Shenmai injection alleviates acute lung injury in a severe acute pancreatitis rat model via heme oxygenase-1 upregulation

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Research article

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Abstract

Background: To determine the effect of Shenmai injection (SMI) on acute lung injury (ALI) induced by severe acute pancreatitis (SAP) in rats.

Methods: Forty male Sprague-Dawley (SD) rats were grouped into 4 categories: SAP group, sham surgery (SS) group, SAP + SMI group, SAP + SMI + Zinc protoporphyrin (ZnPP) group. Rats in the SAP group were intravenously injected with 1.6 ml/kg saline 30 minutes after induction of SAP models. Rats in the SAP + SMI group were intravenously injected with 1.6 ml/kg SMI, while those in the SAP + SMI + ZnPP group rats were intravenously injected with 1.6 ml/kg SMI and 30 mg/kg ZnPP via intraperitoneal injection. Twenty-four hours after SAP induction, the rats were sacrificed. Excised lung tissues were histologically examined, protein concentration in bronchoalvelar lavage fluid (BALF) was measured and lung wet-to-dry (W/D) weight ratio was calculated. The protein and mRNA levels of the tumor necrosis factor (TNF) -α, heme oxygenase (HO) -1 and interleukin (IL) -10 in blood and tissue samples were measured.

Results: SMI treatment attenuated SAP-induced ALI as evidenced by lower scores of lung damage compared with untreated SAP group (p 0.05). SMI also abolished the SAP-induced rise in BALF and W/D ratio protein concentrations (p 0.05). Moreover, SMI treatment increased HO-1, IL-10 levels but decreased TNF-α level in serum and tissue samples (p 0.05). However, inhibition of HO-1 expression by ZnPP led to significant inhibition of all the changes.

Conclusions: SMI can alleviate SAP-induced ALI through HO-1 upregulation.

1. Background

Severe acute pancreatitis (SAP) is a condition characterized by inflammation of the pancreas, and it is a serious pathological condition with a high rate of fatality of nearly 30% and thus requires critical care [1-3]. SAP usually results in systemic inflammatory response syndrome (SIRS) and causes distant organs complications [4]. One of the serious complications caused by SAP is Acute lung injury (ALI). ALI leads to early mortality due to single or multiple organ dysfunctions [5,6]. It is reported that about one third of acute pancreatitis deaths occur in the early stage of hospitalization and are associated with ALI in most cases [5]. It has been observed that early intervention prevents lung injury leading to good prognosis.

Acute lung injury is associated with high inflammatory response, accumulation of activated neutrophil and elevated interstitial edema [7]. Cytokines, such as interleukin (IL) -6, tumor necrosis factor (TNF) -α, and IL-1β, aggravate local pancreatitis as well as cause multiple organ failure [8]. As such, suppressing inflammation is a viable treatment strategy for SAP as this will suppress ALI [9,10].

Heme oxygenase (HO) -1 (also referred to as HSP-32), an inducible isoform of heme oxygenase, catalyzes the degradation of heme into carbon monoxide (CO), iron and biliverdin [11]. Previous studies indicated that HO-1, which protects cells and organs against inflammation-induced injury and oxidative stress, plays an important role in the pathogenesis of SAP and other inflammatory disorders [12-16]. Zinc

protoporphyrin (ZnPP), which is a specific inhibitor of HO-1, as a negative regulator is used in HO-1 related research [17].

Shenmai injection (SMI) is a traditional Chinese medicine comprising *Ophiopogonjaponicas (lilia-ceae),* and *Ginseng Rubra* (*araliaceae*) extracts [18] and is widely used to clinically treat heart diseases, sepsis, osteoarthritis and asthma [19-22]. SMI is generally considered a safe drug [23,24]. Research has shown that SMI suppresses inflammation by decreasing TNF-α production [25]. Based on this knowledge, we speculate that SMI may confer protection against pancreatitis-related lung injury. To date, none has reported whether SMI regulates SAP-induced ALI. This study, therefore, used an established SAP rat model to explore the effect of SMI on SAP associated with ALI and its mechanism of action.

2. Methods

2.1 Animal model

All animal experiments were performed in full compliance with the Shandong Committee guidelines on Animal Care of China and this study was approved by the same committee. Forty healthy male SD rats weighing between 220 and 260g were obtained from the Shandong Experimental Animal Center of Chinese Academy Science. The rats were housed in a room with constant temperature of 25±1 ℃ and adjusted to a 12-hours duration of light. All rats had free access to chow diet and drinking water although prior to surgery, they were given water only for 24 hours.

