Ligustrazine attenuates acute lung injury induced by blunt chest trauma

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ABSTRACT

Objectives: To investigate the effects of ligustrazine on acute lung injury induced by blunt chest trauma.

Methods: This study was performed in the Animal Center of Renmin Hospital of Wuhan University, Wuhan, China between September 2009 and September 2010. Male Sprague-Dawley rats were randomly allocated into 4 groups: sham control group (group C, n=60), ligustrazine treatment group (group C+L, n=60), blunt chest trauma model group (group T, n=60), and the trauma plus ligustrazine treatment group (group T+L, n=60). The lung contusion was induced as previously described. Animals of the T+L group were intraperitoneally injected with ligustrazine. Acute lung injury was evaluated by histopathology of the lung, and apoptosis was determined by terminal dUTP nick-labeling. Pulmonary edema was estimated using Evans blue dye extravasation and wet/dry ratios of lung tissue. The expression of caspase-3, Bcl-2, and Bax in the lung, as well as blood plasma tumor necrosis factor (TNF)-α were also measured.

Results: The ligustrazine treatment significantly attenuated lung injury induced by blunt chest trauma, as shown by decreased apoptosis index, and pulmonary edema (p<0.04). The blood plasma TNF-α level after blunt chest trauma significantly deceased after the administration of ligustrazine (p<0.03). In addition, the ligustrazine treatment significantly alleviated the expression of caspase-3 (p<0.03), and increased the ratio of Bcl-2 to Bax (p<0.03).

Conclusion: Ligustrazine effectively protects lung injury induced by blunt chest trauma, and the protective effects seem to be mediated by attenuation of cell apoptosis via an increased ratio of Bcl-2/Bax and decreased caspase-3 activity.


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Severe blunt chest trauma remains an important injury with high morbidity and mortality, and accounts for nearly one-third of all hospital admissions of acute trauma.\(^1,2\) Lung contusion induced by severe blunt chest trauma is frequently associated with pneumonia, and is also an independent risk factor for the development of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS).\(^3,4\) The clinical syndromes of ALI/ARDS reflect severe inflammatory lung injury, and have a high mortality and morbidity despite sophisticated respiratory support and intensive care.\(^1,4\) Therefore, it is important to investigate the mechanism of ALI induced by blunt chest trauma, and how to protect against ALI.

As an energy-driven process in response to extracellular signals or developmental cues, apoptosis is considered an important factor in the pathogenesis of pulmonary edema secondary to injury.\(^5\) Many studies have sought to improve the understanding of the inflammatory mechanisms that contribute to blunt trauma induced lung contusion,\(^6\) although the regulatory effect of tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) production as well as the molecular mechanism associated with the stimulation of apoptotic cell death are not fully understood. The effect of TNF-\(\alpha\) on apoptosis induced by blunt chest trauma has not yet been elucidated clearly. A variety of therapeutic methods and tools have been used to attenuate inflammation and abnormal apoptosis caused by blunt chest trauma. Ligustrazine, a purified and chemically identified component of a Chinese herbal remedy, has been demonstrated to have protective effects against lung and liver injury after burn and renal ischemia-reperfusion injury in rats, and prevents acute myocardial infarction and cerebrovascular accidents in humans.\(^7,11\) Ligustrazine has also been shown to alleviate hepatic and endothelial cell damage induced by ischemia-reperfusion.\(^8,12,13\) Here, we investigated whether ligustrazine could produce protection against lung injury induced by blunt chest trauma in rats. Several intracellular signaling pathways, such as the caspase cascades and B-cell lymphoma 2 (Bcl-2) family proteins have been demonstrated to be critical in the regulation of apoptosis. Therefore, the expressions of pulmonary caspase-3, Bcl-2 and Bax, and blood plasma TNF-\(\alpha\) were also examined in this study.

**Methods.** *Animal care and use.* The Bioethics Committee of Renmin Hospital of Wuhan University approved this study, and the procedures were carried out according to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH Publication No. 80-23). The study was performed in the Animal Center of the Renmin Hospital of Wuhan University, Wuhan, China between September 2009 and September 2010. Male Sprague-Dawley (SD) rats weighing 200-250 g (8-10 weeks) were used in this study. The Center of Experimental Animals at Wuhan University supplied the rats. Food was withheld for 12 hours before the experiment.

