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Original Article

Glycyrrhizin acid prevent hydrochloric acid-induced inhalational lung injury in mice through inhibition of MAPK pathway

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Abstract: Inhalational lung injury is easy to cause acute respiratory distress syndrome (ARDS), which is one of the most serious complications leading to death or disability in severe patients. Glycyrrhizin acid (GA) has the effects of anti-inflammation, anti-oxidation and immune regulation. This study explored the protective mechanism of GA to inhalational lung injury in mice. Hydrochloric acid (HCL)-induced inhalational lung injury model was used in this study and the mice were divided into control group, HCL group, HCL plus GA group, and GA group. In the HCL group, serum TNF-α, IL-1β, IL-6 and MPO levels and MDA activity were increased, Superoxide Dismutase (SOD) activity was decreased, pulmonary edema of mice was evidenced by HE staining and JNK/P38 signal pathway was activated. After treatment of GA, the TNF-α, IL-1β, IL-6 and MPO levels and MDA activity in serum were decreased, and the SOD activity was increased. Pulmonary edema of mice was alleviated as indicated by HE staining and the expression of JNK/P38 signal pathway was also inhibited. From these results, we can conclude that GA has a protective effect on HCL-induced inhalational lung injury.

Keywords: Glycyrrhizin acid, inhalational lung injury, oxidative stress, MAPK pathway

Introduction

Acute respiratory distress syndrome (ARDS) is caused by multiple factors, associated with a variety of diseases, and has high mortality and disability rates in severe patients [1, 2]. About 11% of inhalational lung injury is caused by reflex aspiration [3]. Hydrochloric acid (HCL)-induced inhalational lung injury is manifested by acute progressive hypoxemia, dyspnea, respiratory distress and even respiratory failure, and may be complicated with pulmonary edema, pulmonary collapse, pulmonary infection and atelectasis. Glycyrrhizin acid (GA) is extracted from licorice, a Chinese herbal medicine, which is reported to have anti-inflammation, hypoglycemic, immune regulation, anti-oxidation and anticancer effects [4, 5]. In this paper, mice model was established to study the protective effect and mechanism of GA on HCL-induced inhalational lung injury.

Materials and methods

Drugs and antibodies

GA was purchased from Sellcek Chemicals (USA) and dissolved in 0.9% NaCl to a concentration of 5 mg/ml before use. The antibodies used for western blot, including anti-P38, anti-p-P38, anti-JNK, anti-p-JNK and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Animals

All animal care and procedures used in this study were in compliance with the guidelines on the use of animals of the Ethics Committee on Experimental Animals of Shanghai Jiaotong University School of Medicine. The experiments were conducted using BALB/c mice (n=32, 5-7 weeks old and weighing 20-25 g, Shanghai

