmonary edema were significantly alleviated compared with the mice in SAP group. In addition, the decreased ratio of W/D were observed after NIM intervene. The expression levels of TNF-α, IL-1β and IL-6 proteins were downregulated following NIM treatment. More, NIM inhibited the expression of COX2 in lung tissues. Taken together, our study demonstrated that NIM was able to protect against ALI induced by SAP via inhibiting inflammation, which will be of novel therapeutic strategies for the clinical treatment of ALI.

Keywords: Acute lung injury, pancreatitis, inflammation, nimesulide

Introduction

Acute pancreatitis (AP) is characterized by acute inflammatory process of the pancreas which is able to induce local peripancreatic tissue and remote organ systems [1]. The incidence of AP is increasing globally with a reported annual incidence rate of 13 to 45 per 100,000 people [2]. Importantly, it is reported that approximately 20% of AP cases develop into severe acute pancreatitis (SAP) which could lead to a systemic inflammatory response syndrome (SIRS) and multisystem organ injury [3]. Acute lung injury (ALI) is one of the most common complications of SAP, which serves as an important death factor in the early stage of SAP with high rates of mortality ranging from 30% to 40% [4, 5]. The excessive generation and release of multiple inflammatory cytokines is considered as the pathogenesis of ALI induced by SAP [6]. Therefore, chemical agents which features anti-inflammatory activity may be beneficial for the treatment of ALI induced by SAP and reducing mortality.

Nimesulide (NIM), a nonsteroidal anti-inflammatory drug which is a cyclooxygenase-2 (COX-2) specific inhibitor, is used in treatment of diverse inflammation associated diseases [7, 8]. It is well documented that NIM could attenuate the injury status during acute lung inflammation induced by lipopolysaccharide [9]. Other anti-inflammatory properties for NIM have been reported such as suppression of the expression of tumor necrosis factor-α and inhibition of matrix metalloproteinase enzymes [10]. However, the effect of NIM on ALI induced by SAP remains to be elucidated.

In our present study, the effect of NIM on ALI induced by SAP was investigated in a mice model. And the objective of the study was to

determine whether NIM protects against ALI and the underlying molecular mechanisms, which will be of critical significance for the clinical treatment of ALI induced by SAP.

Materials and methods

Animals

Male C57BL/6 mice, weight 20-25 g, were obtained from the Model Animal Research Center of the Second Affiliated Hospital of Harbin Medical University (Harbin, China). All animals were reared in temperature-controlled cages with free access to water and standard laboratory food. They were allowed to acclimate to the new environment for at least a week prior to the experiment. All of the study protocols involving animals were approved by the Ethics Committee on Animal Experiments of Harbin Medical University.

Induction of acute pancreatitis and intervention

All animals were divided into four groups randomly (n = 10 in each group), which were marked as control, model, low-dose treatment group (NIM, 3.6 mg/kg BW) and high-dose treatment group (NIM, 7.2 mg/kg BW). Severe pancreatitis was induced by intraperitoneal injection of caerulein hourly for 10 h (50 mg/kg; Sigma-Aldrich, St Louis, MO, USA), and 10 mg/kg LPS was employed to intraperitoneal injection at the last administration of caerulein. Then, mice in treatment groups were administered NIM intragastrically at 3.6 or 7.2 mg/kg while animals in control group and model group received comparable injections of normal saline. Twelve hours after administration, all the mice were sacrificed. Blood samples, pancreatic and pulmonary tissues were collected for following experiments.

Histopathological analysis

Appropriate weight pancreatic tissues and pulmonary tissues were conventionally fixed in 4% paraformaldehyde over night at 4°C and routinely included in paraffin subsequently. Strips of tissue were cut into sheets (at thickness of 5-7 μm) which were then stained with hematoxylin and eosin. Then, all the sections were dehydrated with graded ethanol and xylene. Pathological scores were blindly evaluated by two independent pathologists with a previously established scoring system under a light microscope (Olympus Corp., Tokyo, Japan) using 200X magnification (100 fields per section) [11, 12].

Wet/dry ratio of lung (W/D)

To evaluate the severity of tissue edema, the pulmonary W/D ratio were calculated. Right upper pulmonary lobe was excised immediately after exsanguination. Then, those tissues were blotted dry and weighed to obtain the “wet weight value”. And then tissues were put in a constant temperature oven at 75°C for 48 h and weight again to get the lung dry weight. Finally, the ratio of the wet lung to the dry lung was calculated.