**Surgical procedures.** The rats were anesthetized intraperitoneally with 2% sodium pentobarbital (45 mg/kg). Surgery was performed after loss of the blink and withdrawal reflexes. The rats were then placed in the supine position and allowed to breathe spontaneously. The right jugular vein was cannulated with polyethylene tubing (PE-24G). After the surgical procedures, the rats received an intravenous infusion of 10 ml lactated Ringer’s solution (2 ml/h) to prevent dehydration.

**Experimental protocol and blunt chest trauma model.** After surgical preparation and instrumentation, the animals were randomly allocated into 4 groups: sham control group (group C, \(n=60\)), ligustrazine treatment group (group C+L, \(n=60\)), trauma model group (group T, \(n=60\)), and the trauma plus ligustrazine treatment group (group T+L, \(n=60\)). Each group was further divided equally into 2 subgroups, and one of the 2 groups received Evans blue dye injection. Lung contusion was induced as previously described.\(^4,13\) Briefly, the rats were anesthetized in their supine position and a 0.3 kg weight from a height of 95 cm was dropped through a vertical stainless steel tube onto a platform resting on the chests of rats. Animals of the T+L group were intraperitoneally injected with ligustrazine (80 mg/kg)\(^8,12\) immediately after blunt chest trauma. Lung tissues were collected at each respective time point. Exsanguination and thoracotomy were performed to harvest lung tissue specimens. The right upper lung tissues were fixed in 4% paraformaldehyde, then embedded in paraffin and prepared in slices of 5 \(\mu\)m thickness. Expressions of caspase-3, Bax and Bcl-2, and cell apoptosis were determined by standard assay.

**Histological changes.** The upper lobe of the left lung was stained with hematoxylin and eosin (HE) for standard histological examination. A pathologist blinded to the treatment groups examined at least 2 different sections of each specimen. Alveolar septal wall neutrophil sequestration in the peripheral lung parenchyma was expressed as the mean number of neutrophils per 10 high-power fields (100x). The severity of ALI was scored on a scale of 0 to 4 (0=normal, 1=mild damage, 2=moderate damage, 3=severe damage, and 4=most severe damage) as described previously.\(^14\)

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and examined by a blinded pathologist using a 5-point scale according to combined assessments of alveolar congestion, hemorrhage, infiltration in the airspace or vessel wall, and thickness of alveolar wall/hyaline membrane formation damage.

**Assay of apoptosis by TUNEL staining.** The extent of lung apoptosis was also determined using terminal dUTP nick end-labeling (TUNEL), as described previously. The status of apoptotic cells was determined with an in situ cell apoptosis assay kit (Roche Corporation, Basel, Switzerland). Five high-power fields (x400) were randomly chosen and the numbers of apoptotic cells and total cells were counted. The apoptosis index (AI, %) was calculated with the following formula: AI (%) = number of apoptotic cells/number of total cells x 100%.

**Analysis of pulmonary microvascular permeability.** Pulmonary vascular permeability was estimated using the Evans blue dye method. In brief, Evans blue, 30 mg/kg (Sigma-Aldrich, Beijing, China) was injected intravenously approximately 30 minutes prior to the time at which the right lung was perfused via the pulmonary artery for 2 minutes with physiological saline (37°C) at 0.04 ml/g body weight per minute using an infusion pump to eliminate residual blood, and Evans blue dye from the pulmonary bed as described previously. After infusion, the right lung was excised, rinsed externally with physiological saline, and then placed in a drying oven at 90°C for 24 hours. Dried tissue samples were placed in 2 ml of formamide at 37°C for 24 hours. The dye concentration of the samples was then measured by spectrophotometry at 620 nm. The concentration was expressed in micrograms per gram of dried tissue samples.

**Assessment of pulmonary edema.** At the time points of one, 2, 3, 12, and 24 hours after chest trauma, the lower lobe of the left lung was removed from the thoracic cavity, weighed, and then placed in a drying oven at 90°C for 24 hours. After this drying procedure the specimen was reweighed, and the ratio of the weight before and after drying was calculated. Lung edema was represented by an increase in this ratio.

**Measurement of TNF-α level in plasma.** Blood samples were collected by the intravenous route at baseline (0.5 hour) and one, 2, 3, 12, and 24 hours after blunt chest trauma, the blood was centrifuged to separate the plasma. The plasma concentrations of TNF-α were detected by ELISA according to the kit instructions (American R & D System, Incorporation, Minneapolis, USA).

