Original Article
Study on protective effect and possible mechanisms of swertiamarin against sepsis-induced acute lung injury in rats

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Abstract: This study was designed to investigate the protective effect and possible mechanisms of swertiamarin against sepsis-induced acute lung injury (ALI) in rats. Sepsis was induced by cecal ligation and puncture (CLP). The pulmonary gas exchange function (PaO_2/FiO_2 ratio) and the lung water content [lung wet/dry weight (W/D) ratio] were measured by blood-gas analyzer and analytical balance. The blood capillary membrane permeability indices (neutrophils and lymphocytes counts and protein content) and the levels of inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-10) in bronchoalveolar lavage fluid of rats were determined by light microscopy and Elisa. The oxidative stress parameters [activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and levels of malondialdehyde (MDA) and glutathione (GSH)], the activity of myeloperoxidase (MPO) and the levels of NF-κB p65, p-IKKα/β (Ser 180/Ser 181) and IκBα proteins in lung tissue of rats were determined by Elisa and western blot. The survival rate of rats with CLP-induced ALI were recorded and analyzed by survival analysis method. The results indicated that swertiamarin significantly increased the PaO_2/FiO_2 ratio, the activities of SOD and GSH-Px, the levels of IL-10, GSH and IκBα and decreased the lung W/D ratio, the neutrophils and lymphocytes counts, the protein content, the activity of MPO, the levels of TNF-α, IL-1β, IL-6, MDA, NF-κB p65 and p-IKK α/β (Ser 180/Ser 181). Benefiting from these changes as described above, the survival rate of rats with CLP-induced ALI was significantly increased after treatment with swertiamarin. In conclusion, swertiamarin showed protective effect against CLP-induced ALI in rats, and the mechanisms of action might be related to reducing blood capillary membrane permeability, inhibiting inflammatory response and increasing anti-oxidant ability in lung of rats with CLP-induced ALI.

Keywords: Swertiamarin, cecal ligation and puncture, sepsis, acute lung injury, inflammation, oxidative stress

Introduction

Sepsis, induced by infection, is a kind of systemic inflammatory response syndrome (SIRS) and the complication of severe trauma, burns, ischemia-reperfusion injury and major surgery [1, 2]. Sepsis is a clinical syndrome of high morbidity and mortality and the lead cause of mortality in intensive care units (ICU) [3-5]. It was estimated that each year more than 210,000 people die of severe sepsis in America, and the mortality of sepsis reaches to 30-70% in the globe [6-8]. Although the modern medical technology, new antibacterial drug and organ function support are widely applied to treat sepsis, severe sepsis has not been contained with population aging, bacterial resistance, the

increase of traumatic therapy, etc. [9]. Severe sepsis leads to multiple organs injury, which are eventually developed into multiple organ dysfunction syndromes (MODS). Lung is the most easily damaged organs in all organs, and severe sepsis leads to the acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) [3, 10]. Sepsis-induced ALI is a clinical syndrome of high morbidity and mortality in ICU [11]. At present, there are not special preventive measures to treat sepsis-induced ALI. Supportive treatment (breathing machine) is the lead treatment to prevent sepsis-induced ALI in clinical therapy, but excessive mechanical ventilation also leads to the occurrence of inflammation in lung, which further exacerbates lung injury [11, 12]. The sepsis-induced ALI is directly related
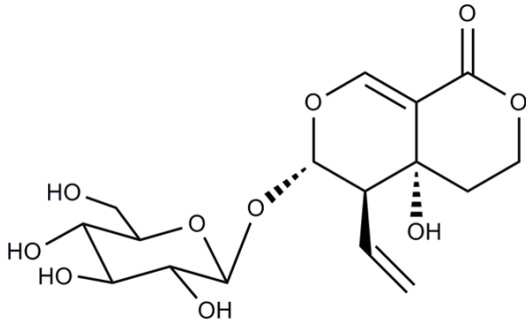
Figure 1. Chemical structure of swertiamarin.

to out of control of inflammatory response in lung [13, 14]. Developing effective anti-inflammatory is a feasible method to reduce the morbidity and mortality of sepsis-induced ALI.

Swertiamarin (**Figure 1**) is widely distributed in the *Swertia* plants (Gentianaceae), such as *S. davidii*, *S. franchetiana*, *S. musosii Franch*, *S. erythrotictai Maxim*, *S. punicea Hemsl*, *S. mileensis*, *S. chirata* and *S. japonica Makino*. It's reported that swertiamarin has the liver protective, anti-oxidant, sedative, analgesic, anti-inflammatory and antimicrobial activities [15-18]. Additionally, it can also be used to eliminate edema, scavenge free radicals and improve diabetic nephropathy [19]. Based on the anti-inflammatory activity of swertiamarin, our team thought that swertiamarin may be used to protect against sepsis-induced ALI. Therefore, the work was designed to investigate the protective effect of swertiamarin on sepsis-induced ALI in rats and the possible mechanisms.

