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Sivelestat sodium hydrate attenuates acute lung injury by decreasing systemic inflammation in a rat model of severe burns

X.-G. XIAO[1], H.-G. ZU[1], Q.-G. LI[1], P. HUANG[1]

The Burn and Plastic Department, The First Affiliated Hospital of the University of Henan Science and Technology, Henan, China

Abstract. – OBJECTIVE: Patients with severe burns often develop acute lung injury (ALI), systemic inflammatory response syndrome (SIRS) often complicates with ALI. Sivelestat sodium hydrate is an effective drug against ALI. However, the mechanisms of this beneficial effect are still poorly understood. In the current study, we evaluate the effects of sivelestat sodium hydrate on systemic and local inflammatory parameters (neutrophil elastase [NE], interleukin [IL]-8, matrix metalloproteinase [MMP] 2 and 9) in a rat model of severe burns and ALI. And to analyze the correlations between expression of NE and IL-8 and acute lung injury.

MATERIALS AND METHODS: 48 Sprague-Dawley (SD) rats were divided into 3 groups: normal control group, severe burns injury group and severe burns treated with sivelestat sodium hydrate group (SSI). The lung water content and PaO_2 were detected in each group. Pathological manifestations in each group were observed for pathology scoring in SD rats with acute lung injury. ELISA was used for detecting expression of NE and IL-8 in serum and BAL specimens of SD rats in each group. RT-PCR was used to detect mRNA expression of NE and IL-8 in lung tissues of each group. Western blotting was used for detecting protein expression of MMP-2 and MMP-9 in lung tissues of each group. SPSS 18.0 was used for statistical analysis.

RESULTS: The PaO_2 was significantly increased after sivelestat sodium hydrate intravenous injection. Pathological score and water content of lung tissue were significantly decreased in SSI group compared with severe burns injury group, slightly higher than that normal control group. NE and IL-8 levels significantly decreased in serum, BAL and lung tissue specimens after sivelestat sodium hydrate intravenous injection; Expression of MMP-2 and MMP-9 were significantly up-regulated in severe burns group and showed no significantly changed after sivelestat sodium hydrate intravenous injection.

CONCLUSIONS: In a rat model of severe burns and ALI, administration of sivelestat sodium hydrate improved symptoms of ALI and significantly decreased inflammatory parameters NE and IL-8.

Key Words:

Sivelestat sodium hydrate, Severe burn, Acute lung injury, Neutrophil elastase, Interleukin-8.

Introduction

Acute lung injury (ALI) is derived from direct or indirect inflammatory damage of acute progressive alveolar capillary membrane[1]. Clinical manifestations are refractory hypoxemia, aggravated severe dyspnea, non-cardiogenic pulmonary edema, etc.[2-4]. The human tissues with severe burn often accompany systemic inflammatory that similar to the infective symptoms, and which regardless of infection is named as systemic inflammatory response syndrome (SIRS). SIRS often complicates with acute lung injury (ALI), clinically represents respiratory distress syndrome adult (ARDS), and which can prevent the rehabilitation of SIRS patients[5-7]. Patients with severe burns are particularly vulnerable to ALI, especially those with upper respiratory tract injury. They may develop hypoxia, organ damage and dysfunction of remote sites[8]. ALI is referred as acute respiratory failure caused by non-cardiac risk factors, and characterized by respiratory distress and refractory hypoxemia[9]. In the previous studies, results showed that ALI was closely associated with the local and systemic inflammatory response[10]. Further findings displayed that elastase and interleukin-8 (IL-8) mediated by neutrophils were participating in the above process. In addition, it had been found that

Corresponding Author: Xiongmu Xiao, MD; e-mail: 25852832@qq.com

matrix metalloproteinase (MMP-2 and MMP-9) also participate in the process of the development of ALI[11].