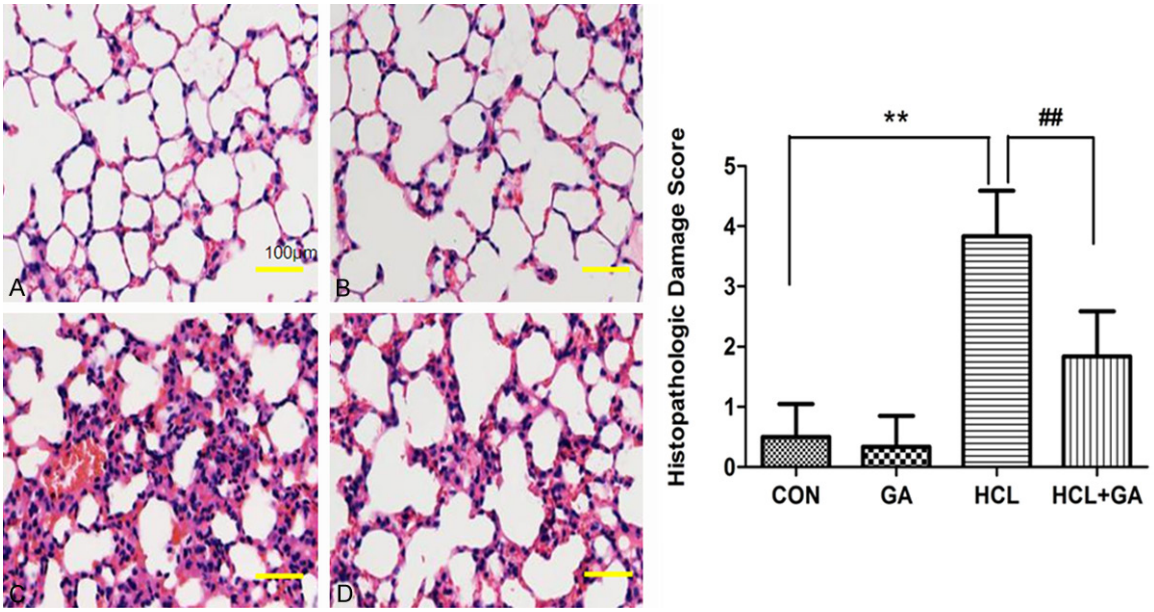

Figure 1. HE staining (200 × magnification) and histopathologic damage score of different groups. HE staining: A: The control group; B: GA group; C: Diffuse pulmonary edema in the HCL group; D: Slighter pulmonary edema in the HCL plus GA group. Bar=100 μm. Histopathologic damage score: data are shown as mean ± SD (n=8), **, p<0.01; ##, p<0.01.

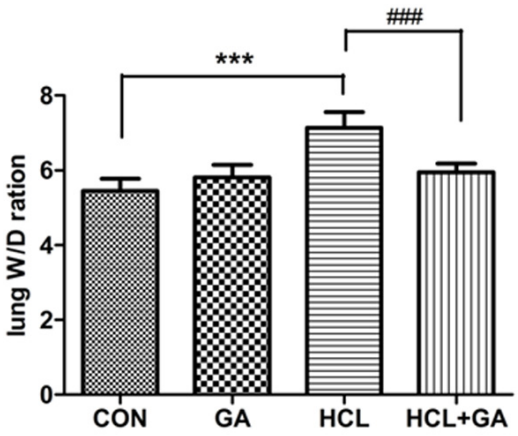

Figure 2. The W/D weight ratios of different groups. Data are shown as mean ± SD (n=8). The W/D weight ratio of the HCL group was significantly higher than that of the control group (***, p<0.001), while the W/D weight ratio of the HCL plus GA group was significantly lower than that of the HCL group (###, p<0.001).

SLAC Laboratory Animal Co. Shanghai, China) that were kept in controlled temperature (22 ± 2°C) and humidity (45%-55%) under a 12:12 h light-dark cycle and fed on standard laboratory diet and water. Thirty-two mice were randomly divided into four groups (n=8 per group): control group, GA (50 mg/kg) group, HCL group, HCL plus GA (50 mg/kg) group.

Acid-induced lung injury

The mice were anesthetized with chloral hydrate (10%, 0.4 mg/kg), and hydrochloric acid (0.1 N HCl, pH 1.5, 2 ml/kg) was intratracheally instilled into lung via a 24-gauge angiocatheter. At 4 h following acid-induced lung injury, blood and lung tissue were harvested [6]. One group of animals was instilled with saline into lung (the control group, n=8).

Treatment with GA

The mice of HCL plus GA group (n=8) were intraperitoneally injected with GA (50 mg/kg) [7]. After 1 h, the mice were anesthetized with 10% chloral hydrate through intraperitoneal injection, and then HCL was intratracheally instilled into lung (the same as HCL group). Blood and lung tissue were harvested after 4 h following the HCL instilling.