**Assay of caspase-3, Bcl-2/ Bax expressions.** Expressions of caspase-3, and Bcl-2/ Bax were determined using an SABC kit (Boster Bio-Engineering Co., Ltd., Wuhan, China). The staining was determined positive when the cytoplasm turned brownish yellow. Five different visual fields under a light microscope (x400) of each respective section were chosen and the mean optical densities (ODs) of caspase-3, Bax, and Bcl-2 positive cells from each section were analyzed with a color image pattern analysis system (HPIAS-1000, Yuanda Technology Corporation, Beijing, China).

**Statistical analysis.** Results were presented as mean ± SD. The data were analyzed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) version 13.0 for Windows. Statistical evaluation of the data was performed by two-way analysis of variance, followed by the t-test as a post hoc test. P<0.05 was considered statistically significant.

**Results.** Change of ALI in different groups. As shown in Figure 1a, blunt chest trauma caused excessive alveolar interstitial edema, hemorrhage, thickening of

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**Figure 1** - Change of acute lung injury in the different groups showing: a) Histological analysis (x100, H&E staining) of lung tissue harvested from control tissue or 3 hours after blunt chest trauma in each group. Blunt chest trauma caused an excessive alveolar interstitial edema, hemorrhage, thickening of the alveolar wall, and infiltration of inflammatory cells into the interstitial and alveolar spaces (middle), which are significantly different to control tissue (group C, left). These features were dramatically diminished in rat treated with ligustrazine. There was minimal edema, hemorrhage, and inflammatory (right). b) Summary of scores of acute lung injury under condition of control, trauma, and treatment of ligustrazine. *p<0.05, **p<0.01 versus control, and #p<0.05 versus trauma by ANOVA and t-test. C (control group; n=60), C+L (control+ligustrazine group; n=60); T (trauma group; n=60); T+L (trauma+ligustrazine group; n=60).
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There was no significant difference between the control group, and the control plus ligustrazine group at each time point. \( p=0.02 \); there was no significant difference between the control group, and the control plus ligustrazine group at each time point. The ALI score in the trauma plus ligustrazine group was lower than that in the trauma group (3.46±0.12 versus 1.24±0.06, \( p=0.02 \)). No statistical difference was found between the trauma plus ligustrazine group, and the control group (Figure 1b).

**Change of apoptosis index in the different groups.** As compared with the control group, the AI in the trauma group was markedly increased 2 hours after trauma and continued to increase over the whole procedure (5.83±0.83 versus 2.97±0.56, \( p=0.02 \)). The AI of the trauma plus ligustrazine group was lower than that of the trauma group (\( p=0.03 \)), but higher than the control group (Figure 2).

**Change of pulmonary edema, and pulmonary microvascular permeability.** The pulmonary microvascular permeability and pulmonary edema formation assessed by Evans blue dye extravasation and wet/dry weight ratios of lung tissues are shown in

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**Figure 2** - Apoptosis index in the different groups showing: a) Changes of apoptosis (TUNEL-positive cells) of lung in each group by TUNEL (TUNELx400). There were almost no cells positive for TUNEL in the control group (group C, left). Trauma induced more TUNEL-positive cells; the nucleus of these cells was brownish yellow or dark brown (Group T, middle). There were a few TUNEL-positive cells after treatment with ligustrazine (right). Arrows show the TUNEL-positive cells. b) Apoptosis index was significantly lower in the ligustrazine-treated group than in the trauma group at every time point. \( *p<0.05 \), \( **p<0.01 \) versus control, and \( #p<0.05 \) versus trauma by ANOVA and t-test. C (control group; n=60), C+L (control+ligustrazine group; n=60); T (trauma group; n=60); T+L (trauma+ligustrazine group; n=60).

**Figure 3** - Evans blue dye concentrations in lung tissue in the different groups. Evans blue dye concentrations were significantly lower in the ligustrazine-treated group than in the trauma group. \( *p<0.05 \), \( **p<0.01 \) versus control, and \( #p<0.05 \) versus trauma by ANOVA and t-test. C (control group; n=60), C+L (control+ligustrazine group; n=60); T (trauma group; n=60); T+L (trauma+ligustrazine group; n=60).