The rats were grouped into four categories (n=10): SAP group, sham surgery (SS) group, SAP + SMI group, SAP + SMI + Zinc protoporphyrin (ZnPP) group. The SAP model was designed by retrogradely injecting the rats with 4% sodium taurocholate (1 ml/kg) via the pancreatic duct following anaesthetization with sodium pentobarbital (40 mg/kg) [26]. Rats in the SAP group were intravenously injected with 1.6 ml/kg saline 30 minutes after induction of SAP. Similarly, rats in the SAP + SMI group were intravenously injected with 1.6 ml/kg SMI 30 minutes after induction of SAP while rats in the SAP + SMI + ZnPP group were intravenously injected with 1.6 ml/kg SMI and 30 mg/kg ZnPP via intraperitoneal injection 30 minutes after induction of SAP. At the end of the surgery, the rats were resuscitated by a single isotonic Sodium Chloride (40 ml/kg) injection and then housed in cages individually. They were only provided with water and sacrificed (spinal dislocation) 24 hours after the operation. All operations follow the experimental workflow (Fig. 1).

2.2 Reagents

All reagents used were bought from internationally recognized suppliers: Sodium taurocholate, sodium pentobarbital, and ZnPP were procured from Sigma Chemical (St. Louis, MO, USA), SMI was purchased from CTQ Pharmaceutical Group Co. Ltd. (Hangzhou, China), and Isotonic sodium chloride was bought from Qilu Pharmaceutical Co. Ltd. (Jinan, China). RIPA buffer, formaldehyde, isopropanol, and ethanol were procured from Dikma Co. (Beijing, China) while Monoclonal antibodies were procured from Abcam (Cambridge, United Kingdom). TRIzol reagent and the fluorescence quantitative RT-PCR kit were bought

from Thermo Fisher Scientifc, Inc. (Waltham, MA, USA). The primer sequences for IL-10, TNF-a, HO-1, and β-actin (Table 1) were prepared by Invitrogen (Carlsbad, CA, USA). ELISA kits were obtained from EIAab Science Co. Ltd. (Wuhan, China).

Table 1

PCR primer sequences (5'-3')

Gene	Forward primer	Reverse primer
HO-1	ACC CCA CCA AGT TCA AAC AG	GAG CAG GAA GGC GGT CTT AG
TNF-α	CCC AAT CTG TGT CCT TCT AAC T	CAC TAC TTC AGC GTC TCG TGT
IL-10	GGC TCA GCA CTG CTA TGT TGC C	AGC ATG TGG GTC TGG CTG ACT G
β-actin	TGG TGG GTA TGG GTC AGA AG	GAC AAT GCC GTG TTC AAT GG

2.3 Histomorphology Examination

Lung tissues were prepared and fixed in 40 g/L formaldehyde. Paraffin-embedded tissues were sectioned into 5μm thick sections and stained with hematoxylin and eosin. The sections were visualized under a light microscope [26]. Histopathology scores were calculated based on 3 randomly chosen tissues. Assessment of the histological results was carried out by an investigator who was blinded to the group allocation. All pathological scores for the lung tissues were analyzed as described by Zhao *et al.* [27]. The following 3 items were considered for scoring ALI: hemorrhage; the alveolar wall thickness or formation of hyaline membrane; and infiltration of neutrophil aggregation in airspace/vessel wall. The severity of lung injury was scored as follows: 0, no evidence of injury; 1, mild injury; 2, moderate injury; and 3, severe injury. All of the evaluations were performed on five fields per section and five sections per organ.

2.4. Protein concentration measurement in bronchoalvelar lavage fluid (BALF)

A bicinchoninic acid (BCA) assay kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to determine the concentration of protein in micrograms per milliliter in BALF.

2.5. Determination of lung wet-to-dry (W/D) weight ratio

The extent of pulmonary edema was determined using the wet to dry lung weight. For the wet weight measurement, the left upper lobe of the lungs was excised, blotted to dry and then weighed. On the other hand, the dry weight was determined after the lung lobe was dehydrated for 24 hours in an 80℃ oven. The ratio of wet to dry weight was obtained by dividing the value of the wet weight with that of dry weight.