Hematoxylin-eosin (H&E) staining

Lung tissue samples were fixed in 10% buffered formalin for 48 h and then dehydrated by washing in ascending grades of ethanol. Samples were then sectioned and embedded in paraffin wax. 5-μm lung sections were prepared for routine H&E stains. H&E stain was used to observe the morphological changes to

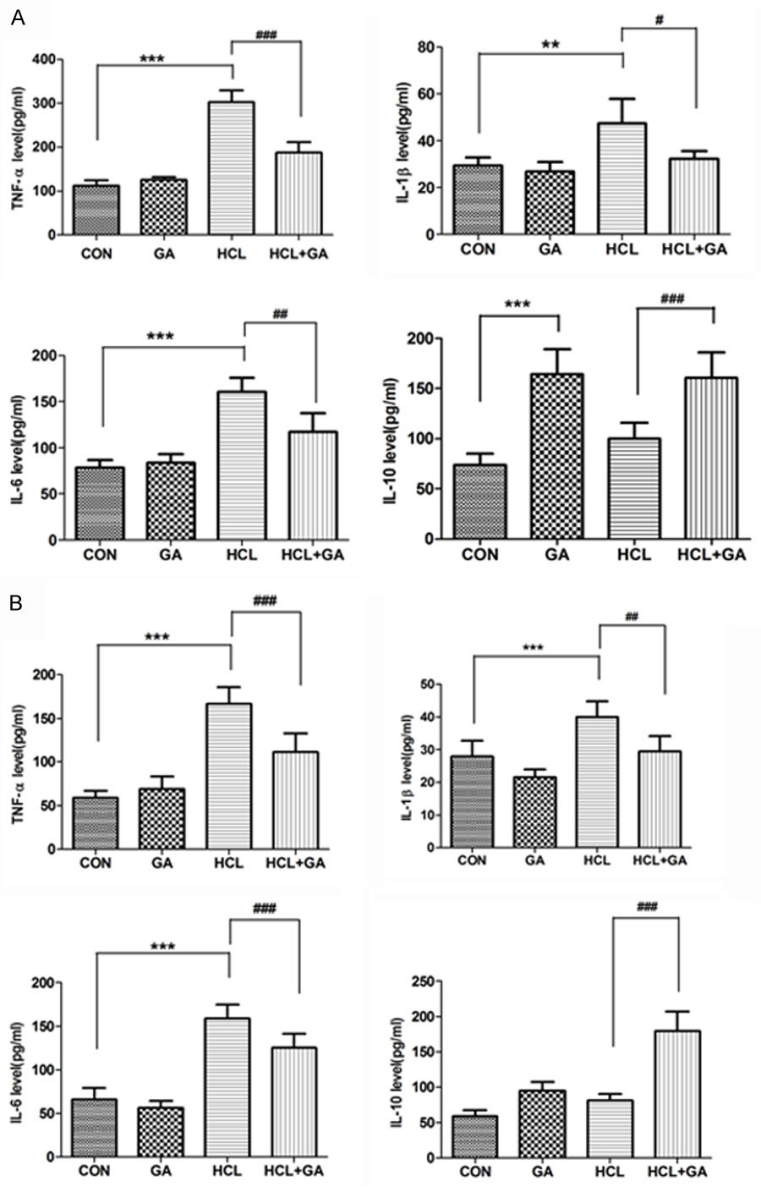

Figure 3. GA reduced the inflammation in hydrochloric acid-induced inhalational lung injury in mice. A: Inflammatory mediators in serum. B: Inflammatory mediators in lung tissue. Data are shown as mean ± SD (n=8). ***, p<0.001; **, p<0.01; ###, p<0.001; ##, p<0.01; #, p<0.05.

the lung tissue sections among the various groups. Under 200× magnification, each section was randomly selected and five fields of view were photographed. The total surface of the slides was scored by two blinded pathologists with expertise in lung pathology. The criteria for scoring lung inflammation was set up as previously described with some modifications [8]: 0, normal tissue; 1, minimal inflammatory change; 2, mild to moderate inflammatory changes (no obvious damage to the lung architecture); 3, moderate inflammatory injury (thickening of the alveolar septae); 4, moderate to severe inflammatory injury (formation of nodules or areas of pneumonitis that distorted the normal architecture); and 5, severe inflammatory injury with total obliteration of the field.