Measurement of serum amylase and lipase levels

The blood samples were centrifuged at 3000 r/min for 10 minutes to obtain the serum. The concentrations of amylase and lipase were measured by the related assay kits. Both above kits were products of Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF-α, IL-1β and IL-6 in serum were measured by ELISA kits according to instructions provided by the manufacturer. The ELISA kits of TNF-α (F3602), IL-1β (F3531) and IL-6 (F3538) were all purchased from Shanghai Xitang Biotechnology Co., Ltd. (Shanghai, China).

Immunohistochemistry analysis of COX2

Paraffin-embedded specimens were cut into 4 μm thick sections and then deparaffinized, rehydrated with a graded ethanol and xylene. Following blocked, slides were incubated with primary antibodies overnight at 4°C. Then slides were incubated with a secondary antibody labeled with HRP followed by staining with Diaminobenzidine (DAB, Beyotime, Shanghai, China) and counterstained with hematoxylin. The immunoreactivity was assessed by optical microscope and brown nuclear staining was considered to be positive protein.

Western blotting analysis

Lung tissue was homogenized on ice in RIPA Lysis Buffer (Beyotime, Shanghai, China). The

protein concentration was measured by a BCA Protein Quantitation kit (Beyotime, Shanghai, China). Then, protein was added with the same volume of SDS-PAGE sample buffer, and subsequently electrophoretically transferred onto nitrocellulose membranes. Following all membranes were put into 5% skim milk and incubated with primary antibody. After washing, membranes were added with a goat anti-rabbit HRP-labeled IgG secondary antibody (ab205718, Abcam, Cambridge, UK) for incubation for 1 h. An enhanced chemiluminescence (ECL) reagent was used for visualization and ImageJ software was employed to analyze the results. Anti-TNF-α (11948T), anti-IL-1β (12242S), anti-IL-6 (12912T), anti-COX-2 (12-282S) and anti-GAPDH (5174S) antibodies were obtained from Cell Signaling Technology (Boston, MA, USA). The protein bands were quantified and normalized to the expression of GAPDH.

Statistical analysis

All results were performed at least three independent experiments. All experimental results were presented as mean ± SEM. Quantitative data were compared using one-way analysis of variance and the Student's t-test. A significance level of $P < 0.05$ was adopted for all analyses.

Results

NIM treatment attenuated pancreatic pathological and functional injury in SAP mice

As shown in **Figure 1A**, the pancreatic tissues in control group presented normal structure and little histopathological changes. However, light microscopy revealed that mice with caerulein induced pancreatitis showed typical pancreatitis manifestations, such as interstitial edema, neutrophil infiltration, and necrosis. In addition, the result of histopathology score was in accordance with above (**Figure 1B**). At the same time, serum levels of amylase and lipase were decreased significantly following treatment with NIM (**Figure 1C** and **1D**). These date indicated that NIM attenuated pancreatic pathological and functional injury in SAP.

NIM treatment decreased the levels of inflammatory factor in serum of SAP mice

As presented in **Figure 2A-C**, the levels of TNF-α, IL-1β and IL-6 in serum were increased notably in SAP group. Following treatment with NIM, the levels of TNF-α, IL-1β and IL-6 were decreased. Above date indicated that NIM treatment decreased the levels of inflammatory factor in serum of SAP mice.

NIM treatment alleviated histology injury of lung in model of SAP

To assess the effect of pathologic histology of NIM on SAP in rats, the histologic changes in lungs were examined. HE staining of the lung tissues showed that tissues from control group exerted normal structures without histopathologic changes. In comparison with control group, lung tissues from the model group showed a severe pathologic abnormality, including pulmonary interstitial hyperemia, edema and hemorrhage, infiltration of inflammatory cells into alveolar space, and alveolar collapse (**Figure 3A**). In addition, the ratio of W/D is one of the most significant indexes that reflects lung edema formation. As showed in **Figure 3B**, the lung W/D ratio was greatly increased in the model group. After intervention with NIM, the ratio of W/D was evidently reduced, which was in accordance with the results of histology injury of lung. These results suggested that NIM treatment alleviated histology injury of lung in model of SAP.