Materials and methods

Chemicals and reagents

Swertiamarin was purchased from National Institutes for Food and Drug Control (purity > 98%). Dimethyl sulphoxide (DMSO) was obtained from Sigma (Sigma, Germany). BCA protein quantitation kit was purchased from Abnova (Abnova, USA). TNF-α, IL-1β, IL-6, IL-10, myeloperoxidase (MPO), superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH) and glutathione peroxidase (GSH-Px) Elisa kits were purchased from Lengton (Shanghai, China), Biodee (Beijing, China) and Beyotime Biotechnology (Shanghai, China). Primary antibodies against NF-κB p65, p-IKKα/β (Ser 180/Ser 181), IκBα and β-actin and horse-radish peroxidase (HRP)-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, USA).

Animals

Male Sprague-Dawley rats (200 ± 20 g) were obtained from the Shandong University Laboratory Animal Center (Jinan, China). Animals were maintained under standard laboratory conditions (temperature: 25 ± 1°C, light 12 h/dark 12 h cycle) and had free access to food and water. All experiments were conducted in accordance with the international guidelines for care and use of laboratory animals. The experimental protocols were performed with the approval of the Animal Care and Use Committee of Qilu Hospital of Shandong University, Jinan, China (protocol number: QLH 2013049).

Grouping, administration and modeling

Rats were randomly divided into 6 groups (*n* = 10): sham, control, vehicle, low-dose swertiamarin (L-Swe) at 50 mg/kg, medium-dose swertiamarin (M-Swe) at 100 mg/kg and high-dose swertiamarin (H-Swe) at 200 mg/kg groups. Swertiamarin was dissolved in 1% (w/v) DMSO. Thirty minutes before CLP, the treatments were administrated intraperitoneally. Additionally, to study the effect of swertiamarin on the survival rate of rats with cecal ligation and puncture (CLP)-induced ALI, another 60 rats were randomly divided into 6 groups (*n* = 10) and treated as described above.

According to the reported method of Chaudry et al. [20], sepsis was induced by CLP with a modification. Briefly, rats were anesthetized with chloral hydrate anesthesia (400 mg/kg) by intraperitoneal injection (i.p.). After the abnormal fur was shaved, a 2-cm midline abdominal incision was made to expose the cecum and then the cecum was withdrawn through the incision. So that the intestinal continuity was maintained, the cecum was ligated below the ileocecal junction using a 3-0 silk suture. Using an 18-gauge needle, the cecum was punctured twice and then a spot of cecal contents was extruded through the puncture wound to confirm patency of the holes. After the bowel was returned to the abdomen, the incision was closed and then normal saline (24 mL/kg, i.p.) was administrated to supply body fluid loss of rats. In sham group, the cecum and bowel of
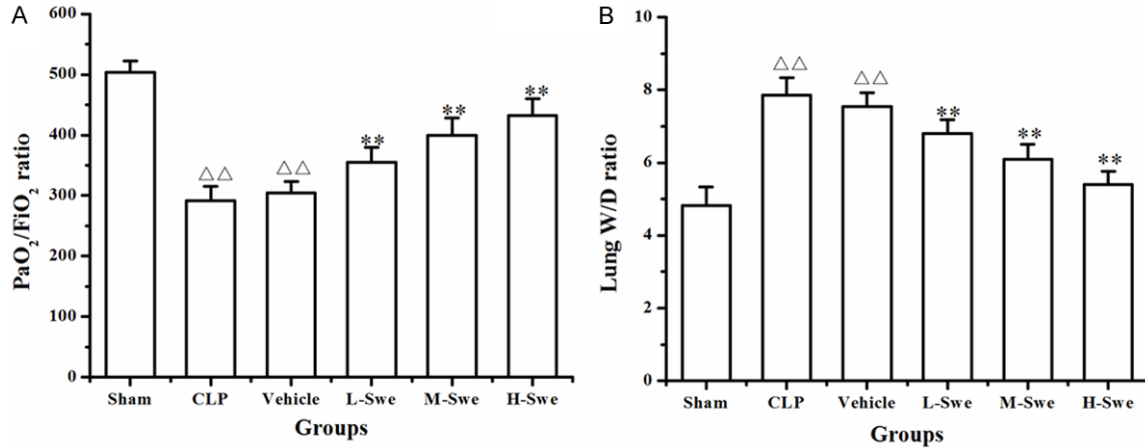
Figure 2. Swertiamarin increased the pulmonary gas exchange functions (PaO_2/FiO_2 ratio) and decreased the lung water content (lung W/D ratio) in rats with CLP-induced ALI. A and B. PaO_2/FiO_2 ratio and lung W/D ratio. $^{\triangle\triangle}P < 0.01$, compared with the sham group; $**P < 0.01$, compared with the vehicle group.

rats were exposed and massaged as described above, but the cecum was not ligated and punctured.

Arterial blood gas analysis

Rats were anesthetized with chloral hydrate anesthesia (400 mg/kg) by i.p. at 12 h after CLP. The abdominal cavity of rat was opened, and blood samples were collected by the abdominal aorta. About 0.5 mL blood was obtained and used to analyze the arterial blood gas. Then the partial pressure of oxygen (PaO_2) and fraction of inspiration oxygen (FiO_2) were determined using Stat Profile Critical Care Xpress Analyzer (Nova, USA).