Sivelestat sodium is a specific elastase inhibitor, and is a synthetic heterocyclic compound. In addition, sivelestat sodium is used to improve the symptoms of ALI and reduce the length of hospital stay[12]. However, it is still poorly understood that the molecular mechanism of sivelestat sodium participated in the process of improving the symptoms of ALI caused by severe burn.

In this study, ALI model of SD rats induced by severe burning will be established to observe and detect pathological changes in lung tissue of SD rats. We will verify whether sivelstat sodium can alleviate the symptoms of ALI by reducing the inflammatory response in a rat model of severe burn. Then, we further explore the expression of NE, IL-8, MMP-2 as well as MMP-9 in serum, BAL and lung tissues will also be detected to investigate the effect on lung injury induced by severe burning, thus providing new ideas for prevention and treatment of ARDS.

Materials and Methods

Animals, Equipment and Reagents

48 healthy clean grade Sprague Dawley (SD) rats with weighing 220 - 250 g were fed under standard laboratory environment for one week. The SD rats were provided by Experimental Animal Center of Second Military Medical University. The Heraeus centrifuge was from Kendro Laboratory (Hamburg, Germany), whereas the GEM Premier 3000-type blood gas analyzer was purchased from Instrumentation Laboratory (Bedford, MA, USA). The goat anti-MMP-2 and anti-MMP-9 polyclonal antibodies were purchased from Abcam (Cambridge, UK). The NE and IL-8 ELISA kits were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Injectable sivelestat sodium hydrate was provided by Haitian Pharmaceutical Technology Development (Shanghai, China).

Grouping and Model Establishment

The SD rats were intraperitoneally anaesthetized with 3% pentobarbital sodium in 2 days before the experiment. Then, the SD rats neck and back were shaved and disinfected, and incubated at external jugular vein and connected to a continuous intravenous infusion device. Anaesthesia with 3% pentobarbital sodium injection was repeated immediately before the experiment. The SD rats were randomly divided into 3 groups: normal control group, severe burns group and severe burns treated with sivelestat sodium hydrate group (SSI). The SD rats were burnt at the back using the constant burning apparatus in 98º C for 12 s in SSI group, which resulted in a third degree burn of approximately 30% TBSA, and then the SD rats were intravenously injected into sivelestat sodium hydrate (100 mg/kg weight). At the same time, the SD rats were exposed to burn without the treatment in severe burns group. The SD rats were treated with mock burning using a room temperature water bath and did not receive fluid resuscitation in normal control group. After the experiment, all the SD rats received Ringer's lactate solution (2 mL/kg/hour) to restore.

Specimen Collection and Detection

Arterial blood (0.5 mL) was obtained from left common carotid artery at 1, 3, 6, 24 and 48 hours after the experiment and was used for blood gas analysis. 2 mL venous blood were centrifuged (13,000 rpm, 15 min, 4°C) to obtain supernatants, which were kept at -70º C. Lung tissues was obtained from SD rats that were euthanized by exanguinations. Right upper lungs were used to determine the water content, while small pieces of the right middle lobe were used for assessing MMP-2 and MMP-9. Left lungs were placed in a 10% formaldehyde solution for subsequently histopathological examination. 3 SD rats were randomly picked in each group, we obtained bronchoalveolar lavage (BAL) from injecting pre-chilled saline (15 mL/kg of body weight) in each group of SD rats. After instillation and aspiration for 3 times, the recoveries of BAL were more than 90%. The BAL was placed for 15 min at room temperature, and then centrifuged with 13000 rpm for 15 min at 4º C. The supernatants of each group were stored at -70º C.

Detection of Lung Tissue Sections Using HE Staining

The lung tissue was taken out from stationary liquid (10% formaldehyde solution) and douched using saline. The liquid on the surface was wiped by filter paper carefully. Then it was dehydrated with 95%-100% alcohol in automatic hydroextractor overnight. Pathological tissue embedding machine was used to slice the lung tissue into slices of 3 μm. Then the slice was tweezered on-

to glass slide gently, the folds were stretched. The ready-made slice was dewaxed twice using xylene, each time for 10 min. Afterwards it was douched by saline. The resultant slice was stained for 5 min by haematoxylin. The glass slide was douched and then 0.5% eosin was used to stain for 30s. The slice was dehydrated with alcohol again and dealcoholized with xylene. Neutral gum was dropped on it and cover ship was replaced. The resultant slice was observed under light microscope and photographed.