2.6 Measurement of IL-10, HO-1, and TNF-α

Serum and tissues were harvested and stored at -80 ℃ awaiting assessment. ELISA tests were performed to determine the concentration of IL-10, TNF-α, and HO-1 in serum while the protein expression of TNF-α, IL-10, and HO-1 in lung tissues were analyzed using the Western blot assay [28]. Protein extract (15ug) of whole tissue was resolved in 12% Bis-Tris polyacrylamide gel and the protein bands transferred onto a nitrocellulose membrane. The membrane was blocked was done for 1 hour using TBS (Tris-buffered saline) supplemented with Tween 20 and skimmed milk (5%). The membrane was then incubated with monoclonal antibodies anti-IL-10, anti-TNF-α and anti-HO-1 for 24 hours after which it was washed thrice. The membrane was then incubated with a conjugated secondary antibody (horseradish peroxidase) and washed several times. Autoradiography was used to visualize the proteins. The intensity of the bands was determined using Image Analysis software (Bio-Rad, Hercules, CA) and the density of each sample was normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

In addition, RT-PCR was conducted to measure the mRNA level of IL-10, TNF-α, and HO-1 in tissues on the ABI7900 instrument (Applied Biosystems, USA). Total RNA was extracted using the TRIzol buffer and then reverse-transcribed to cDNA. The cDNA was used as a template in PCR reactions to measure the expression level of TNF-α, IL-10, and HO-1. The expression of β-actin was used for data normalization. The PCR reaction conditions used were; 15 seconds of denaturation at 95℃, 20 seconds of annealing at 60℃, and 30 seconds of extension at 72℃ [26].

2.7 Statistics

Data analysis was done using SPSS 16.0 and values are presented as mean ± SEM. Multiple groups were compared using the one-way (ANOVA) analysis of variance then by Tukey's test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Histomorphology and Histopathologic scores

Hemorrhage, interstitial inflammatory cell infiltration, and pulmonary edema were more pronounced in the lungs of rats in the SAP group compared to rats in the SS group. These parameters were weaker in the SAP + SMI group, but were aggravated in SAP + SMI + ZnPP group (Fig. 2). Further analysis revealed that the lungs' histopathologic scores were higher in the SAP group relative to the SS group (p 0.05). The histopathologic scores were higher in the SAP group relative to the SAP + SMI group (p 0.05). However, the histopathologic scores were significantly higher in the SAP + SMI + ZnPP group than in the SAP group and SAP + SMI group (p 0.05) (Fig. 2).

3.2. SAP-induced vascular permeability and lung edema

Vascular permeability and pulmonary edema were determined by measuring the ratio of W/D of the lungs and BALF protein concentration from groups with different treatments. Results showed that SMI

significantly reduced the SAP-induced rise in the W/D ratio (Fig. 3-A & 3-B) (p<0.05) and BALF protein concentrations (p<0.05). However, these effects were not observed in the SMI + ZnPP group.

3.3. Influence of SMI on HO-1 expression

The level of HO-1 in the serum and tissues of the SAP group was higher than in the SS group (p<0.05). Similarly, HO-1 concentration in the serum and tissues of the SAP + SMI group was significantly higher than in the SAP group (p<0.05). However, the concentration of HO-1 in serum and tissues was lower in the SAP + SMI + ZnPP group than in the SAP group (Fig. 4) (p<0.05).

3.4. Impact of SMI on the level of IL-10 and TNF-α

The IL-10 and TNF-α levels were higher in the SAP group than in the SS group (p<0.05). However, the level of TNF-α was higher in the SAP group than in the SAP + SMI group (p<0.05) but was higher in the SAP + SMI + ZnPP group than in the SAP group (p<0.05). On the other hand, the level of IL-10 was higher in the SAP + SMI group (p<0.05) but lower in the SAP + SMI + ZnPP group when compared to that in the SAP group (Fig. 5) (p<0.05).

4. Discussion

Severe acute pancreatitis (SAP) is a systematic disorder characterized by pancreatic necrosis, cytokine activation, SIRS and multiple organ dysfunction syndromes (MODS) [29,30]. MODS is the main cause of SAP-related deaths [30]. ALI is the most frequent form of organ failure in patients with SAP and accounts for 60-70% mortality of patients within the first week of infection [31]. Thus, it is imperative to develop effective treatment, initiate early intervention and prolong hospital stay for ALI patients to minimize deaths [32]. Studies have suggested that SAP induces ALI by triggering overproduction of inflammatory mediators by macrophages, neutrophils and other cells that form part of the immune system [7,10,33].