Measurement of lung wet/dry weight (W/D) ratio

After blood was obtained, the heart and lung tissues were taken out immediately from the chest, and the right main bronchus was clamped. The right upper pulmonary lobe was excised from lung, blotted dry on filter paper and weighed to obtain the wet weight by analytical balance. Then it was placed in an oven at 80°C for 24 h and weighed to obtain the dry weight.

Determination of blood capillary membrane permeability indices and inflammatory cytokines in bronchoalveolar lavage fluid (BALF) of rats

The left lung was lavaged 4 times with instillation of Hanks balanced salt solution (0.5 mL

each time), and the BALF was collected by a polyethylene tube inserted into the trachea. The recovery rate of bronchoalveolar lavage was higher than 90%. Then total BALF was promptly centrifuged at 4000 rpm for 10 min at 4°C. The deposit (cell pellet) of BALF was collected for determining blood capillary membrane permeability indices (neutrophils and lymphocytes counts), and the supernatant of BALF was stored at -80°C and used to determine blood capillary membrane permeability index (protein content) and levels of inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-10).

The cell pellets were re-suspended in normal saline (100 μL), centrifuged onto slides by a Shandon cytospin (Thermo, USA) at 500 rpm for 4 min and stained with Wright-Giemsa staining for 8 min. The neutrophils and lymphocytes counts were quantified by counting 200 cells per slide at 40× magnification on an Olympus BH-2 light microscopy (Olympus, Japan). According to the manufacturer's instructions, the protein concentrations were determined using BCA protein quantitation kit, and the levels of inflammatory cytokines were determined using corresponding Elisa kits.

Determination of activity of MPO and oxidative stress parameters in lung tissue of rats

The tissues of right lower pulmonary lobe were weighed and homogenized (1:10, w/v) in phosphate buffer (0.1 M, pH 7.4) with an ice bath, and then the homogenate was centrifuged at 10000 rpm for 10 min at 4°C. The activity of MPO, oxidative stress parameters (activities of
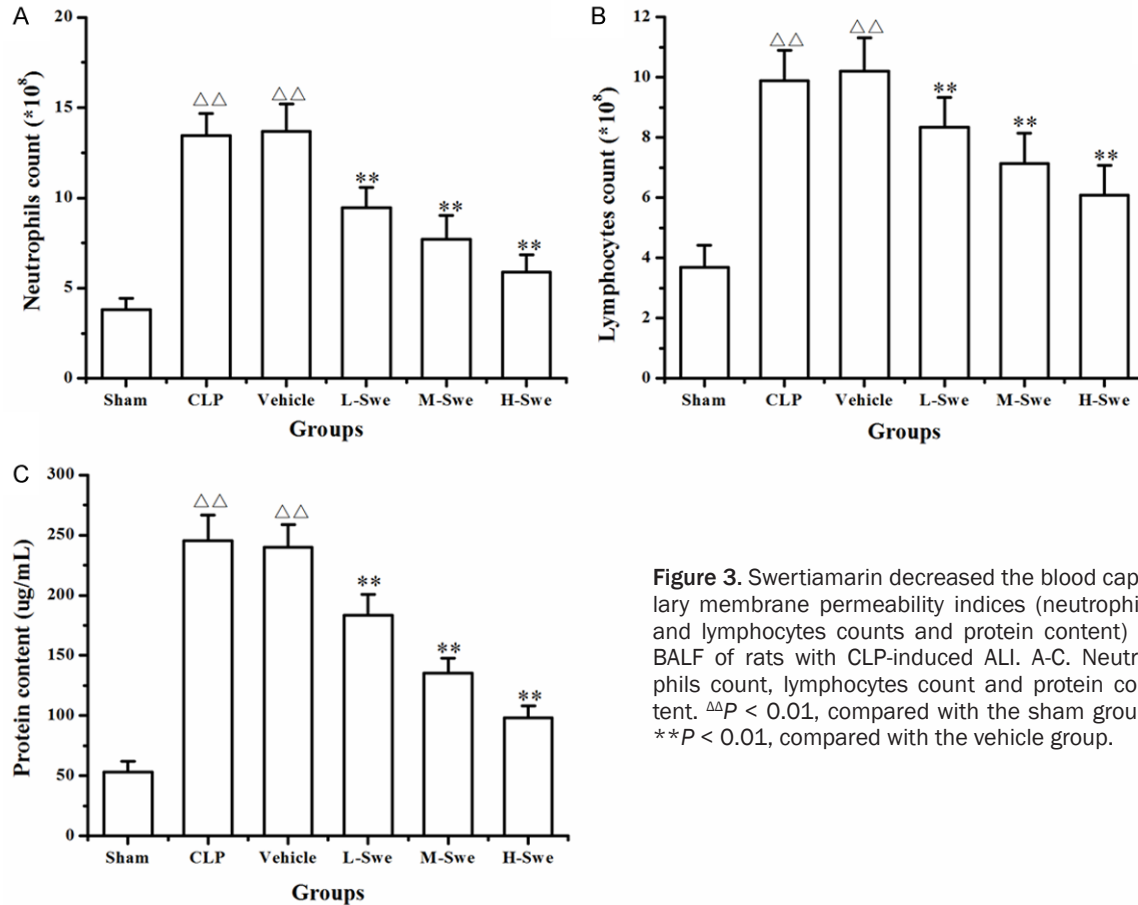
Figure 3. Swertiamarin decreased the blood capillary membrane permeability indices (neutrophils and lymphocytes counts and protein content) in BALF of rats with CLP-induced ALI. A-C. Neutrophils count, lymphocytes count and protein content. $^{\triangle\triangle}P < 0.01$, compared with the sham group; $**P < 0.01$, compared with the vehicle group.