Lung Wet/Dry (W/D) weight ratio

Lung W/D weight ratio was determined to assess the lung edema. The fresh right middle lobe was measured using an electronic scale and then dried in an oven at 85°C for 48 h until the weight remained constant. Dry weights were measured, and the W/D ratios were calculated.

Measurement of IL-6, IL-1β, IL-10 and TNF-α levels

IL-6, IL-1β, IL-10 and TNF-α levels in blood and lung tissue were assayed by ELISA according to the manufacturer's instructions (JRDUN Biotechnology Co., Ltd, Shanghai, China). Concentrations were calculated by generating a standard curve using standard proteins.

Detection of MPO and SOD activities and MDA concentration

Lung samples were homogenized with 0.9% normal saline and then centrifuged. Superoxide dismutase (SOD) activity, myeloperoxidase (MPO) activity, and the malondialdehyde (MDA) concentration were determined using commercial kits produced by JRDUN Biotechnology (Shanghai, China). MPO activity and SOD activity were measured by colorimetry. MPO activity is expressed as units/g of protein and SOD activity is expressed as units/mg of protein. The MDA concentration is expressed as mM/mg of protein (mM/mgprot).

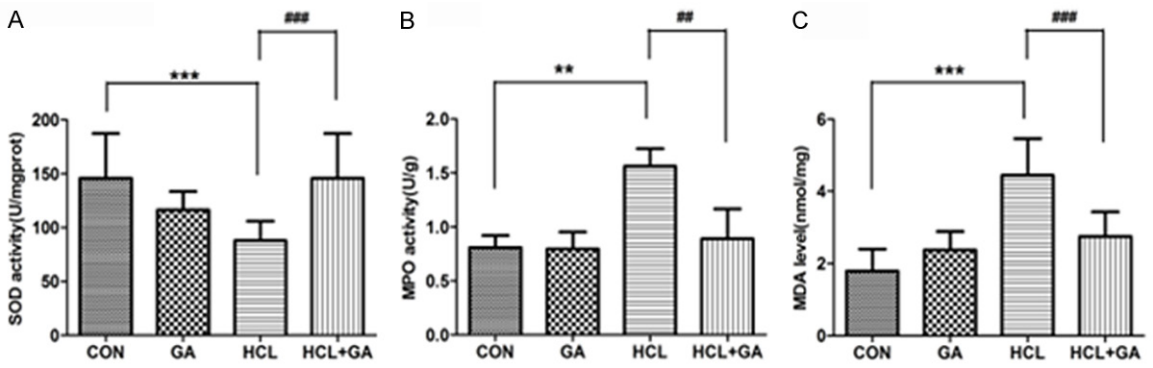

Figure 4. Effect of GA on oxidative stress damage induced by lung injury. A. SOD activity (U/mg); B. MPO activity (U/g); C. MDA level (mmol/mg). Data are shown as mean ± SD (n=8). **, p<0.01; ***, p<0.001; ##, p<0.01; ###, p<0.001.