Detection of PaO_2

Arterial blood was obtained and was used for blood gas analysis according to previous report[13].

Detection of Wet/Dry Ratio (W/D) and the Water Content in Lung Tissues of SD Rats

Right anterior lobe of the lung was taken out from rat after it was sacrificed. The blood on the surface was rapidly wiped with filter paper. Then the tissue was put on electronic balance for weighing, which was named wet weight of lung tissue (*W*). Afterwards, the weighed lung tissue was baked until constant weight at 70°C and the lungs were placed on a filter paper to weighed on electronic balance, which was named dry weight (*D*). The wet/dry ratio of lung tissue = W/D. The lung water content was calculated as [wet weight – dry weight] / wet weight × 100%.

Lung Injury and Pathological Score

Pathological score was obtained using the Gloor pathology score in a double-blind manner. The score consisted of 0 points (normal), 1 (mild), 2 (moderate), 3 (severe), or 4 (especially severe) points ranked according to three indicators (alveolar hemorrhage, alveolar edema, and neutrophil infiltration). 2 pathology experts observed lung tissue slice under 10×40 high-power microscope. 10 different visual fields were randomly selected in lung slice for scoring. The average value was the final result (Table I).

Detection of NE and IL-8 Content by ELISA

The kit and SD rats serum sample were defrosted at room temperature (RT). Washing liquor, test plasma, standards, and horseradish peroxidase-labeled streptavidin were diluted into required concentration. 50 μL standard substance, reference substance and test plasma were respectively added into each well of ELISA plate and then sealed with closure plate membrane for incubation for 2 hours. Afterwards the liquid in the plate was abandoned and 300 μL washing liquor was added into each well. The liquid in the plate was abandoned again. The plate was dried by filter paper. The above process was repeated 6 times. 100 μL horseradish peroxidase-labeled streptavidin was added into each well. The plate was sealed with new closure plate membrane for 45 min of incubation in shaking table. Afterwards the liquid in the plate was abandoned and 300 μL washing liquor was added into each well. The liquid in the plate was abandoned again. The plate was dried by filter paper. The above process was repeated 6 times. 100 μL TMB (3,3',5,5'-Tetramethylbenzidine) was added into each well in dark room to incubate for 15 min at RT. The plate frame was knocked gently to mix the liquid thoroughly. The wells turned from colorless to yellow green. The OD value was assayed using automatic microplate reader at wavelength of 450 nm during 30 min. The abscissa represented the concentration of standard substance. The ordinate represents the corresponding OD value. Standard curve was generated by regression fitting in computer. The concentration of test antibody in test serum was calculated according to the standard curve.

Table I. Pathological score of ALI (scoring criteria of Ge ZJ).

Score	Observation indexes: Alveolar edema	Pulmonary interstitial edema	RBC leak cell infiltration	Inflammatory	Hyaline membrane formation	Extent of Atelectasis
0	no	no	no	no	no	no
1	<25%	<25%	<25%	small amount	sometimes	sometimes
2	25%-50%	25%-50%	25%-50%	multiple	small amount	light - moderate
3	50%-75%	50%-75%	50%-75%	clustered	significant	severe

Detection of NE and IL-8 mRNA Expression Using RT-PCR

RNA extraction reagent Trizol was used to extract total RNA in lung tissue of SD rats in each group. The quality of RNA was detected using Nano Drop-2000 (Thermo Scientific, Waltham, MA, USA). The purity of RNA was estimated according to the ratio at 260 nm and 280 nm. We acquired the sequence of NE and IL-8 from NCBI database and designed the RT-PCR primer using Primer Bank. Glyceroaldehyde 3-phosphate dehydrogenase (GADPH) was the normalized internal reference gene.