Previous studies have implicated TNF-α (a protein produced by activated lymphocytes and macrophages) in the developmental mechanisms of SAP [34]. Once activated, TNF-α intensifies the production and expression of IL-8 and IL-6 leading to the generation of an inflammatory signaling cascade [35]. This is a common phenomenon during organ failure. On the contrary, IL-10 produced by stellate cells, hepatocytes, T helper-2 (Th2) cells, and macrophages confers protection in various inflammatory disorders [36]. IL-10 decreases the synthesis of pro-inflammatory cytokines TNF-α, IL-2, and IL-3 thereby averting SAP-induced MODS [37,38]. This study revealed that anti-inflammatory cytokines are lower than the pro-inflammatory cytokines in patients who develop ALI [39].

HO-1 is an enzyme that regulates the breakdown of heme to biliverdin, iron, and CO [11]. Previous studies indicated that HO-1, which protects cells and organs against inflammation-induced injury and oxidative stress, plays an important role in the pathogenesis of SAP and other inflammatory disorders [12-16]. This study revealed that HO-1 induction during the early stages of SAP development modulated systemic inflammatory response by suppressing TNF-α and augmenting IL-10 [26].

Nature is a rich source of lead molecules for drug discovery and development [40]. Phytoconstituents represent a promising class of therapeutic agents with wide acceptability not only based on folk practices but sound presence of pharmacological and molecular evidences [41]. SMI is a patented drug used in China for hospitalized patients. Previous studies found out that SMI reduced the concentration of TNF-α, IL-8, and IL-6 in serum [42] and ameliorated oxidative damage [19]. Advances in SMI research have improved the clinical application of SMI in the treatment of inflammatory diseases [20-22]. Our results show that SAP-induced ALI was significantly attenuated by SMI treatment as evidenced by the lower lung damage severity scores. In the same line, SMI significantly reduced the SAP-induced rise in the W/D ratio and protein concentrations of BALF. These results suggest that SMI suppresses inflammatory response by reducing the production of cytokines thus preventing lung damage.

Our results also reveal that intravenous SMI injection in rats exposed to SAP treatment significantly increased HO-1 in plasma and lung tissues. This resulted in the suppression of inflammatory reaction and oxidative damage thus preventing injury to the lungs. On the contrary, injection of ZnPP (a specific HO-1 inhibitor) alleviated the increase in HO-1 expression caused by injection of ZnPP thereby aggravating lung tissue inflammation and injury. Mechanistically, the effect of SMI on SAP-induced ALI involves HO-1 upregulation which inhibits systemic inflammatory response and lung injury by balancing pro-inflammatory and anti-inflammatory factors.

To enrich the quality of our research, we plan to include other endpoints such as pulmonary alveolar leakage of fluorescent tagged macromolecule, the oxygen saturation by pulse oximetry, number of dead cells in alveolar space, and level of secreted cytokines (e.g., IL-6 and IL-8). Effective management of SAP requires a holistic approach. It is worth noting that there was no data to confirm the survival benefits of SMI, and explore the exact mechanisms of SMI therapy in this study. Further investigations are therefore needed to address these limitations. A better understanding on the role of HO-1 in ALI will require inclusion of an arm of only SAP + ZnPP to determine whether this will produce worse results than the SAP only group. Moreover, given that male rats present stable hormone levels than female rats, we chose male rats for the present study. Therefore, our results do not explain whether the protective effect of SMI on rats with SAP is affected by sex hormones.

5. Conclusions

Our results reveal that SMI alleviates SAP-induced ALI by activating HO-1 signaling, decreasing TNF-α expression and increasing IL-10 levels in the SAP rat model. ZnPP, on the other hand, significantly inhibited these effects. SMI blunts systemic inflammatory response and lung injury and thus is a novel promising therapeutic agent against SAP-induced ALI.

Abbreviations

SMI: Shenmai injection; ALI: acute lung injury; SAP: severe acute pancreatitis; BALF: bronchoalvelar lavage fluid; SD: Sprague-Dawley; ZnPP: Zinc protoporphyrin; GAPDH: glyceraldehyde-3-phosphate

dehydrogenase; HO: heme oxygenase; TNF: tumor necrosis factor; IL: interleukin; SIRS: systemic inflammatory response syndrome; MODS: multiple organ dysfunction syndromes; Th2: T helper-2.