SOD and GSH-Px and levels of MAD and GSH) in the supernatant of homogenate were determined using Elisa kits according to the manufacturer's instructions.

Western blot

The total proteins of lung tissues were extracted, and its concentration was determined using BCA protein quantitation kit. Total proteins (about 40 μg) were separated by SDS/PAGE and then transferred on a PVDF membrane. After blocking 5% fat-free milk, PVDF membranes were incubated with homologous primary antibodies at 4°C overnight and subsequently with HRP-conjugated secondary antibody for 1 h. The NF-κB p65, p-IKKα/β (Ser 180/Ser 181) and IκBα proteins were detected immediately by chemiluminescence. Additionally, antibody directed against β-actin was used to assess proteins loading.

Survival of CLP-induced ALI rats

After treatment with swertiamarin and CLP, rats were followed for 72 h with survival time defined

as hours, and the survival time of each rat was recorded.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) ($n = 10$). The differences among different groups were analyzed by using a one-way ANOVA (LSD test) on SPSS (version 21.0). Survival analysis was carried out on SPSS (version 21.0). Kaplan-Meier method and Log-rank test were separately used to illustrate survival rate among groups and analyze the differences among different groups. The differences were recognized as statistically significant at $P < 0.05$.

Results

Swertiamarin improved pulmonary gas exchange function (PaO_2/FiO_2 ratio) of rats with CLP-induced ALI

As shown in **Figure 2A**, the PaO_2/FiO_2 ratio in CLP and vehicle groups was significantly reduced ($P < 0.01$), compared with the sham
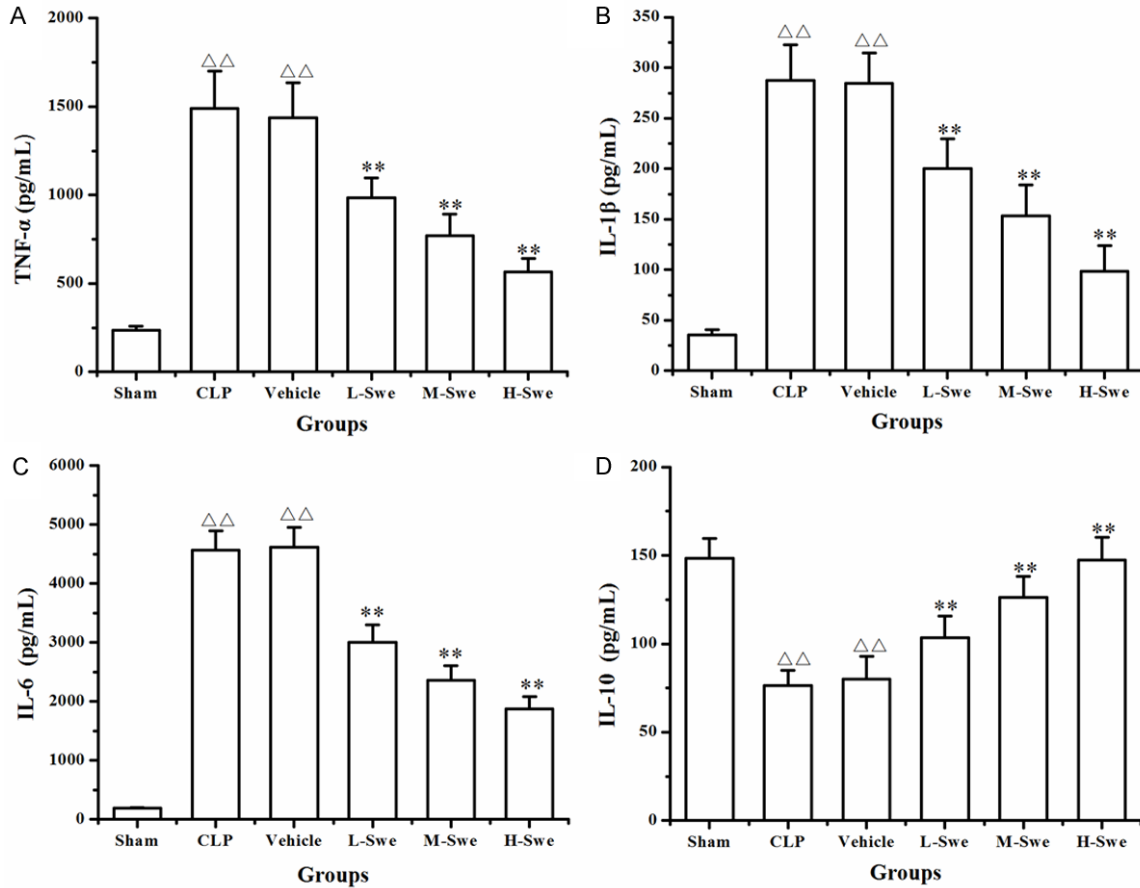
Figure 4. Swertiamarin reduced the levels of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and increased the level of anti-inflammatory cytokine (IL-10) in BALF of rats with CLP-induced ALI. A-D. TNF-α, IL-1β, IL-6 and IL-10. $^{\triangle\triangle}P$ < 0.01, compared with the sham group; $**P$ < 0.01, compared with the vehicle group.