Detection of MMP-2 and MMP-9 Protein Expression Using Western Blotting

100-mg piece of right middle lobe tissue were homogenized on ice for 10 min after ultrasonication. Next the resultant mixture was centrifuged at 4°C, 12000 rpm for 15 min. The supernatant was collected for detecting protein concentration using protein assay kit. Loading buffer of 1/5 volume was added to the proteins and boil at 100°C for 5 min. 20 μg protein sample was taken for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membranes by semi-dry method. Afterwards 5% skimmed milk powder / PBST was used for sealing at RT 2-4 h. Anti MMP-2 (1:500), MMP-9 (1:500) and GADPH (1:1000) primary antibody was incubated at 4°C refrigerator for overnight. HRP labelled secondary antibody used for incubation at RT for 2 h. ECL luminescent liquid was added analyze in chemiluminescence imaging system. The representative image was shown.

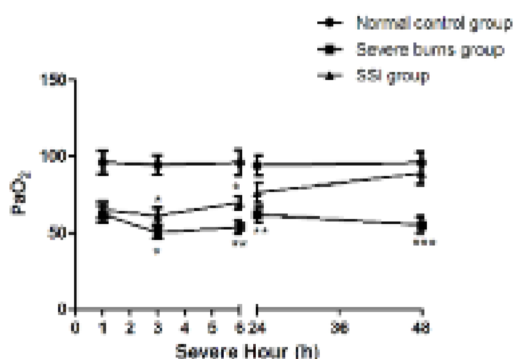

Figure 1. The PaO2 values in normal control group, severe burns group and SSI group. $^{*}p < 0.05$, $^{**}p < 0.01$.

Statistical Analysis

Statistical analysis was conducted using SPSS14.0 (SPSS Inc., Chicago, IL, USA). The data were expressed as mean ± SEM and were compared using ANOVA test. Multiple comparisons were done using the LSD-t test. The data that did not meet normal distribution and homogeneity of variance were analyzed with the Kruskal-Wallis test, with multiple comparisons done using the Nemenyi test. PaO_2 values at different time points were compared using the repeated measures ANOVA. At the p value of $<$ 0.05, the differences were considered statistically significant.

Results

The PaO_2 was Significantly Increased After Sivelestat Sodium Hydrate Intravenous Injection

The oxygenation index were 292.2, 240.9, 272.1, 289.5 and 281.9 mmHg in severe burnt injury group SD rats at 1 h, 3 h, 6 h, 24 h and 48 h post injury, respectively. The results suggested that the existence of pulmonary dysfunction in severe burn injury group. The PaO_2 value was significantly lower in severe burns group and SSI group than that of the normal control group ($p < 0.05$) at different time point. Moreover, the PaO_2 value was significantly lower in severe burns group compared with SSI group at 3 h, 6 h, 24 h and 48 h post injury ($p < 0.01$), the PaO_2 in SSI group began to fell sharply at 2 h post injury, then gradually recovered (Figure 1).

Pathological Score was Significantly Decreased After Sivelestat Sodium Hydrate Intravenous Injection

For depth study of pathological conditions after sivelestat sodium hydrate intravenous injection, we carefully observed the lung tissue after SD rats were sacrificed and counted the data according to Ge ZJ assessment criteria (Table I). We found structure clear, alveolar walls thin and smooth, alveolar septa consistent in lung tissue of normal control group. In severe burns group, symptoms including alveolar space narrowing or disappearing, significantly increasing inflammatory cells in cavity, pulmonary hyperemia, pulmonary mesenchyme edema, diffuse inflammatory cell infiltration were observed. While in Group SSI, symptoms including alveolar space slightly narrowing, part alveolar cavity thickening, pulmonary hyperemia, mild edema in pul-