Declarations

Ethics approval and consent to participate

Experiments based on animals were performed in full conformity with the Shandong Committee guidelines on Animal Care of China and were approved by the committee. Approval Number: SCXK(LU)20140007.

Consent to publish

Not applicable.

Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

LK and FHZ conceived and designed the study. FHZ and YL drafted the manuscript. HH and KLF performed the experiments. WD and WHL performed the statistical analysis. All authors have read and approved the final manuscript.

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Figures

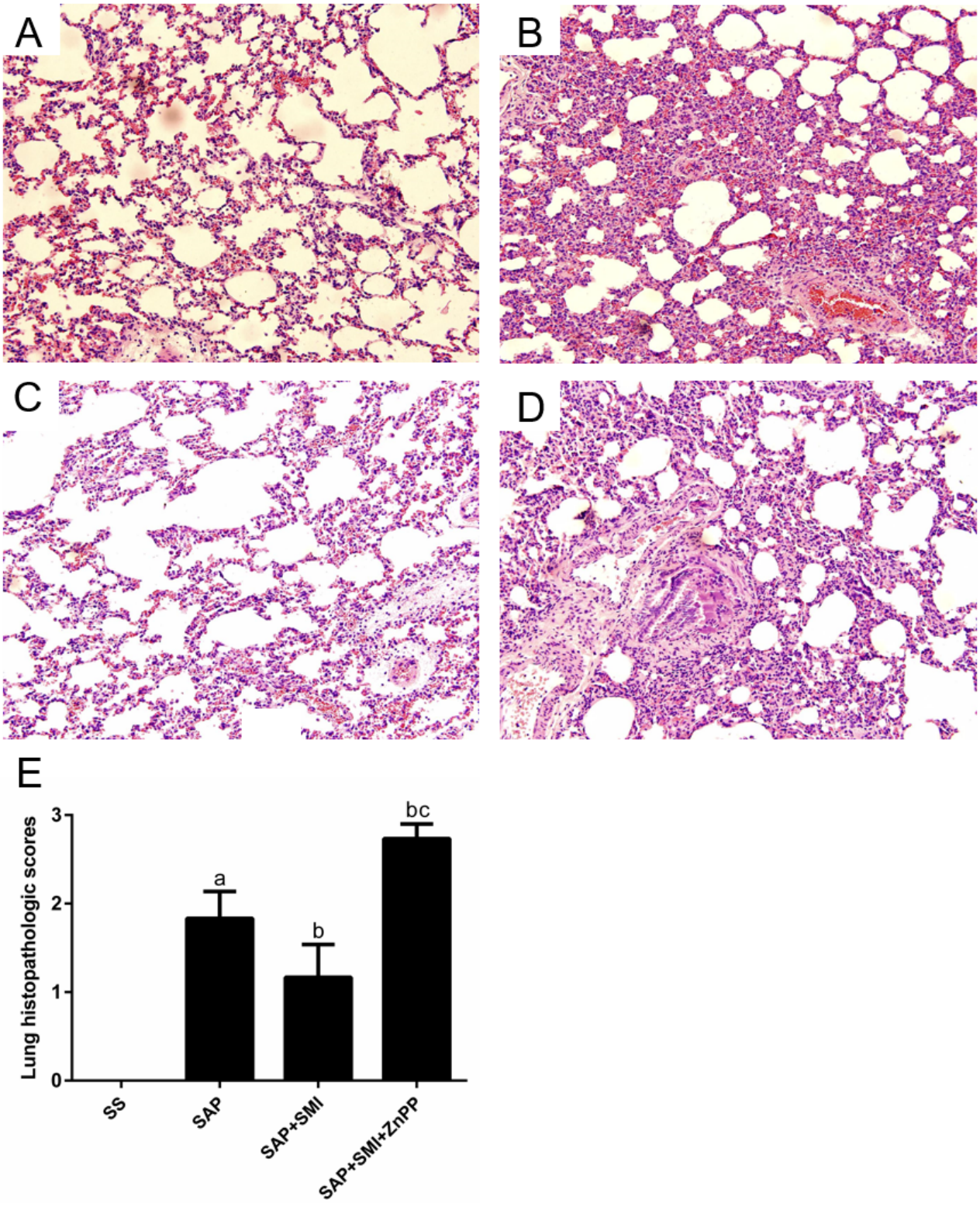

Figure 1

