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Original Article

Changes in oxidative stress indices in sulfur mustard ($1LD_{50}$)-induced pulmonary injuries via different routes

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Abstract: *Objective:* Sulfur mustard (SM) is an important chemical warfare agent. The mechanisms underlying SM toxicity have not been completely determined. However, oxidative stress and the following damage to macromolecules have been considered one of the crucial steps in SM toxicity. In the current study, an animal model of SM was established by equal toxicity dose ($1LD_{50}$)-induced acute pulmonary injuries in rats. This study used two methods to directly compare oxidative stress indices in serum enzymes and alveolar septum. *Methods:* Male Sprague-Dawley rats were randomly divided into intraperitoneal SM, intraperitoneal propylene glycol, tracheal SM, tracheal propylene glycol, and normal groups. SM-induced serum enzyme levels and protein-related expression in the alveolar septum were measured by ELISA and immunohistochemistry methods. *Results:* Serum SOD, CAT, and GSH-Px levels were increased in the intraperitoneal SM group, compared with the tracheal SM group. Positive expression ratios of CuZn-SOD, Mn-SOD, PON-1, and ApoA1 proteins in the alveolar septum of the intraperitoneal SM group were increased, compared with the tracheal SM group. *Conclusion:* Under SM ($1LD_{50}$), there were significantly higher serum enzyme levels and protein-related expression levels in the alveolar septum of rats injected with SM intraperitoneally, compared with SM administered by intratracheal instillation. Differences in oxidative stress indices associated with SM ($1LD_{50}$)-induced pulmonary injuries at the molecular level are dependent on the route of exposure.

Keywords: Sulfur mustard, lethal dose 50, lung injury, oxidative stress, rat

Introduction

Sulfur mustard (SM) is an alkylating and vesicating agent that has been used for 100 years as a chemical weapon [1, 2]. In its pure form, SM is a viscous and colorless fluid. SM enters the body through the eyes, skin, respiratory tract, and gastrointestinal tract, causing acute and delayed effects on different body organs [3, 4]. Different cellular mechanisms and molecular pathways are responsible for damage to body tissues, such as DNA damage, oxidative stress, apoptosis, and inflammation [5, 6].

The lungs are one of the major target organs of SM-associated injury. Mechanisms underlying SM-induced pulmonary injuries have not been completely determined and there are no medications with treatment efficacy, thus morbidity and mortality rates are high [7]. It has been reported that SM can induce pulmonary oxidative stress reactions by depleting cells of intracellular antioxidants, including glutathione, and increasing lipid peroxidation, thioredoxin, and the mitochondrial membrane potential, resulting in increased production of reactive oxygen species (ROS) and loss of antioxidant enzyme activity, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), thioredoxin reductase (TrxR), and glutathione S-transferase (GST) [8, 9].

Animal experiments have confirmed that SM-induced pulmonary injuries are not limited by the respiratory tract and can occur in multiple ways [10-13]. In addition, SM is a dose- and time-dependent toxic agent. Therefore, a positive correlation between dose and time and the degree of SM-induced pulmonary injury has been observed [14, 15]. Mechanisms underlying SM-induced inflammation and oxidative