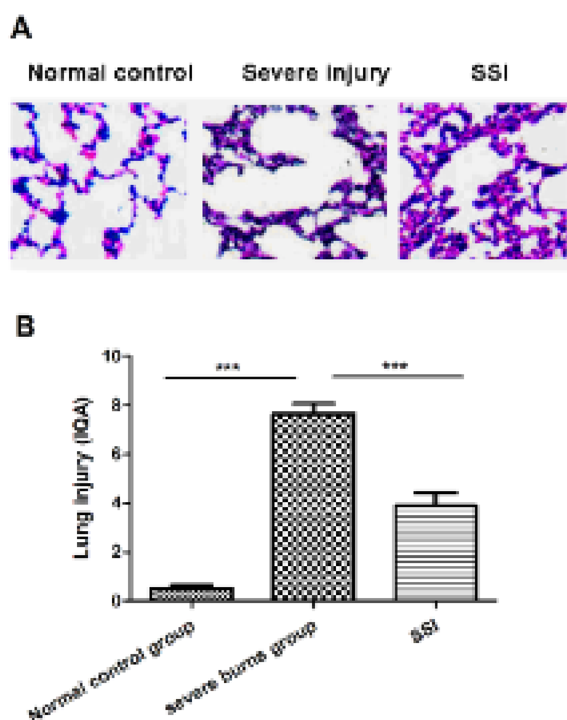

Figure 2. The Pathological character of ALI in each group. ***(A)***. HE staining of lung tissue in SD rats with ALI of each group; ***(B)***. Pathological scores in SD rats with ALI of each group (p <0.001). The score in normal control group was significantly lower than that severe burns Group (p <0.001). The score in SSI Group was significantly lower than that severe burns Group (p <0.001).

monary mesenchyme, small focal inflammatory cell infiltration were observed (Figure 2A). These results suggested that pathological score significantly decreased after sivelestat sodium hydrate intravenous injection compared with severe burns group (p <0.01), and slightly increased in SSI group compared with normal control group (p <0.01). The results were illustrated in Figure 2B.

Water Content of Lung Tissue was Significantly Decreased After Sivelestat Sodium Hydrate Intravenous Injection

For investigating water content changes of lung tissue in SD rats after sivelestat sodium hydrate intravenous injection, we analyzed wet/dry ratio in each group and found that *W/D* in severe burns group and SSI groups were significantly higher than that in normal control group (p <0.01). Moreover, *W/D* in SSI group was significantly lower than that in normal severe burns group (p <0.05) (Figure 3). At the same time, we analyzed the water content of lung tissues in each group and found that water content of lung tissue in SSI group was significantly lower than that in severe burns group, slightly higher than normal control group (p <0.01). These results indicating that sivelestat sodium hydrate alleviated to lung injury in some extent.

NE and IL-8 Levels were Significantly Decreased in both Serum and BAL Specimens After Sivelestat Sodium Hydrate Intravenous Injection

To explore NE and IL-8 mRNA levels induced by sivelestat sodium hydrate intravenous injection in SD rats, we detected serum and BAL specimens composition changes in SD rats. ELISA was used to detect content of NE and IL-8 in serum and BAL specimens. The results showed that NE levels of serum and BAL specimens in severe burns group were significantly higher compared with control group (p <0.01) (Figure 4A). Moreover, NE levels of serum and