The lung histopathologic scores and alterations. (A) = SS group; (B) = SAP group; (C) = SAP + SMI group; (D) = SAP + SMI + ZnPP group; (E) = Histopathologic scores. Data are shown as means ± SEM). ap0.05 relative to the SS group; bp0.05 relative to the SAP group; cp0.05 relative to the SAP + SMI group (n = 10). Histopathologic scores of lungs show significant decrease after SMI treatment under conditions of SAP. ZnPP suppressed this effect.

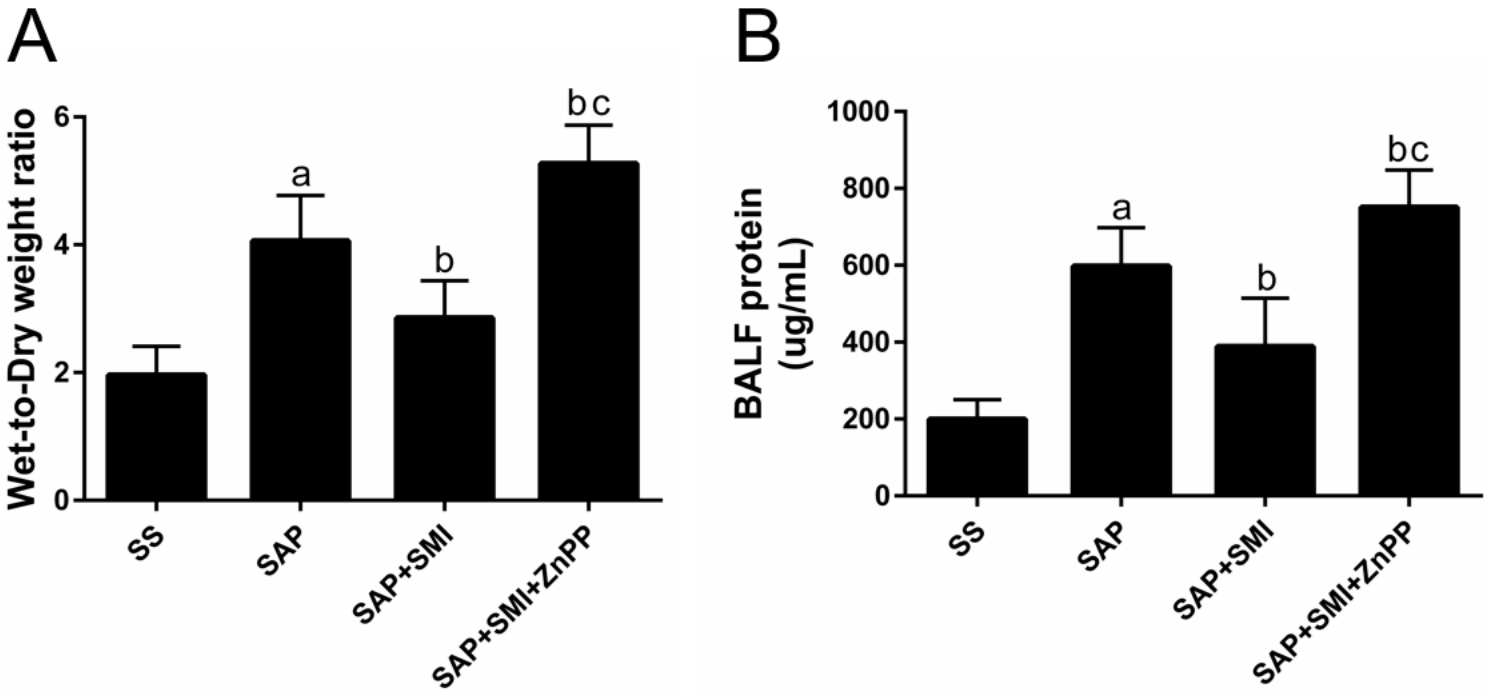

Figure 2

The W/D ratio of the lung and BALF protein concentration. Data are shown as means ± SEM. ap0.05 relative to the SS group; bp0.05 relative to the SAP group; cp0.05 relative to the SAP + SMI group (n = 10).