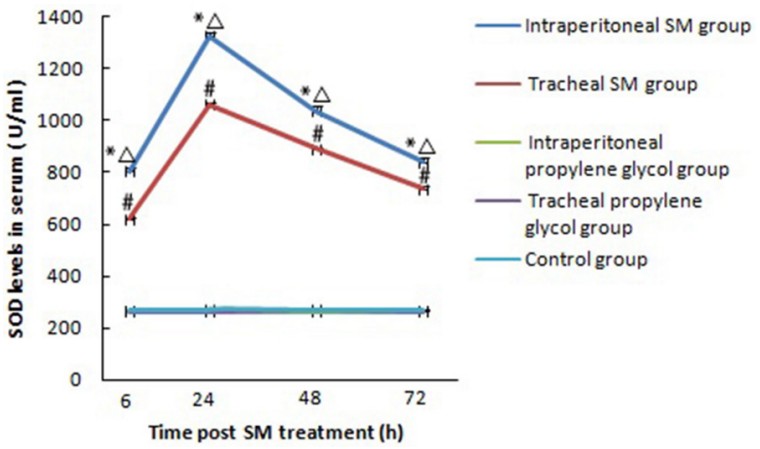

Figure 1. Serum SOD levels in rats with SM ($1LD_{50}$)-induced acute pulmonary injury via intraperitoneal injections and intratracheal instillation. Rats of the tracheal SM group were injected intratracheally with diluted SM ($1LD_{50}$ = 2 mg/kg), the rats of intraperitoneal SM group were injected intraperitoneally with diluted SM ($1LD_{50}$ = 8.2 mg/kg). X ± s, n = 8. $^{*}P$ < 0.05, compared with intraperitoneal propylene glycol control group. $^{\#}P$ < 0.05, compared with tracheal propylene glycol control group. $^{\Delta}P$ < 0.05, compared with tracheal SM group. Intraperitoneal propylene glycol control group, tracheal propylene glycol control group compared with corresponding normal control group.

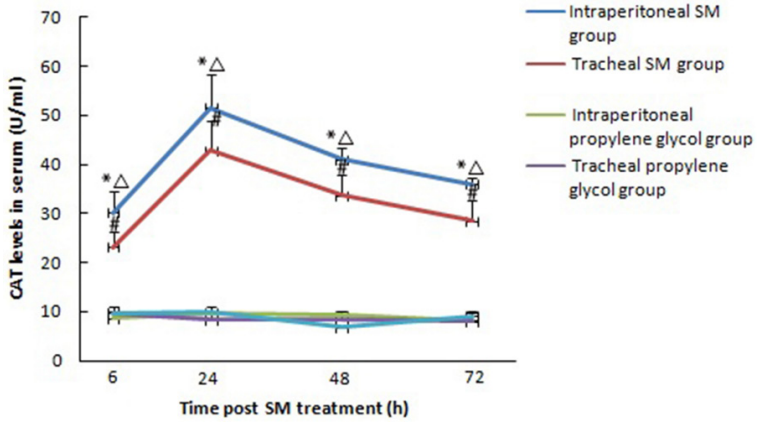

Figure 2. Serum CAT levels in rats with SM ($1LD_{50}$)-induced acute pulmonary injury via intraperitoneal injections and intratracheal instillation. See **Figure 1** for the rat treatment. X ± s, n = 8. $^{*}P$ < 0.05, compared with intraperitoneal propylene glycol control group. $^{\#}P$ < 0.05, compared with tracheal propylene glycol control group. $^{\Delta}P$ < 0.05, compared with tracheal SM group. Intraperitoneal propylene glycol control group, tracheal propylene glycol control group compared with corresponding normal control group.

stress have interdependence and reciprocal causation, playing a key role in the mechanisms leading to pulmonary injury [16, 17].

The present study conducted preliminary experiments by calculating the equal toxicity dose ($1LD_{50}$), by Horn's method, and establishing a rat model of SM ($1LD_{50}$)-induced acute pulmonary injury in rats via two administration routes. This study compared differences in serum enzyme levels and protein-related expression levels in alveolar septa, as a function of these different SM administration methods. Furthermore, this study investigated the molecular mechanisms underlying SM-induced acute pulmonary injury to provide a theoretical basis for targeted treatment.

Materials and methods

Ethical approval of the study protocol was obtained from the Animal Research Ethics Committee of the 89th Hospital of People's Liberation Army, Weifang, China.