**Figure 4** - Lung tissue wet/dry (W/D) weight ratios in the different groups. Wet/dry weight ratios of lung tissue were significantly lower in the ligustrazine-treated group (T+L group) than in the trauma group at the same time point. \( *p<0.05 \), \( **p<0.01 \) versus control, and \( #p<0.05 \) versus trauma by ANOVA and t-test. C (control group; n=60), C+L (control+ligustrazine group; n=60); T (trauma group; n=60); T+L (trauma+ligustrazine group; n=60).

**Figure 5** - Blood plasma tumor necrosis factor (TNF)-\( \alpha \) level in the different groups. The expression of TNF-\( \alpha \) began to increase at one hour, and peaked at 3 hours after blunt chest trauma, then gradually declined at 12 and 24 hours after blunt chest trauma. Ligustrazine significantly decreased the plasma level of TNF-\( \alpha \). \( *p<0.05 \), \( **p<0.01 \) versus control, and \( #p<0.05 \) versus trauma by ANOVA and t-test. C (control group; n=60), C+L (control+ligustrazine group; n=60); T (trauma group; n=60); T+L (trauma+ligustrazine group; n=60).
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The mean Evans blue dye concentration in the trauma group was significantly higher than those in the control group ($p=0.002$). Ligustrazine treatment significantly reduced the blunt chest trauma induced pulmonary microvascular dysfunction ($p=0.04$). Similarly, the mean wet/dry weight ratio of lung tissues in the trauma group was significantly higher than those in the control group ($p=0.003$). Ligustrazine administration significantly decreased the blunt chest trauma related pulmonary edema ($p=0.04$).  

**Change of plasma TNF-α levels in the different groups.** As shown in Figure 5, the expression of TNF-α began to increase at one hour, and peaked 3 hours after blunt chest trauma. This then gradually declined at 12 and 24 hours after blunt chest trauma (382.3±52.5 versus 134.5±39.1, $p=0.001$). Ligustrazine significantly decreased the plasma level of TNF-α ($p=0.03$). There was no significant difference between the trauma group, and the trauma plus ligustrazine group at 12 and 24 hours after blunt chest trauma.

**Change of caspase-3, and the ratio of Bcl-2/Bax in the different groups.** As shown in Figures 6 & 7, the expression of caspase-3, Bax, and Bcl-2 were determined by mean optical densities (OD) value. The expressions of caspase-3 (a) were significantly lower in the ligustrazine-treated group (group T+L) than in the trauma group (group T) at the same time point. The ratio of Bcl-2 to Bax (d) increased significantly in the ligustrazine-treated group (group T+L) compared with the trauma group (group T) at the same time point. *$p<0.05$ versus control, $p<0.05$ versus trauma by ANOVA and t-test.

C (control group; n=60), C+L (control+ligustrazine group; n=60); T (trauma group; n=60); T+L (trauma+ligustrazine group; n=60).
trauma \( (p=0.02, \text{ Figures 7a, 7c, and 7d}) \). Ligustrazine decreased the expression of Caspase-3 and Bax, but enhanced the increase of Bcl-2, thus, the ratio of Bcl-2/Bax markedly increased during the entire experiment over 24 hours (0.716±0.018 versus 1.292±0.033, \( p=0.03 \), Figure 7b).

Discussion. This study demonstrates that ligustrazine provided protection against apoptosis and ALI induced by blunt chest trauma in rats. The main findings are as follows: 1) Ligustrazine causes a substantial amelioration of lung injury induced by blunt chest trauma. 2) Ligustrazine attenuated apoptosis in the lung caused by blunt chest trauma. 3) Ligustrazine decreased the expression of TNF-\( \alpha \), increased the Bcl-2/Bax ratio and decreased the cleaved Caspase-3 level. All these data support the view that ligustrazine can attenuate inflammation and apoptosis caused by blunt chest trauma in rats.

We confirmed that severe blunt chest trauma can induce ALI and apoptosis, an important factor in the pathogenesis of pulmonary edema secondary to injury. The AI in the lung tissue rose significantly after blunt chest trauma, and the lung tissues were significantly damaged after blunt chest trauma under a light microscope. The lung injury and AI induced by trauma were alleviated by the ligustrazine treatment. Evans blue dye extravasation and the wet-to-dry weight ratio of lung tissue were used in the current study to assess the changes in the microvascular permeability. They were significantly higher when the animals received blunt chest trauma, and recovered to normal after ligustrazine treatment. All of these results indicate that ligustrazine can attenuate ALI and apoptosis in the lung, and reduce the pulmonary microvascular dysfunction and pulmonary edema induced by the blunt chest trauma.