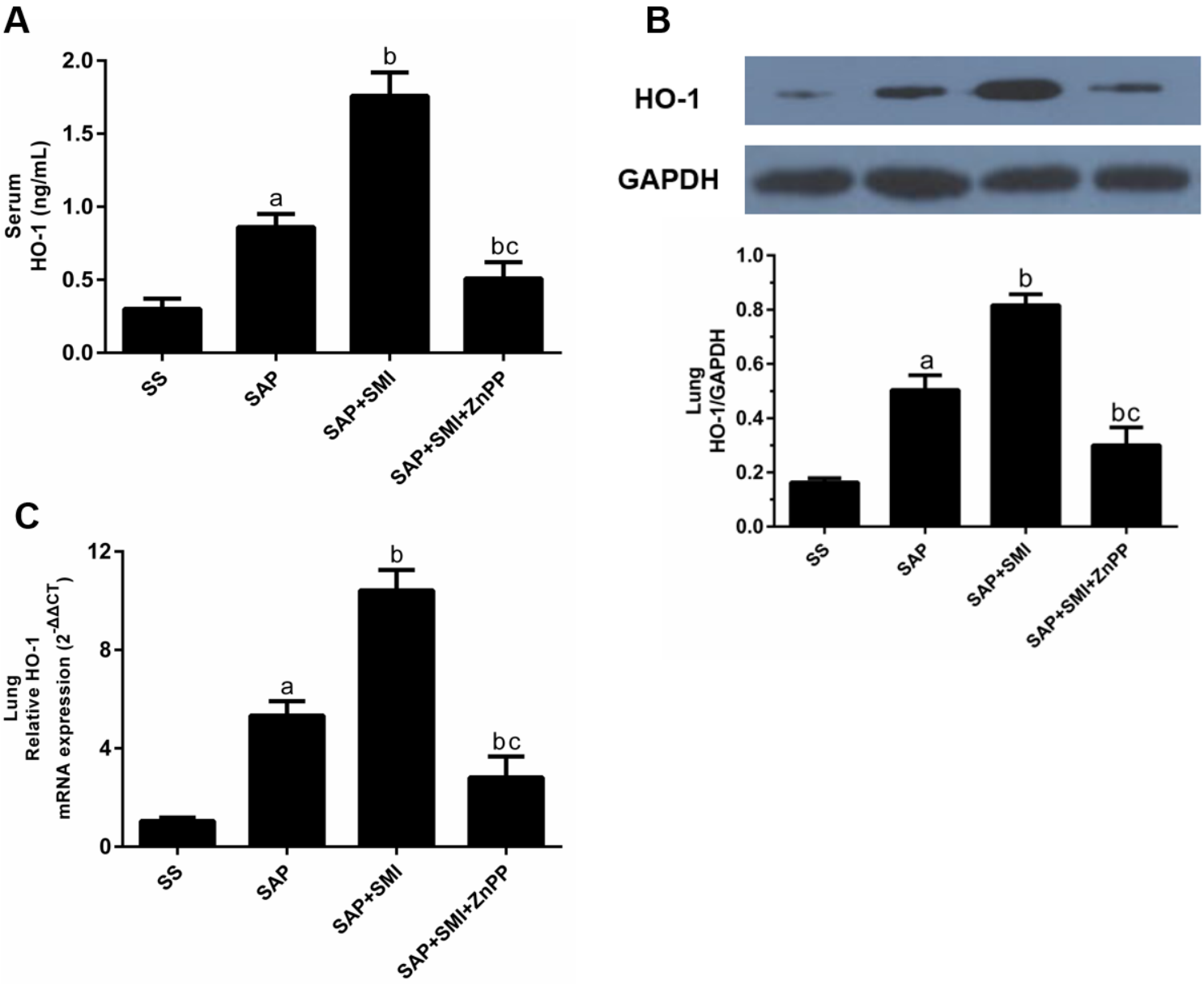

Figure 3

The HO-1 expression levels. (A) = Serum (ELISA); (B) = Lung (Western blot); (C) = Lung (RT-PCR). Data are shown as means ± SEM. ap□0.05 relative to the SS group; bp□0.05 relative to the SAP group; cp□0.05 relative to the SAP + SMI group (n = 10).