Reagents and instruments

Reagents were sourced from the following providers. SM was provided by the Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences (Beijing, China). The 1, 2-propylene glycol solution was provided by Tianjin Zhiyuan Chemical Co. Ltd (Tianjun, China). SOD, CAT, and GSH-Px ELISA kits were provided by Hangzhou Lianke Biotech Co. Ltd (Hangzhou, China). Goat serum was provided by Beijing Zhongshan Jinqiao Biotech Co. Ltd (Beijing, China). CuZn-superoxide dismutase (CuZn-SOD), Mn-superoxide dismutase (Mn-SOD), paraoxonase-1 (PON-1), and apolipoprotein-1 (ApoA1) immunohistochemical kits, as well as secondary (biotin-labeled goat anti-rabbit IgG) and tertiary antibodies (horseradish enzyme-labeled streptavidin), were provided by Beijing Bioss Biotech Co. Ltd (Beijing, China). Finally, the instruments used in this study included an automatic biochemical immune analyzer (COBAS 8000 type; Roche, Basel, Switzerland), a cold light source (AXEL-

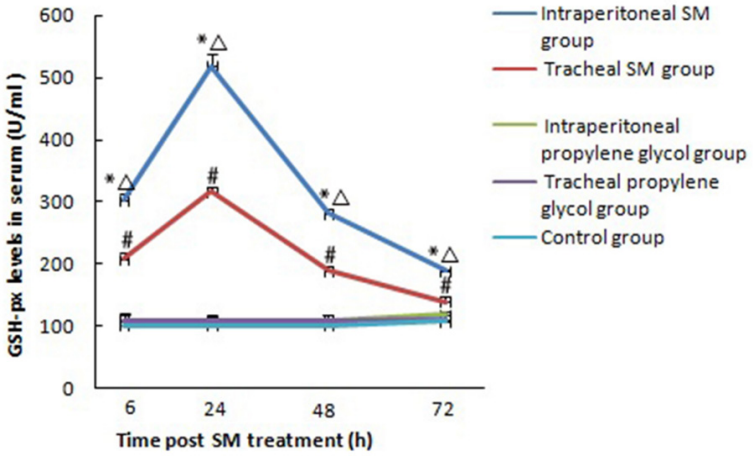

Figure 3. Serum GSH-Px levels in rats with SM ($1LD_{50}$)-induced acute pulmonary injury via intraperitoneal injections and intratracheal instillation. See **Figure 1** for the rat treatment. X ± s, n = 8. $^{*}P < 0.05$, compared with intraperitoneal propylene glycol control group. $^{\#}P < 0.05$, compared with tracheal propylene glycol control group. $^{\Delta}P < 0.05$, compared with tracheal SM group. Intraperitoneal propylene glycol control group, tracheal propylene glycol control group compared with corresponding normal control group.

300 type; Olympus, Tuttlingen, Germany), a light microscope (BX51 type; Olympus, Tokyo, Japan), and Image Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA).

Experimental animals and groups

One hundred eighty-six Sprague-Dawley rats (male, 280-300 g, 15 weeks, specific pathogen-free) were purchased from the Experimental Animal Center of the Chinese PLA Military Academy of Medical Sciences (certification No. 0015902, Beijing China). An acute pulmonary injury model was established in a preliminary experiment using intraperitoneal injections and intratracheal instillation of SM, in a total of 50 rats, aiming to calculate an equal toxicity dose ($1LD_{50}$) by Horn's method. One hundred thirty-six rats were then randomly put into groups as follows: intraperitoneal SM group, n = 32; intraperitoneal propylene glycol control group, n = 32; tracheal SM group, n = 32; tracheal propylene glycol control group, n = 32; and normal group, n = 8. SM (96% pure) was diluted in propylene glycol for later use.

Establishment of the animal model via intratracheal instillation. A sub-tegumental dose of atropine (0.05 mg/kg) was administered and, 30 minutes later, ketamine hydrochloride (100 mg/kg) was used as an anesthetic. SM [0.1 mL per rat (0.98 LD_{50} = 2 mg/kg)] was instilled into the tracheas of rats in the SM group, while propylene glycol (0.1 mL per rat) was instilled into the tracheas of those in the propylene glycol control group.

Establishment of the animal model via intraperitoneal injection was as follows. Rats were anesthetized, as described above. Next, SM [0.1 mL per rat (0.96 LD_{50} = 8 mg/kg)] was administered intraperitoneally, while propylene glycol (0.1 mL per rat) was administered to the intraperitoneal propylene glycol control group. The normal group received no treatment.