group, which indicated the CLP-induced ALI model was successfully established. The PaO_2/FiO_2 ratio between CLP group and vehicle group was not significantly different, which showed that it was not affected by vehicle (1% DMSO). After treatment with swertiamarin (50, 100 and 200 mg/kg), the PaO_2/FiO_2 ratio was significantly increased (P < 0.01), compared with the vehicle group.

Swertiamarin decreased lung water content (lung W/D ratio) of rats with CLP-induced ALI

As shown in **Figure 2B**, the lung W/D ratio in CLP and vehicle groups was significantly increased (P < 0.01), compared with the sham group, which indicated the CLP-induced ALI model was successfully established. The lung W/D ratio between CLP group and vehicle group was not significantly different, which suggested that it was not affected by vehicle (1%

DMSO). After treatment with swertiamarin (50, 100 and 200 mg/kg), the lung W/D ratio was significantly reduced (P < 0.01), compared with the vehicle group.

Effects of swertiamarin on blood capillary membrane permeability indices and inflammatory cytokines in BALF of rats with CLP-induced ALI

As shown in **Figures 3** and **4**, the neutrophils and lymphocytes counts, the protein content and the levels of TNF-α, IL-1β and IL-6 in CLP and vehicle groups were significantly (P < 0.01) increased, and the level of IL-10 in CLP and vehicle groups was significantly (P < 0.01) decreased, compared with the sham group, which indicated the CLP-induced ALI model was successfully established. The neutrophils and lymphocytes counts, the protein content, the levels of TNF-α, IL-1β, IL-6 and IL-10 between
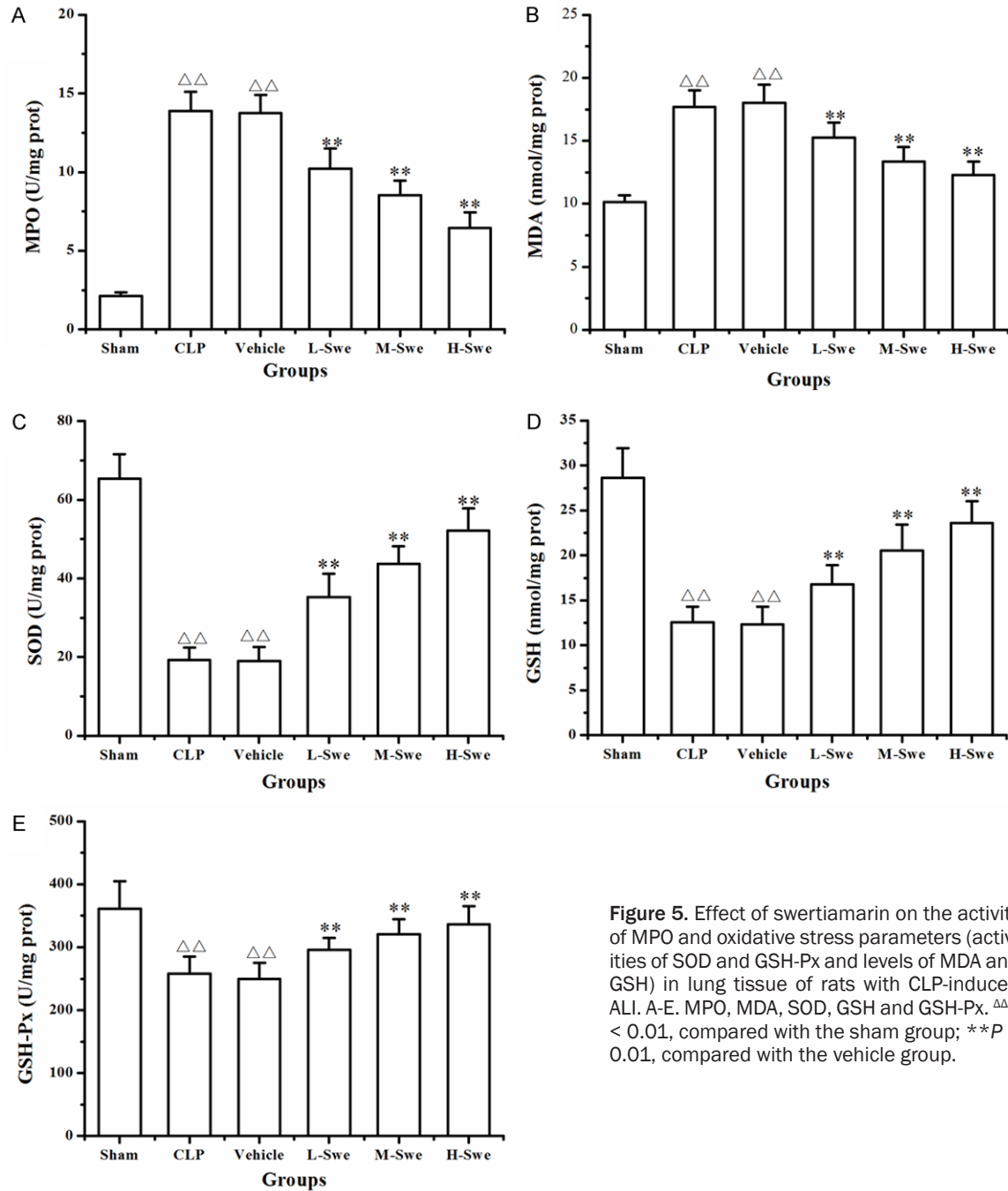
Figure 5. Effect of swertiamarin on the activity of MPO and oxidative stress parameters (activities of SOD and GSH-Px and levels of MDA and GSH) in lung tissue of rats with CLP-induced ALI. A-E. MPO, MDA, SOD, GSH and GSH-Px. $^{\Delta\Delta}P$ < 0.01, compared with the sham group; **P < 0.01, compared with the vehicle group.