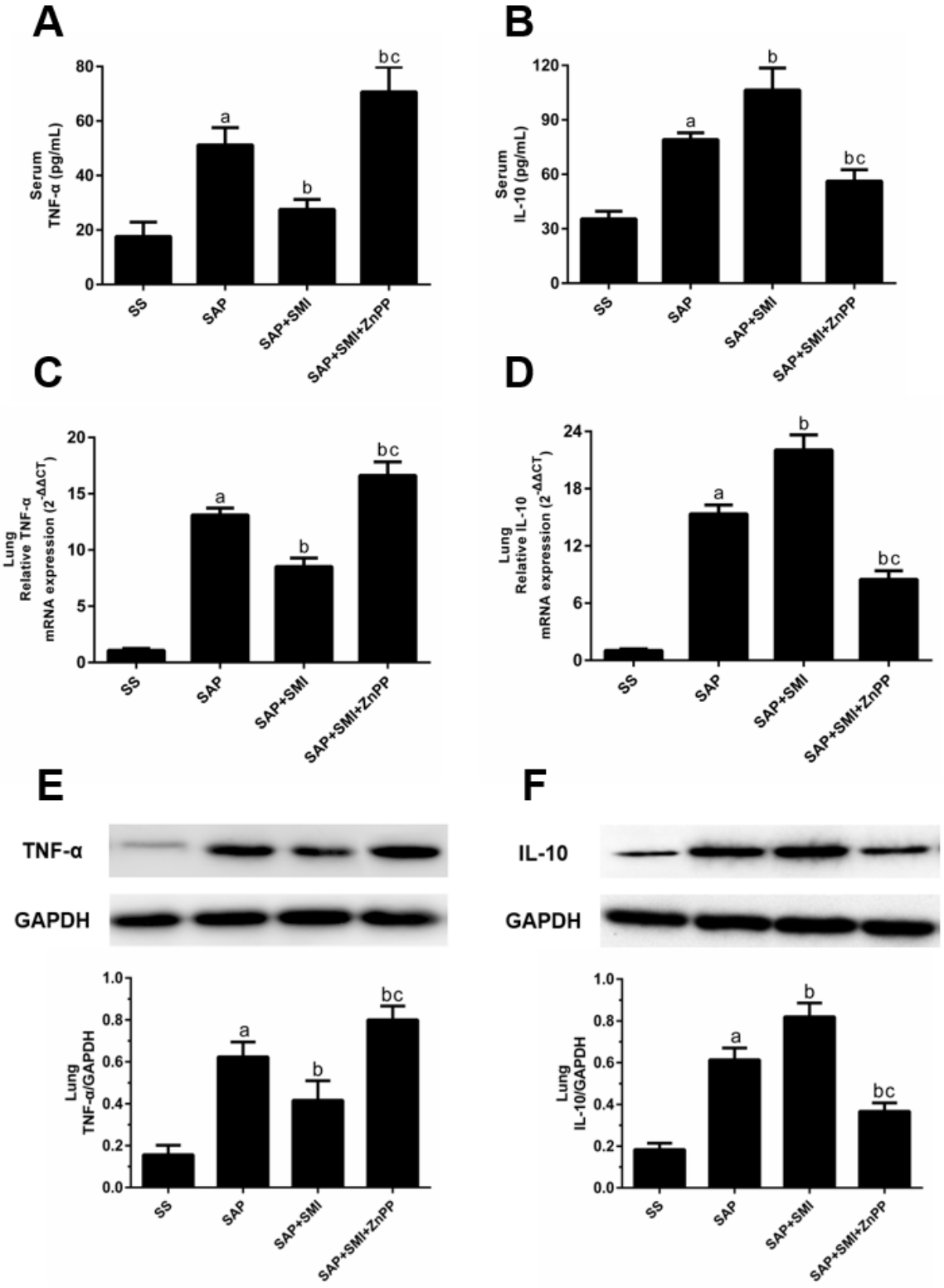

Figure 4

The IL-10 and TNF-α expression levels. TNF-α: (A), (C), (E); IL-10: (B), (D), (F); Serum: (A), (B); Lung: (C), (D), (E), (F). Data are shown as means ± SEM. ap0.05 relative to the SS group; bp0.05 relative to the SAP group; cp0.05 relative to the SAP + SMI group (n = 10).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ARRIVEchecklist.docx
- AdditionalFiles.pdf