Measurement of serum oxidative stress indexes: Intracavitary puncture of the hearts and collection of blood samples (2 mL/rat) from the rats in the SM and propylene glycol groups were performed at 6, 24, 48, and 72 hours. Samples were placed in a 37°C water bath for 1 hour, stored at 4°C overnight, and centrifuged at 2000 × g for 20 minutes. The supernatant was then decanted into a sterile vial and stored at -80°C until further use. Serum SOD, CAT, and GSH-Px were measured using an automatic biochemical immune analyzer.

Immunohistochemistry

CuZn-SOD, Mn-SOD, PON-1, and ApoA1 expression was measured by immunohistochemical staining (streptavidin-peroxidase method). Tissue samples were embedded in paraffin, sectioned, and treated by conventional xylene dewaxing, followed by antigen retrieval. Rabbit anti-rat monoclonal antibodies against CuZn-SOD, Mn-SOD, PON-1, and ApoA1 (20 μL/slice) were then added. Following incubation with primary antibodies (1:800, 1:800, 1:400, 1:400, and 1:400, respectively), slices were then incubated with secondary (biotin-labeled goat anti-rabbit IgG) and tertiary antibodies (horseradish enzyme-labeled streptavidin). Finally, 3, 3'-diaminobenzidine staining was employed. Slices were counterstained with hematoxylin and slides were sealed with conventional resin.