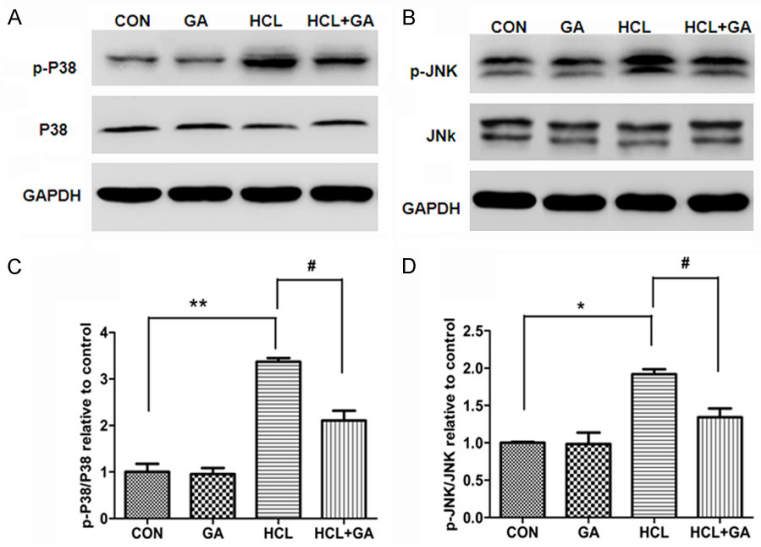

Figure 5. GA inhibits the activation of JNK and p38 MAPK pathways. The contents of p-P38 and p-JNK1/2 in lung tissue were detected by Western Blot and were analyzed using gray value. GAPDH was used as negative control. The results were expressed as mean ± SD (n=8). *, p<0.05; **, p<0.01; #, p<0.05.

Western blot analysis

Lung tissues were homogenized in cold RIPA buffer (Beyotime, Jiangsu, China) by an ultrasonic vibrator and a mechanical homogenizer. Isolation of nuclear and cytoplasmic protein extraction were kitted according to the manufacture's instruction (Beyotime). Proteins (40 μg) were separated by 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes (Millipore Corp., Bedford, MA, USA). After being blocked with 5% non-fat dry milk in PBS, and incubated with anti-P38 (1:1000), anti-p-P38 (1:1000), anti-JNK (1:1000), anti-p-JNK (1:1000), anti-GAPDH (1:1500) antibodies, respectively, at 4°C overnight, the membranes were incubated with a secondary horseradish peroxidase-conjugated IgG (1:1000, Santa Cruz Biotechnology) for 1 h at room temperature, followed by ECL detection reagent (Beyotime). The optical densities of the bands were scanned and quantified by Genesnap and Genetools software (Syngene).

Statistical analysis

All data are expressed as the means ± SD. Unpaired Student *t*-test was employed for analyzing histopathologic damage score and lung Wet/Dry weight ratio. One-way analysis of variance followed by Student-Newman-Keuls test was used to analyze the concentration of inflammatory cytokines, MPO activity, SOD activity, MDA concentration and JNK pathway and P38 MAPK pathway. A *p* value<0.05 was considered statistically significant. Statistical analyses were conducted with SPSS22.0.

Results

GA alleviates pulmonary edema

As shown in **Figure 1**, in the control group and GA group, the lung tissue was intact with clear

lobular and alveolar cavity, and no edema or inflammatory cell infiltration was observed in the interstitium. In the HCL group, diffuse pulmonary edema, thickened alveolar septum and neutrophil infiltration were observed in the lung tissue. In the HCL plus GA group, slight pulmonary edema, slightly thickened alveolar septum and mild neutrophil infiltration were observed in the lung tissue, which was much milder than the HCL group.

The histopathologic damage score of the HCL group was significantly higher than that of the control group (3.83 ± 0.75 vs. 0.5 ± 0.548, $p<0.01$), and the score was significantly decreased after treatment of GA (1.83 ± 0.75 vs. 3.83 ± 0.75, $p<0.01$). As shown in **Figure 2**, the W/D weight ratio of the HCL plus GA group was significantly lower than that of the HCL group ($p<0.001$). From these results, GA can alleviate HCL-induced inhalational lung injury in mice.

GA reduces inflammatory mediators

As shown in **Figure 3A**, in HCL group, HCL significantly increased serum TNF-α, IL-1β and IL-6 levels of mice compared with the control group (***: $p<0.001$), while after the treatment of GA, the levels of the inflammatory mediators induced by HCL were decreased. The IL-10 level was not increased in the HCL group, but in HCL plus GA group, IL-10 level in serum was increased significantly (###: $p<0.001$ vs. HCL group). The changes of the inflammatory mediators in lung were consistent with that in serum (**Figure 3B**).