NIM treatment downregulated the expression of TNF-α, IL-1β and IL-6 in lung tissues in model of SAP

To investigate the potential mechanism of NIM protected against ALI induced by SAP, the expression of TNF-α, IL-1β and IL-6 in lung tissues were measured by Western blot. As presented in **Figure 4A**, the levels of TNF-α and IL-1β were increased in SAP model group. Following treatment with NIM, both expression of TNF-α and IL-1β were decreased obviously. And the expression of IL-6 shown the same results with above (**Figure 4B**). These results indicated that NIM protect against ALI induced by SAP via downregulated the expression of TNF-α, IL-1β and IL-6.

NIM treatment inhibited the expression of COX2 in lung tissues in model of SAP

To the best of our knowledge, NIM serves as a cyclooxygenase-2 (COX-2) specific inhibitor. Therefore, we detected the expression of COX2 in lung tissues in model of SAP. As presented in

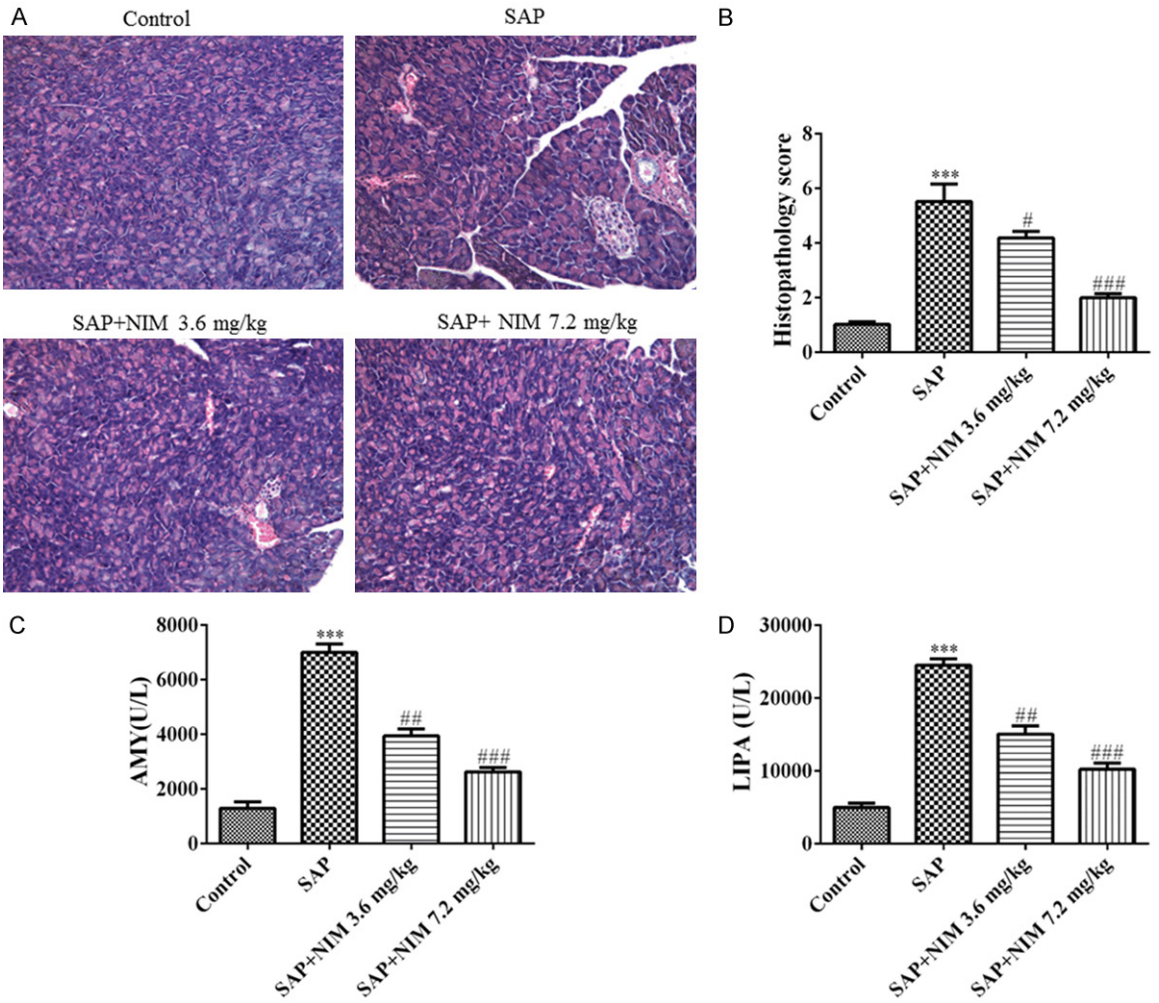

Figure 1. NIM improved the pancreatic histological lesions and decreased the content of serum amylase and lipase in SAP-induced ALI mice. (A) Representative images of H&E staining from each experimental group at 12 h after administration. (magnification, ×200). (B) The histopathology score of pancreatic histological changes. The levels of (C) serum amylase and (D) lipase were measured by the related assay kits. $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. SAP. H&E, hematoxylin and eosin; NIM, Nimesulide; SAP, severe acute pancreatitis; ALI, acute lung injury.

Figure 5A, the expression of COX2 were increased in model compared with control in Immunohistochemistry analysis. Following treatment with NIM, the levels of COX2 was decreased significantly. Moreover, the result of Western blot was in accordance with Immunohistochemistry analysis (**Figure 5B**). Above date indicated that NIM treatment inhibited the expression of COX2 in lung tissues in model of SAP.

Discussion

In the present study, we established ALI model induced by SAP by intraperitoneal injection caerulein and LPS. We found that treatment with NIM improved injury of pancreatic and pulmonary tissues, which was attribute to inhibition of TNF-α, IL-1β, IL-6 and COX2 expression. Our study manifests the use of NIM as a potential therapeutic for the clinical treatment of ALI induced by SAP.

Mounting evidence supported that rapid increase of serum amylase and lipase activities were the biochemical markers of SAP, which were commonly applied to evaluate the process of SAP in clinical patients [13]. The release of above enzymes led to the damage of pancreatic acinar cells and contributes to the inflamma-