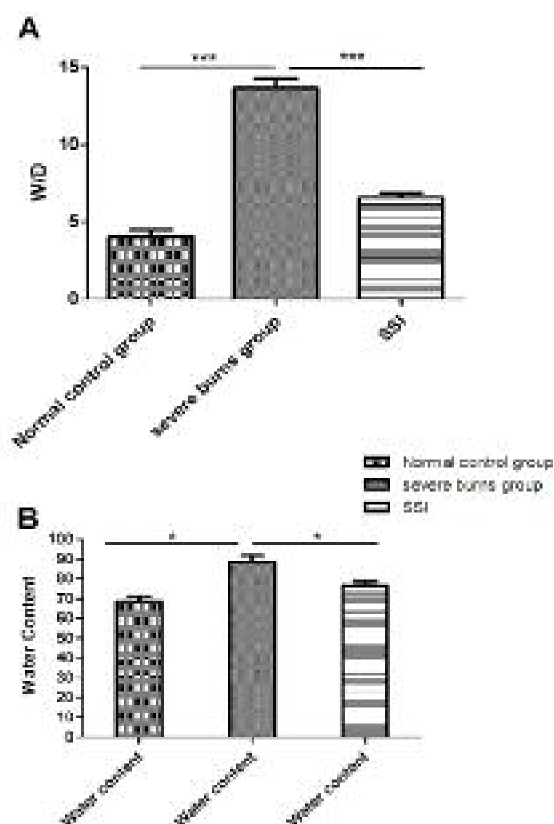

Figure 3. W/D and water content of lung tissue in each group. ***(A)***. W/D of lung tissue in each group; ***(B)***. The water content of lung tissue in each group. $^{*}p$ <0.05, $^{***}p$ <0.001.