GA shows antioxidant effect (MDA+SOD, MPO)

To evaluate the effect of GA on HCL-induced oxidative stress damage, we measured SOD activity, MPO activity and MDA level. As shown in **Figure 4**, SOD activity in the HCL group was significantly lower than that in the control group (**Figure 4A**: ***: $p<0.001$, HCL group vs. control group), -after the treatment of GA, the SOD activity was increased. (**Figure 4A**: ###: $p<0.001$, HCL plus GA group vs. HCL group). MPO activity of the HCL group was higher than that of the control group (**Figure 4B**: ***: $p<0.001$, HCL group vs. control group), while MPO activity in the HCL plus GA group was decreased (**Figure 4B**: ##: $p<0.01$,HCL plus GA group vs. HCL group). MDA level in the HCL group was increased (**Figure 4C**: ***: $p<0.001$ vs. control group) while the MDA level in the HCL plus GA group was decreased (**Figure 4C**: ###: $p<0.001$ vs. HCL group).

Effect of GA on JNK and P38 MAPK pathways

JNK and P38 proteins were selected to study the mechanism of GA improving HCL-induced inhalational lung injury. As shown in **Figure 5**, the level of p-JNK and p-P38 were elevated in the HCL group compared with the control group, so inhaling HCL can activate JNK pathway and P38 MAPK pathway, and after treatment of GA, the -p-JNK level and p-P38 level were obviously decreased in the HCL plus GA group compared with the HCL group. We found GA can inhibit the JNK pathway and P38 MAPK pathway.

Discussion

Reflux aspiration often occurs in the process of anesthesia induction and recovery as well as in the patients suffering from loss of consciousness such as cerebrovascular disease. Pulmonary aspiration of gastric acid may cause acute chemical lung injury. The early lung injury is caused by the action of gastric acid on alveolar epithelial and pulmonary capillary. The late lung injury is mainly related with neutrophils-mediated immune responses: the neutrophils gather and infiltrate into lung and extrapulmonary tissues, release reactive oxygen species and enzymes, which induce injury of tissue [9]. About one third of patients showed severe clinical symptoms, 30% of whom will develop high mortality of ARDS [10, 11]. In this study, mice model of HCL-induced lung injury via airway inhalation was used to mimic ARDS. This animal model leads to direct lung injury, characterized by damage of integrity of alveolar capillary, pulmonary edema, and increased airway resistance and diffusion dysfunction caused by alveolar hemorrhage and decrease of alveolar surfactants, which are similar to pathological manifestations of patients [12].

In this study, GA was used to protect lung from HCL-induced lung injury. GA was extracted from licorice, a traditional Chinese Medicine herb. As the main active ingredient of licorice, GA has extensive pharmacological effects, such as anti-inflammation, hypoglycemic activity, anti-oxidant, anti-tumor, anti-bacteria and anti-virus effects [4, 5]. GA shows anti-inflam-

mation effect by inhibiting the expression of inflammatory cytokines and neutrophil infiltration [13]. IL-1β is an important proinflammatory cytokine at the early stage of inflammation, and IL-6 is a multi-effect cytokine with extensive biological activities in acute and chronic inflammation, vascular diseases and tumor diseases [14]. These cytokines such as TNF-α, IL-1β and IL-6 may cause lung inflammation, facilitate interaction between neutrophils and special cytokines and release reactive oxygen metabolites, which become worse with increase of neutrophils [15-17]. Higher TNF-α, IL-1β and IL-6 levels in serum or lung lead to higher incidence and severity of ARDS, resulting in higher mortality rate [16, 18]. In this study, HCL significantly increased TNF-α, IL-1β and IL-6 levels in serum and lung tissue of mice as compared with the control group (**Figure 3**, *p<0.001*). As evidenced by HE staining and histopathologic damage score analysis (**Figure 1**), HCL induced diffuse edema and extensive neutrophils infiltration in lung of mice as well as a high damage score. As compared with the control group, the W/D ratio of the HCL group was significantly increased (**Figure 2**, $p<0.001$). These results indicated that inhalation of HCL through airway can cause acute lung inflammation and acute pulmonary edema. After treatment of GA (HCL plus GA group), the TNF-α, IL-1β and IL-6 levels in serum and lung tissue of mice were decreased (**Figure 3**, *p<0.001*). Lung edema and neutrophil infiltration were also observed in the mice of HCL plus the GA group but they were less severe with a lower score when compared with the HCL group (**Figure 1**). The W/D ratio of the HCL plus GA group was higher than that of the control group (**Figure 2**, $p<0.05$) but lower than that of the HCL group (**Figure 2**, $p<0.001$). To sum up, lung edema, neutrophil infiltration and release of inflammation mediators remained in mice pre-treated with GA but were obviously alleviated when compared with the HCL group, indicating that GA can inhibit release of inflammation mediators, neutrophil infiltration, alleviate inflammation response and lung edema. The results of GA group were similar to those of the control group, suggesting that GA is safe to mice.