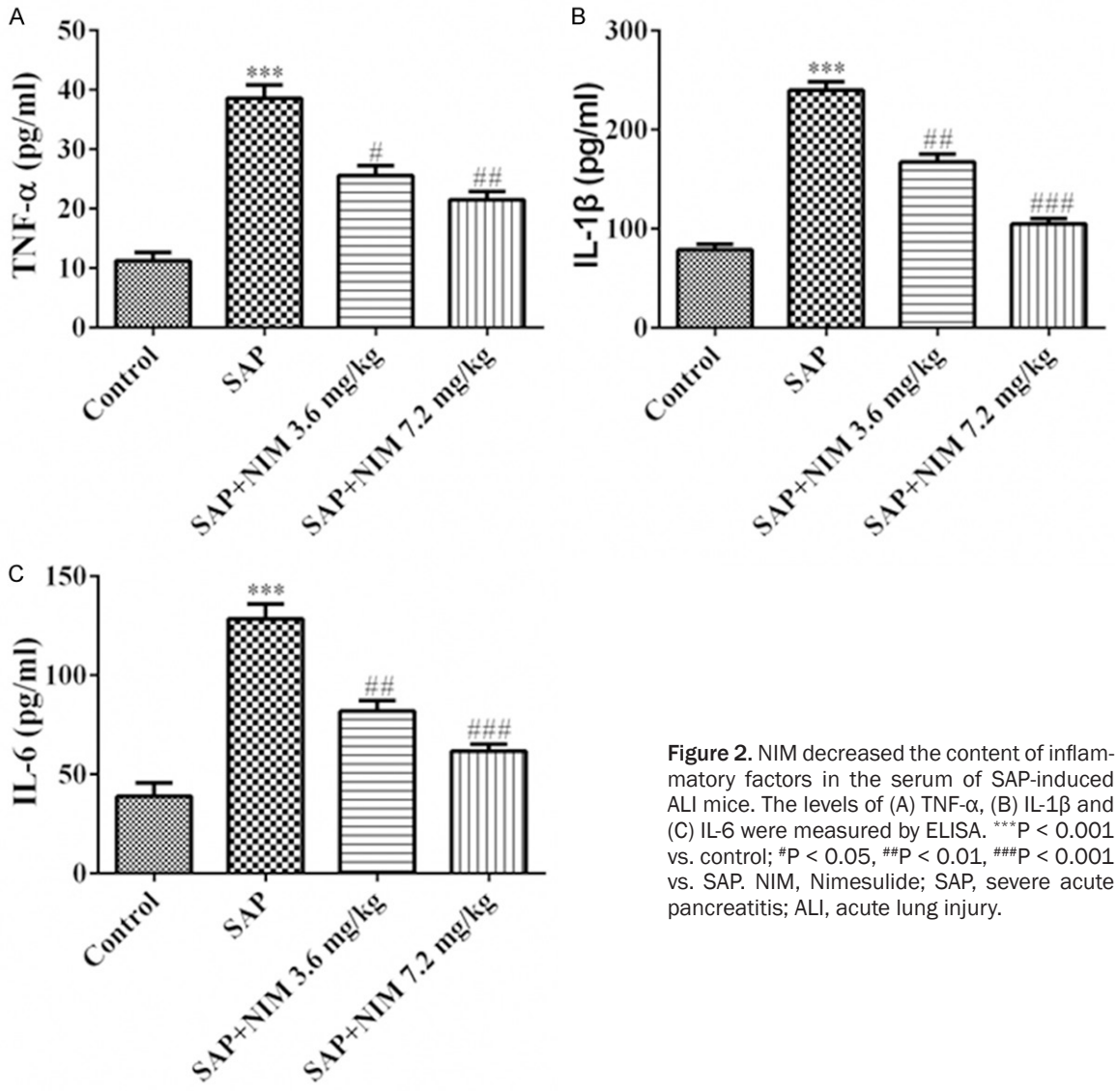

Figure 2. NIM decreased the content of inflammatory factors in the serum of SAP-induced ALI mice. The levels of (A) TNF-α, (B) IL-1β and (C) IL-6 were measured by ELISA. $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. SAP. NIM, Nimesulide; SAP, severe acute pancreatitis; ALI, acute lung injury.

tory response during the progress of SAP [14]. In the present study, caerulein combined with LPS obviously enhanced the levels of serum amylase and lipase activities, and typical pathological damages were observed in the model group, which were in concordance with the previous study [15]. Above results demonstrated that the SAP model was established successfully. And this increase was suppressed by NIM suggesting the protective effect of NIM against SAP.

Accumulating evidences shows that inflammatory cytokines play crucial roles in the outcome of SAP, in particular by triggering multi-organ dysfunction syndrome [16, 17]. Emerging studies have demonstrated that the contribution of inflammation to AP-caused acute lung injury [18]. The most common cause of death in patients with acute pancreatitis is pulmonary impairment, so the focus of nursing care for patients with acute pancreatitis should be early identification of lung injury and active control of lung injury symptoms. In our study, the expressions of inflammatory cytokines including TNF-α, IL-1β, and IL-6 were significantly elevated in lung tissues of mice that underwent pancreatitis. High levels of inflammatory cytokines have been reported to contribute to severe pulmonary histopathology by damaging intercellular tight junctions. The obviously alveolar wall thickening and inflammatory cell infiltration in the alveolar spaces in model group were observed. Moreover, we found that pretreat-