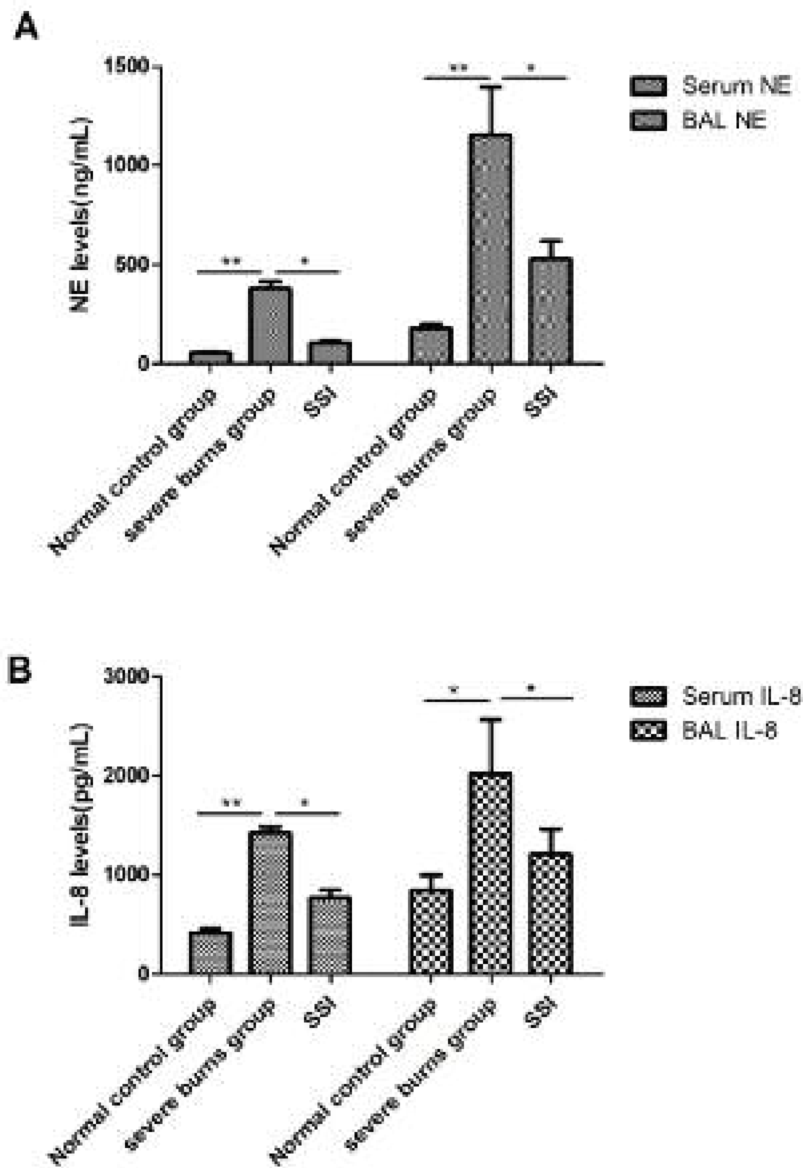

Figure 4. Detection of NE and LI-8 levels in serum and BAL specimens by ELISA, data are mean ± SEM. $^{*}p$ <0.05, $^{**}p$ <0.01. ***(A)***. Detection of NE levels in serum and BAL specimens (ng/mL); ***(B)***. Detection of IL-8 levels in serum and BAL specimens (pg/mL).

BAL specimens in SSI group was significantly lower than that in severe burns group, and slightly higher than that in normal control group (p <0.01) (Figure 4A). The IL-8 levels of serum and BAL specimens in SD rats each group has the same tendency (Figure 4B). These results demonstrated that lung injury alleviated along with the extending time of sivelestat sodium hydrate intravenous injection. This data were consistent with the previous results of our pathological observation.

NE and IL-8 mRNA Levels were Significantly Decreased After Sivelestat Sodium Hydrate Intravenous Injection

To explore the relationship between the expression of NE, IL-8 mRNA and sivelestat sodium hydrate intravenous injection in SD rats, we studied the mRNA expression of NE and IL-8 by RT-PCR in normal control group, severe burns injury group and SSI group rats. The results showed that NE and IL-8 mRNA expression in severe burns group were significantly higher

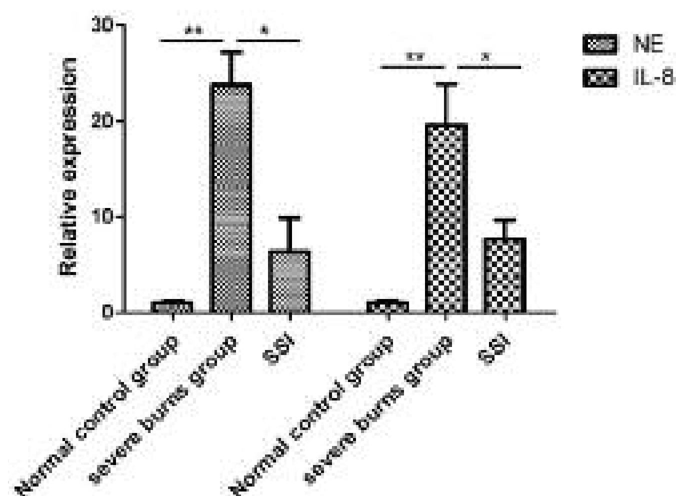

Figure 5. Detected the NE and LI-8 mRNA expression of lung tissue in SD rats by RT-PCR. $^{*}p$ <0.05, $^{**}p$ <0.01.

compared with control group (p <0.01) (Figure 5). Moreover, NE and IL-8 mRNA expression in SSI group was significantly lower than that in severe burns group, and slightly higher than that in normal control group (p <0.01) (Figure 5). NE and IL-8 mRNA expression of lung tissues were consistent with which were in serum and BAL specimens after sivelestat sodium hydrate intravenous injection.

Expression of Mmp-2 And Mmp-9 were Significantly Up-Regulated in Severe Burns Group and Showed no Significantly Changed After Sivelestat Sodium Hydrate Intravenous Injection

To explore the relationship of MMP-2 and MMP-9 expression induced by severe burns injury and sivelestat sodium hydrate intravenous injection in SD rats, we detected the expression of MMP-2 and MMP-9 in normal control group, severe burns injury group and SSI group rats. The results showed that expression of MMP-2 and MMP-9 in severe burns group were significantly higher compared with control group (p <0.01) (Figure 6). Moreover, expression of MMP-2 and MMP-9 in SSI group was no significantly changed compared with severe burns group (Figure 6). The results demonstrated that expression of MMP-2 and MMP-9 was not correlated with sivelestat sodium hydrate intravenous injection.