As a multifunctional negative regulator, IL-10 is involved in biological regulation of immune cells, inflammatory cells, tumor cells and some other cells and inhibits production of IL-1, IL-6, IL-8, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) in the inflammatory response, thus showing an anti-inflammatory effect [19]. Therefore, IL-10 is an anti-inflammatory factor and play important roles in down-regulation of the inflammatory response and inhibition of inflammatory mediators. The results of this study showed that the IL-10 levels in serum and lung tissue of mice in the HCL plus GA group was higher than that in the HCL group (**Figure 3**, $p<0.001$), demonstrating the anti-inflammatory of GA.

MDA is an aldehyde substance produced in free radical-induced lipid peroxidation, which can indirectly reflect the degree of oxidative damage [20]. SOD is an important enzyme for the defense of superoxide anion in the inner and outer environment [21, 22]. Increase of MDA level and decrease of SOD activity can lead to oxidative stress response, resulting in cell damage and even cell death. MPO is involved in many processes that regulate inflammatory control, and its activity changes represent the function and activity of neutrophils [22, 23]. The results of this study showed that HCL increased MDA level and MPO activity and decreased SOD activity, indicating that HCL led to increased lung inflammation and pulmonary edema in mice. After treatment of GA, MDA level and MPO activity decreased and SOD activity increased, suggesting that GA reduced the lung inflammation by improving responses to oxidative stress (**Figure 4**, $p<0.001$). Zhao et al. demonstrated that GA could inhibit oxidative stress to relieve sepsis-induced acute lung injury [7].

Mitogen-activated protein kinase (MAPK) is a group of serine-threonine protein kinases that can be activated by different cytokines, neurotransmitters, hormones and so on. MAPK includes extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK), P38-MAPK and ERKS/BMKI [24]. The most important signal pathways are JNK and P38 MAPK. JNK signal pathway plays a vital role in the development and progression of various diseases such as cell apoptosis and stress response [25]. P38 MAPK can be activated by heat shock, pro-inflammatory cytokines (TNF-

α, IL-1), LPS and other stress responses. Activated P38 MAPK influences cell proliferation and differentiation and synthesis of cytokines by up-regulating the expression and biological activity of certain transcription factor genes [24, 26, 27]. It has been demonstrated that the activation of P38 MAPK signal pathway is related with the development of seizure-induced lung injury [28, 29]. In this study, inhaling HCL induced increase of p-JNK and p-P38 level, (**Figure 5**), indicating that HCL-induced inhalational lung injury was associated with activation of JNK and P38 MAPK signal pathways. The expression of JNK and P38 signal pathways was decreased after treatment of GA (**Figure 5**), indicating that GA could inhibit the activation of JNK and P38 signal pathways. So, JNK and P38 signal pathways are important molecular mechanisms in protection of inhalational lung injury by GA.

In conclusion, our study demonstrated that GA can improve hydrochloric acid-induced inhalational lung injury, inhibit cell apoptosis of lung tissue by inhibiting JNK and P38 signal pathways, inhibit release of inflammatory factors, inhibit pulmonary inflammatory response and oxidative stress and improve pulmonary edema. Therefore, GA may serve as a potential therapeutic agent for the clinical treatment of inhalational lung injury.

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Disclosure of conflict of interest

None.

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