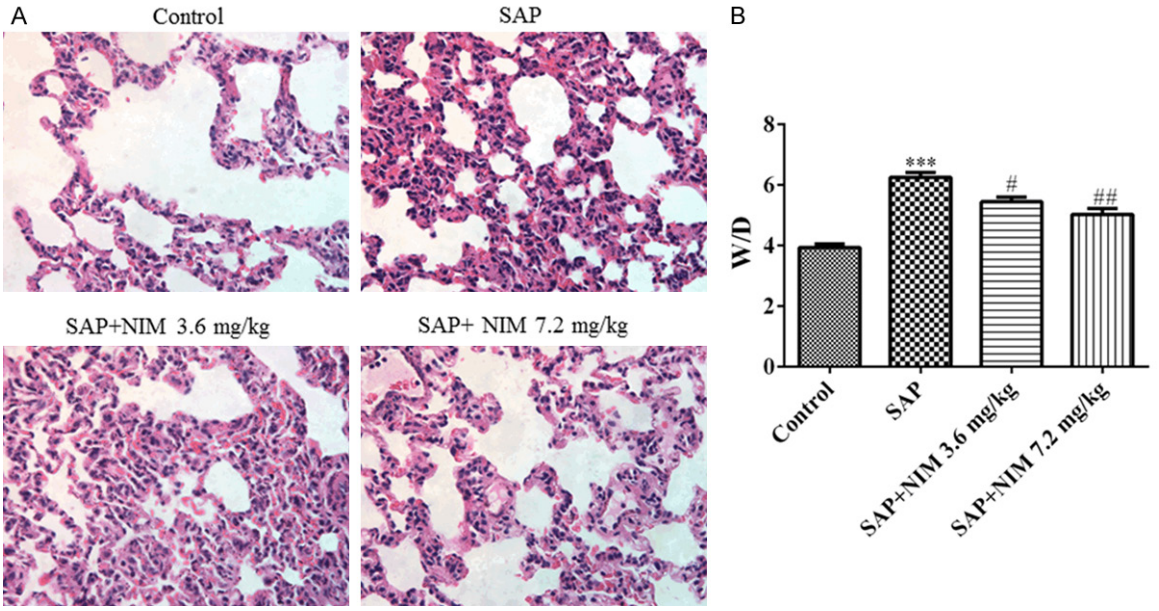

Figure 3. NIM improved the lung tissue pathological changes and edema in SAP-induced ALI mice. A. Representative images of HE staining from each experimental group at 12 h after administration. (magnification, ×200). B. NIM attenuated lung W/D ratio in SAP-induced ALI mice. ***P < 0.001 vs. control; #P < 0.05, ##P < 0.01 vs. SAP. NIM, Nimesulide; HE, hematoxylin and eosin; SAP, severe acute pancreatitis; W/D: Wet/dry ratio of lung; ALI, acute lung injury.

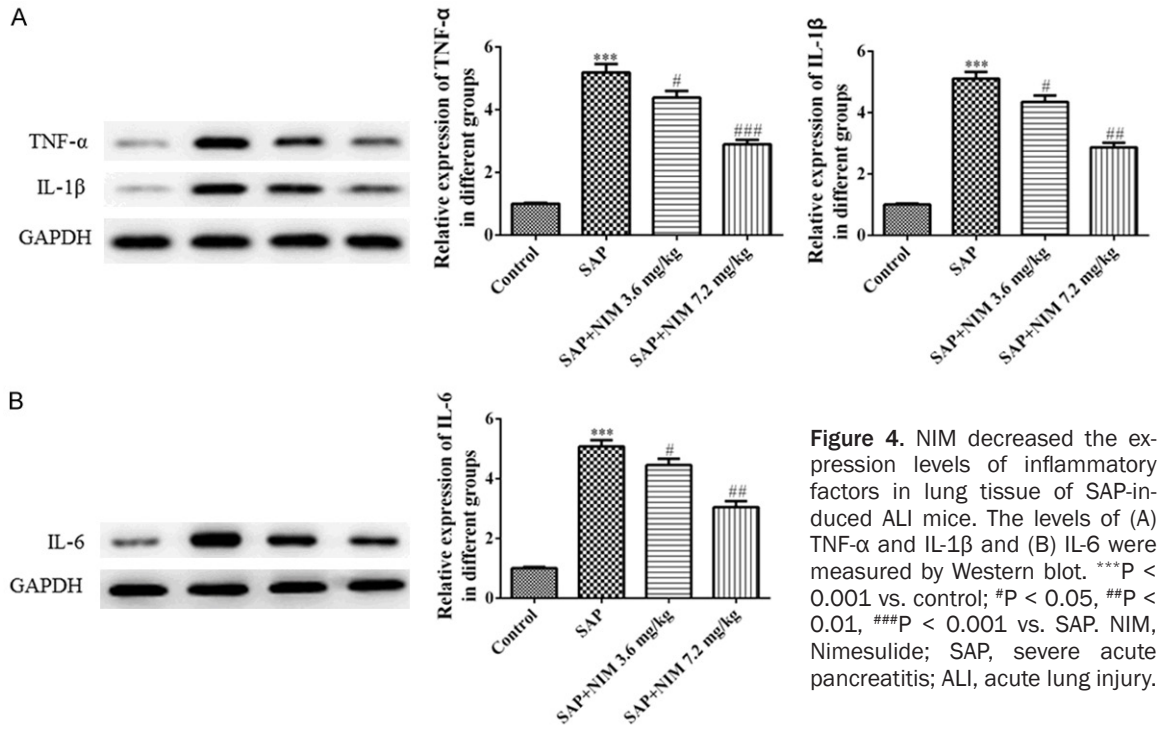

Figure 4. NIM decreased the expression levels of inflammatory factors in lung tissue of SAP-induced ALI mice. The levels of (A) TNF-α and IL-1β and (B) IL-6 were measured by Western blot. ***P < 0.001 vs. control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. SAP. NIM, Nimesulide; SAP, severe acute pancreatitis; ALI, acute lung injury.