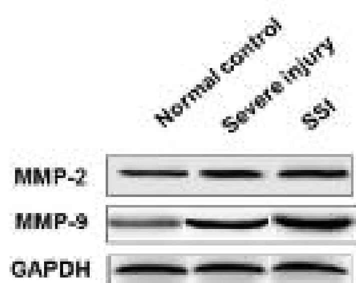

Figure 6. Detection the expression of MMP-2 and MMP-9 of lung tissue in SD rats by western blotting, the representative image was shown.

Discussion

The genesis and development of ALI involves in many pathophysiological processes, including inflammatory response, oxidative stress, apoptosis, etc.[14-16]. Inflammatory response and oxidative stress are thought to be the earliest pathophysiological mechanism[17,18]. Studies[19] have found that the ALI manifested as infiltration and activation of inflammatory cells such as neutrophilic granulocytes, lung macrophages etc. at the cellular level, as well as vascular endothelial cells (EC) damage. At the molecular level, it manifested as activation of tumor necrosis factor (NF-KB) and overexpression of proinflammatory cytokines, adhesion molecules, chemokines, etc., accompanied by production of multiple inflammatory mediators[20,21]. The inflammatory cascade reaction formed of inflammatory mediators led to wide lung injury.

Severe burns can lead to secondary ALI. Some severe patients even develop Acute Respiratory

Distress Syndrome (ARDS), which was reported to have a prevalence of up to 12%-17%. Once ARDS is developed, mortality can reach 40%-70%[22]. Principal mechanisms of ARDS include damage to lung tissue caused by complement, inflammatory cytokines, metabolites of plasmin, and reactive oxygen species released by inflammation-triggered neutrophils and endothelial cells. In addition, microthrombosis causes imbalance of ventilation and perfusion, and hypoxemia[23]. Therefore, pathogeneses of both ALI and ARDS are mainly associated with systemic inflammatory response syndrome.

Sivelestat sodium hydrate is a synthetic heterocyclic compound used in the treatment of ALI[24]. In our study, we have established a SD rat model of severe burns. Various manifestations of ALI were evident in this model: (i) blood gas analysis showed a decrease of PaO_2, (ii) assessment of lung water content indicated a development of traumatic wet lung as a consequence of pulmonary edema, and (iii) histopathological examination showed hyperemia. In addition, we have observed a large number of inflammatory cells infiltrating the pulmonary interstitial and alveolar wall, as well as some alveolar wall rupture. NE is an elastase released from neutrophils that decomposes extracellular matrix, causes damage to pulmonary microvascular system, promotes pulmonary oedema and inflammatory cells infiltration, reduces lung compliance by destruction of pulmonary surfactant, and facilitates infection by increasing pathogen adherence[25]. IL-8 is an inflammatory cytokine generated by monocyte-macrophage cells. It promotes neutrophil adhesion and exudation, which results in accumulation of neutrophils in lung tissue[26]. Studies demonstrate a close relationship between NE and IL-8. NE induces monocyte-macrophage cells to produce IL-8, and newly recruited neutrophils release more NE, which results in a vicious cycle[27]. However, these pathological changes have been rectified by the treatment with sivelstat sodium hydrate. Our data showed that NE and IL-8 levels were significantly decreased in serum, BAL and lung tissue specimens after sivelestat sodium hydrate intravenous injection. The result suggested that beneficial effects of this compound on systemic and local markers of inflammation (i.e., NE and IL-8 in serum and BAL). We also detected the effects of sivelestat sodium hydrate on MMP-2 and 9. Expression of MMP-2 and MMP-9 were significantly up-regulated in severe burns group and showed no significantly changed after sivelestat sodium hydrate intravenous injection. The data demonstrated that lack of direct effects of this compound on MMP.

Conclusions

In a rat model of severe burns and ALI, administration of sivelestat sodium hydrate improved symptoms of ALI and decreased inflammatory markers NE and IL-8.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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