CLP group and vehicle group were not significantly different, which indicated that they were not affected by vehicle (1% DMSO). After treatment with swertiamarin (50, 100 and 200 mg/kg), the neutrophils and lymphocytes counts, the protein content and the levels of TNF-α, IL-1β and IL-6 was significantly reduced (P < 0.01), and the level of IL-10 was significantly increased (P < 0.01), compared with the vehicle group.

Effects of swertiamarin on activity of MPO and oxidative stress parameters in lung tissue of rats with CLP-induced ALI

As shown in **Figure 5**, the activity of MPO and the level of MAD in CLP and vehicle groups were significantly increased (P < 0.01), and the activities of SOD and GSH-Px and the level of GSH in CLP and vehicle groups were significantly decreased (P < 0.01), compared with the sham
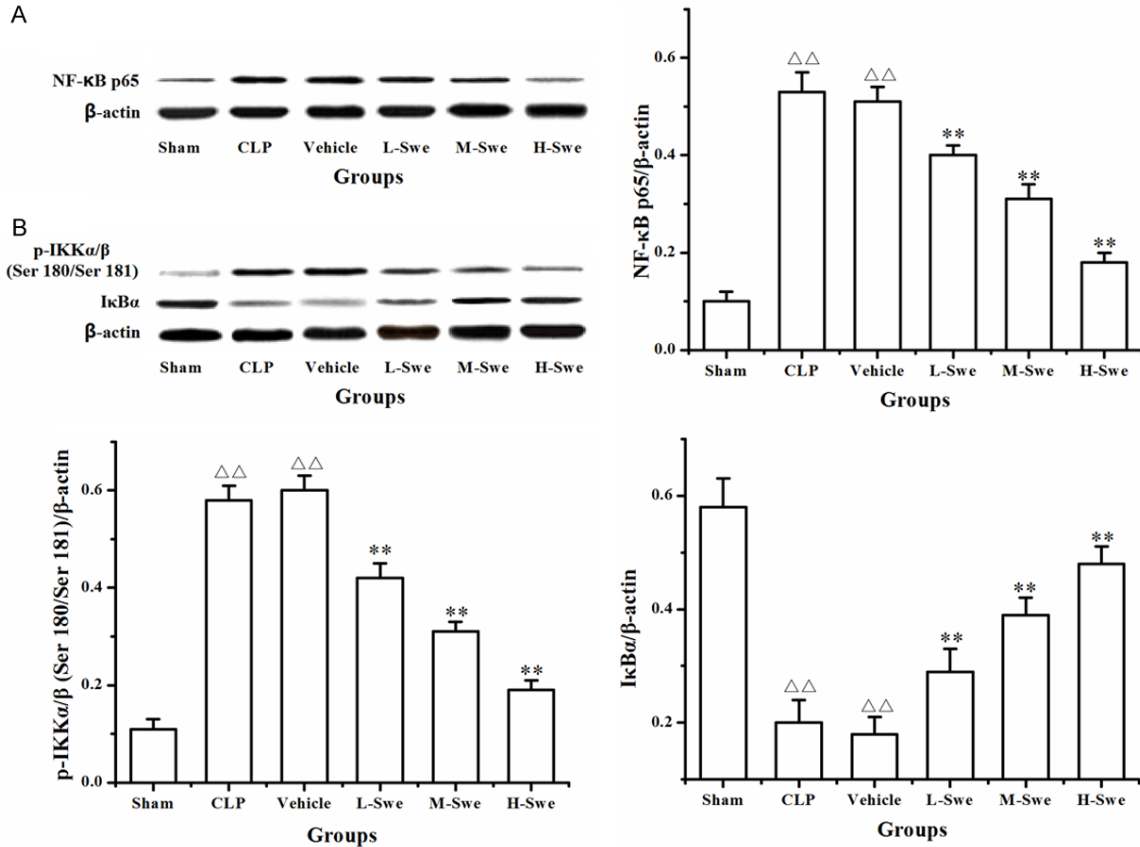
Figure 6. Swertiamarin decreased the levels of NF-κB p65 and p-IKKα/β (Ser 180/Ser 181) proteins and increased the level of IκBα protein in lung tissue of rats with CLP-induced ALI. A. NF-κB p65 protein. B. p-IKKα/β (Ser 180/Ser 181) and IκBα proteins. $^{\triangle\triangle}P < 0.01$, compared with the sham group; $**P < 0.01$, compared with the vehicle group.