ment with NIM significantly reduced the upregulated expressions of TNF-α, IL-1β, and IL-6 in lung tissues and amended the histopathological injury of lung. Results of the present study were in line with the findings that NIM affects protected against acute lung inflammation in

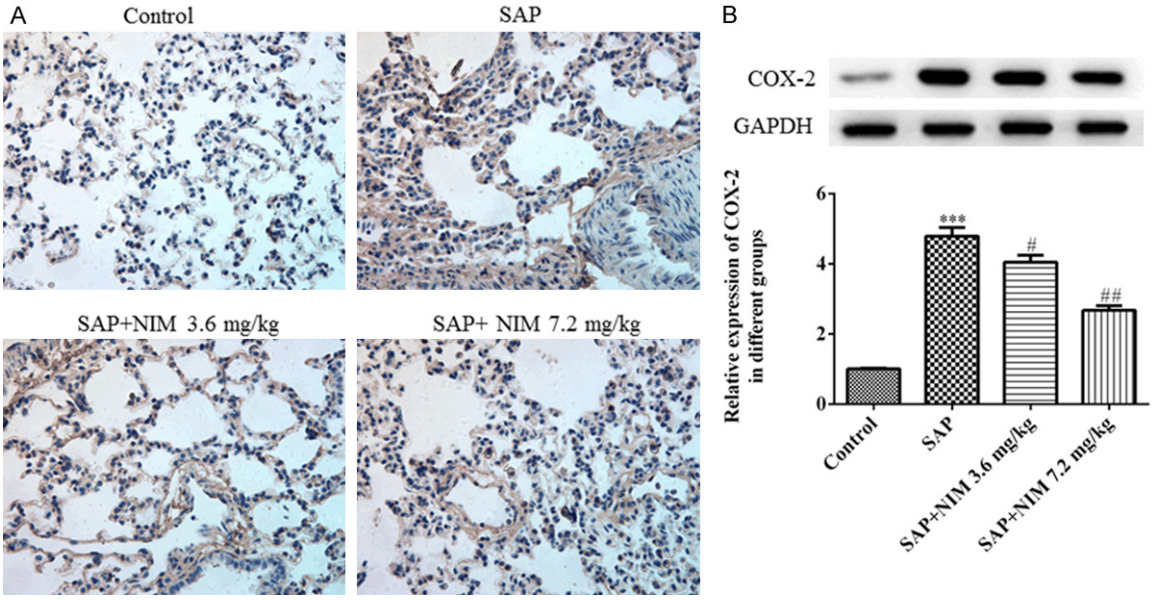

Figure 5. NIM inhibited the expression of COX-2. A. The expression of COX-2 was measured by immunohistochemistry analysis. (magnification, ×200). B. The expression of COX-2 was detected using Western blot. $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. SAP. NIM, Nimesulide; SAP, severe acute pancreatitis; ALI, acute lung injury.

rats induced by LPS [9]. Above date manifested that NIM may protect against SAP-induced ALI by inflammation suppression.

NIM is recognized as a COX-2 specific inhibitor which is used in treatment of diverse inflammation associated diseases [19]. It has been reported that NIM could suppress LPS-induced iNOS expression in alveolar macrophages [20]. And the inhibition of COX-2 exerted anti-inflammatory effects in lung tissues of acute respiratory distress syndrome [21, 22]. In our study, the level of COX-2 was increased in lung tissue of mice that underwent SAP, whereas the level of COX-2 was decreased markedly following treatment with NIM.

Conclusion

For the first time, our study investigated the effect of NIM on SAP-induced ALI. Our study results showed that NIM protected lung tissue against SAP-induced inflammatory responses and COX2 expression, which would significantly increase the survival rate of SAP-induced ALI mice eventually, and could be of critical significance for the clinical treatment of ALI.

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Disclosure of conflict of interest

None.

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