The protective effect of ligustrazine on ALI may benefit from its antioxidant properties. Several studies have shown that ligustrazine has strong effects of scavenging cytotoxic oxygen free radicals. Some reports revealed that ligustrazine reduced mice renal ischemia/reperfusion injuries partially by decreasing the generation of reactive oxygen species (ROS), inhibition of apoptosis and overexpression of TNF-\( \alpha \). Ligustrazine can also remove oxygen free radicals by inactivating xanthinoxidase and raising superoxide dismutase activity.

We found that ligustrazine can attenuate apoptosis induced by ALI in this study. Apoptosis is a highly regulated and intrinsic cell-suicide program, which is important in both physiological and pathological conditions. Apoptosis induced by traumatic ALI involves the release of a large number of inflammatory mediators including TNF, IL-1, IL-6, IL-8, IL-10, and platelet activating factor. The TNF-\( \alpha \) not only plays a dynamic and pivotal role in the beginning of the apoptosis, but also is the central mediator and the trigger of apoptosis. In our study, the serum TNF-\( \alpha \) concentration began to increase at one hour, and almost peaked at 2 hours after blunt chest trauma, this subsequently decreased, and returned to normal at 24 hours after blunt chest trauma. The results indicate that the severity of apoptosis, and the lung injury induced by trauma began to intensify at 2 hours and peaked 3 hours after trauma. Therefore, the release of TNF-\( \alpha \) after the traumatic lung injury might play a dynamic and pivotal role in the induction of the apoptosis and ALI. Administration of ligustrazine resulted in a dramatic decrease of TNF-\( \alpha \) and apoptosis, suggesting that ligustrazine can inhibit the production and release of interleukin of lung cells and indirectly inhibit cell abnormal apoptosis.

The caspase cascades and Bcl-2 families are demonstrated to be critical in the regulation of apoptosis. As a distal executioner, caspase-3 can transform from a proenzyme to the active form by apoptotic triggers-induced caspase-cascade activation, and then mediate the apoptotic cascade. Both Bcl-2 and Bax belong to the Bcl-2 gene family, which are crucial in determining cell fate in the apoptotic pathway. The Bcl-2 has been suggested to act as an anti-oxidant and prevent cell death by its anti-oxidizing effects, or by suppressing the production of oxygen free radicals. The death promoting protein Bax counteracts the anti-apoptotic effects of Bcl-2 by forming a heterodimer with Bcl-2. When the expression level of Bax increases, the effect of Bcl-2 is antagonized, thus promoting apoptosis. Therefore, the ratio of Bcl-2 to Bax, rather than the levels of the individual proteins, is considered to be critical in determining the survival/death of cells.

In this study, we showed that activation of caspase-3 in lung cells accompanied a prominent decrease of Bcl-2 and increase of Bax after blunt chest trauma, which is accompanied with the increase of apoptosis index. It is suggested that decreased levels of Bcl-2 may accelerate caspase-3 activation during apoptosis induced by trauma in the lung. Consistent with its anti-apoptotic effect to lung injury, ligustrazine treatment decreased expression of caspase-3 and Bax, while increasing the expression of Bcl-2. Furthermore, the ratio of anti-apoptotic Bcl-2 to pro-apoptotic Bax was prominently increased. These results imply that changes in the expressions of caspase-3, Bcl-2 and Bax, especially the ratio of Bcl-2 to Bax intimately influenced the development and severity of apoptosis induced by blunt chest trauma.

Although the current study indicated that the changes of caspase-3, Bcl-2, and Bax are important for the treatment of ligustrazine on apoptosis induced by blunt chest trauma, further detailed studies need to be
performed to analyze the effects of ligustrazine treatment on caspase-3 activity and cellular translocation of Bax.

We conclude that ligustrazine provides beneficial action against apoptosis and ALI induced by blunt chest trauma. The beneficial effects of ligustrazine can be attributed to its anti-apoptosis and anti-inflammatory properties with a mechanism that inhibits the production of TNF-α, increases the ratio of Bcl-2 to Bax, and decreases the expression of caspase-3. We propose that ligustrazine may be useful in enhancing the tolerance of lungs against apoptosis and injury in situations where the lung tissues are subject to blunt chest trauma.

References