group, which indicated the CLP-induced ALI model was successfully established. The activities of MPO, SOD and GSH-Px and the levels of MAD and GSH between CLP group and vehicle group were not significantly different, which suggested that they were not affected by vehicle (1% DMSO). After treatment with swertiamarin (50, 100 and 200 mg/kg), the activity of MPO and the level of MAD were significantly reduced ($P < 0.01$), and the activities of SOD and GSH-Px and the level of GSH were significantly increased ($P < 0.01$), compared with the vehicle group.

Swertiamarin reduced levels of NF-κB p65 and p-IKKα/β (Ser 180/Ser 181) proteins and increased level of IκBα protein in lung tissue of rats with CLP-induced ALI

As shown in **Figure 6**, the levels of NF-κB p65 and p-IKKα/β (Ser 180/Ser 181) in CLP and vehicle groups were significantly increased ($P < 0.01$), and the level of IκBα in CLP and vehicle

groups was significantly decreased ($P < 0.01$), compared with the sham group, which indicated the CLP-induced ALI model was successfully established. The levels of NF-κB p65, p-IKKα/β (Ser 180/Ser 181) and IκBα between CLP group and vehicle group were not significantly different, which suggested that they were not affected by vehicle (1% DMSO). After treatment with swertiamarin (50, 100 and 200 mg/kg), the levels of NF-κB p65 and p-IKKα/β (Ser 180/Ser 181) was significantly reduced ($P < 0.01$), and the level of IκBα was significantly increased ($P < 0.01$), compared with the vehicle group.

Swertiamarin increased survival rate of rats with CLP-induced ALI

As shown in **Figure 7**, the survival rate of rats with CLP-induced ALI in CLP and vehicle groups were significantly decreased ($P < 0.01$), compared with the sham group, which indicated the CLP-induced ALI model was successfully established. The survival rate of rats with CLP-
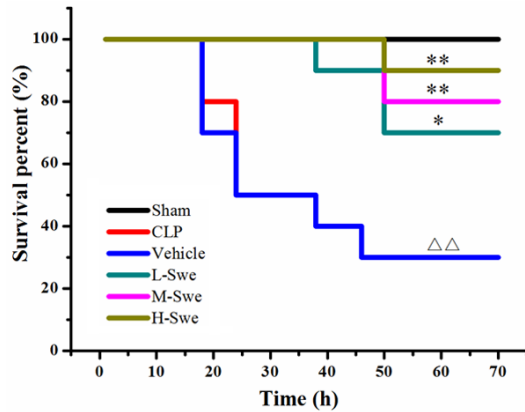
Figure 7. Swertiamarin increased the survival rate of rats with CLP-induced ALI. $^{\triangle\triangle}P < 0.01$, compared with the sham group; $*P < 0.05$, $**P < 0.01$, compared with the vehicle group.

induced ALI was not significantly different between CLP group and vehicle group, which indicated that it was not affected by vehicle (1% DMSO). After treatment with swertiamarin (50, 100 and 200 mg/kg), the survival rate of rats with CLP-induced ALI was significantly increased ($P < 0.05$ or 0.01), compared with the vehicle group.

Discussion

Sepsis-induced ALI is a clinical syndrome of high morbidity and mortality in ICU [11]. Therefore, it's important to prevent ALI for treating sepsis. Generally, CLP is recognized as a method to induce sepsis [21]. Hence, in the present study, CLP was used to induce sepsis and then CLP-induced sepsis further induced the occurrence of ALI. CLP-induced ALI can lead to the out of control of inflammatory response, the infiltration of inflammatory cells and the release of inflammatory medium generated by activated inflammatory cells in lung [13, 14], which can induce the reduction of lung-blood barrier function, the increase of blood capillary basement membrane permeability, etc. [22]. The main pathophysiological characteristics of ALI are the reduction of lung volume, the imbalance of ventilation/bloodstream, the decrease of lung compliance, etc. [23].

ALI can lead to the reduction of the pulmonary gas exchange, and the PaO$_2$ is a simple and effective index to evaluate the pulmonary gas exchange function, but PaO$_2$ is easily disturbed

by FiO$_2$ [24]. Therefore, the PaO$_2$/FiO$_2$ ratio was used to evaluate the pulmonary gas exchange function in the present study. Swertiamarin improved the pulmonary gas exchange function of rats with CLP-induced ALI by increased the PaO$_2$/FiO$_2$ ratio (**Figure 2A**).

Inflammation is the essential reason of ALI. The edema, the increase of blood capillary membrane permeability, the infiltration of inflammatory cells and the release of inflammatory medium are a few meaningful physiological and pathological responses for inflammation [25, 26]. The lung W/D ratio is an accepted indicator used to evaluate the edema [27]. After treatment with swertiamarin, the lung W/D ratio was significantly decreased (**Figure 2B**), which suggested that swertiamarin inhibited the edema in lung of rats with CLP-induced ALI. The increase of blood capillary membrane permeability can lead to the infiltration or exudation of inflammatory cells and protein from blood capillary to pulmonary alveoli or interstitial. The neutrophils and lymphocytes are two important inflammatory cells, which can be used to reflect the infiltration of inflammatory cells [28]. After treatment with swertiamarin, the neutrophils (**Figure 3A**) and lymphocytes (**Figure 3B**) counts and the protein content (**Figure 3C**) in BALF were significantly decreased, which suggested that swertiamarin reduced the blood capillary membrane permeability in lung of rats with CLP-induced ALI. The transmembrane migration, gather and activation of neutrophils in lung tissue induce the respiratory burst, and oxygen can be converted to reactive oxygen species (ROS) by NADPH and NADH oxidases [29-31]. ROS can damage the lung parenchyma cells, the blood capillary basement membrane, etc., which leads to severe lung edema. MPO exists mainly in neutrophils and is generally considered as the migration, gather and activation markers of neutrophils in lung tissue [32, 33]. After treatment with swertiamarin, the activity of MPO (**Figure 5A**) was significantly decreased, which suggested that swertiamarin reduced the migration, gather and activation of neutrophils in lung tissue of rats with CLP-induced ALI. SOD is the only anti-oxidant enzyme, used to remove superoxide, and its activity reflects the anti-oxidant ability of organism [23]. MDA reflects the severity of the body cells attacked by oxygen free radicals [34]. GSH-mediated hydrogen peroxide reduction reaction, which can protect the structure and

function of cytomembrane, is catalyzed by GSH-Px [23, 34]. Therefore, SOD, GSH and GSH-Px can protect cell against oxidative stress damage. After treatment with swertiamarin, the level of MDA (**Figure 5B**) was significantly decreased, and the activities of SOD (**Figure 5C**) and GSH-Px (**Figure 5E**) and the level of GSH (**Figure 5D**) were significantly increased in lung tissue of rats with CLP-induced ALI. These results suggested that swertiamarin decreased the lung edema of rats with CLP-induced ALI by reducing blood capillary membrane permeability and increasing anti-oxidant ability.

Moreover, the anti-inflammatory molecular mechanisms of swertiamarin were investigated by determining the levels of inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-10) in BALF and the levels of NF-κB p65, p-IKK α/β (Ser 180/Ser 181) and IκBα in lung of rats. TNF-α, IL-1β and IL-6 were pro-inflammatory cytokines, and IL-10 was anti-inflammatory cytokine [35]. NF-κB is an important nuclear transcription factor, which is related to growth, differentiation, inflammation, immunoreactions, etc. [36]. Generally, the NF-κB dimer is combined with specific inhibiting protein (IκB) and separated in the cytoplasm of resting cell. IκB kinase (IKK) can be activated by ischemia reperfusion, TNF-α, etc. IKK can phosphorylate IκB, and then p-IκB (IκBα in especial) is degraded by ubiquitin. Further, NF-κB is activated, and activated NF-κB (NF-κB p65 in especial) is sent to the nucleus to combine with κB binding site, which can induce the transcription of inflammatory factor-related mRNA and protein synthesis. Finally, inflammation is induced [37]. The IKK/NF-κB pathway plays an important role in inflammation. The level of p-IKK α/β (Ser 180/Ser 181) can reflect the activity of IKK [38]. After treatment with swertiamarin, the levels of TNF-α (**Figure 4A**), IL-1β (**Figure 4B**), IL-6 (**Figure 4C**), NF-κB p65 (**Figure 6A**) and p-IKK α/β (Ser 180/Ser 181) (**Figure 6B**) were significantly decreased and the levels of IL-10 (**Figure 4D**) and IκBα (**Figure 6B**) was significantly increased, which suggested that swertiamarin inhibited the inflammation in lung of rats with CLP-induced ALI by inhibiting the activation of IKK/NF-κB pathway and the expressions of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and increasing the expression of anti-inflammatory cytokine (IL-10). Finally, benefiting from the change as described above, the survival rate of CLP-induced ALI rats was significantly increased after treatment with swertiamarin.

In conclusion, swertiamarin exhibited protective effect against CLP-induced ALI in rats and increased the survival of rats with CLP-induced ALI, and the mechanisms might be related to reducing blood capillary membrane permeability, inhibiting inflammatory response and increasing the anti-oxidant ability in lung of rats with CLP-induced ALI. Further investigation into the mechanism of swertiamarin on ALI needs to be undertaken.

Disclosure of conflict of interest

None.

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