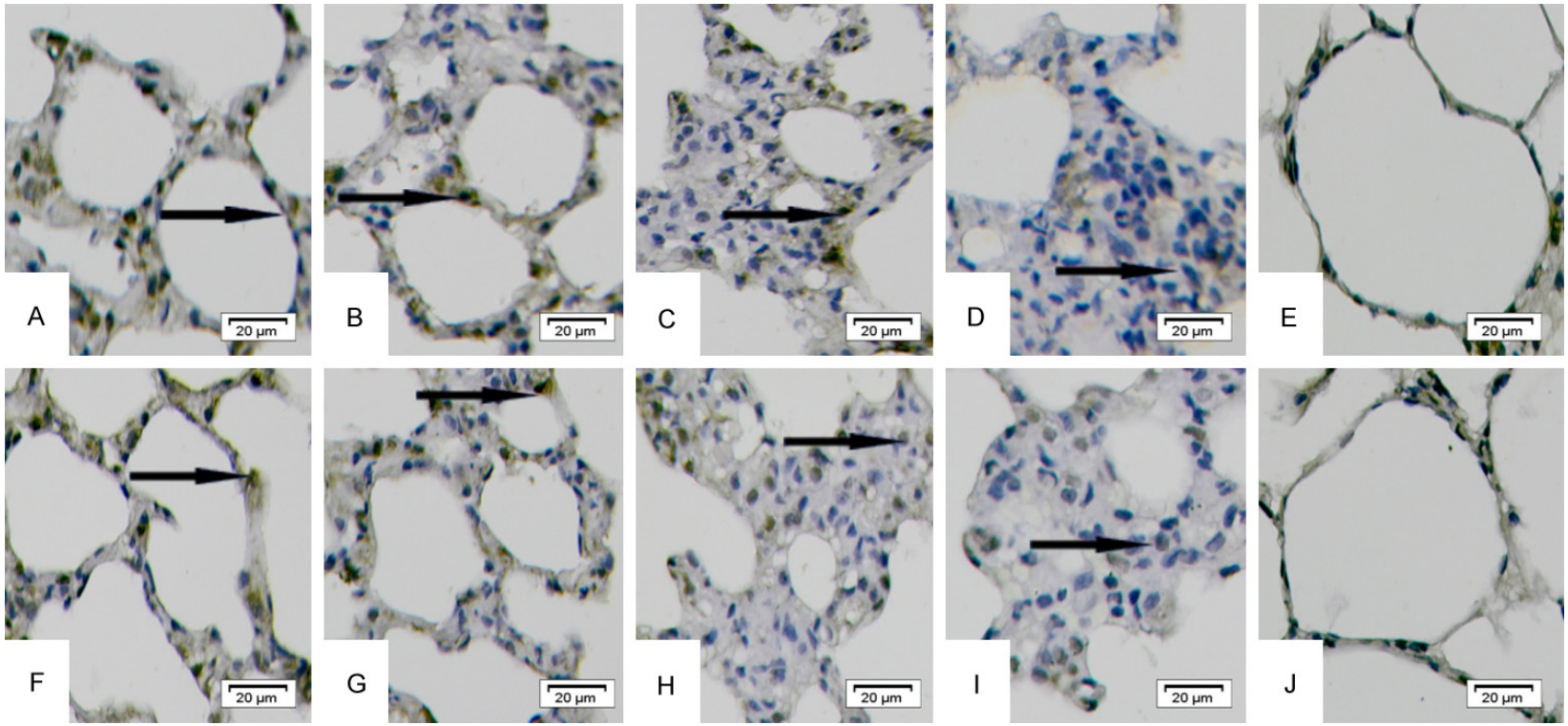

Figure 4. Expression of CuZn-SOD proteins in the alveolar septum of rats with SM ($1LD_{50}$)-induced acute pulmonary injury via intraperitoneal injections and intratracheal instillation at different time points. See **Figure 1** for the rat treatment. (A-D) show the positive expression at 6, 24, 48, 72 hours intraperitoneal SM group; (F-I) show the positive expression at 6, 24, 48, 72 hours in tracheal SM group; (E) and (J) show the negative expression in normal group (The arrow indicates positive expression, × 400, magnification, Bars = 20 μm).

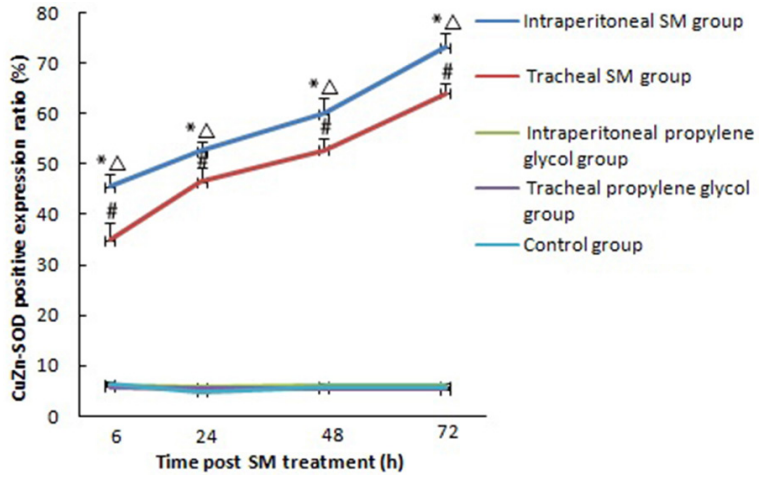

Figure 5. Positive expression ratios of CuZn-SOD proteins in the alveolar septum of rats with SM ($1LD_{50}$)-induced acute pulmonary injury via intraperitoneal injections and intratracheal instillation at different time points. See **Figure 1** for the rat treatment. X ± s, n = 8. $^{*}P < 0.05$, compared with intraperitoneal propylene glycol control group. $^{\#}P < 0.05$, compared with tracheal propylene glycol control group. $^{\Delta}P < 0.05$, compared with tracheal SM group. Intraperitoneal propylene glycol control group, tracheal propylene glycol control group compared with corresponding normal control group.

For negative controls, phosphate-buffered saline (PBS) was used instead of respective primary antibodies. Known positive sections were used as positive controls.

Microscopic image analysis

Image Pro Plus 6.0 Pathological Cell Image Analysis System was used to analyze CuZn-SOD, Mn-SOD, PON-1, and ApoA1 immunohistochemical staining from each group. All measurement parameters were manually selected and the abundance of positive cells and strongly positive cells was measured. A field of view was selected for each interval of a high-powered field of vision (× 400) to be observed. At least five high-power fields of vision were examined from each slice, along with positive cell ratios of the alveolar septum (= positive cells/total cells in five high power fields × 100%). Mean values were also calculated.

Statistical analyses

Data analyses were carried out using SPSS 17.0 software (SPSS, Inc., IBM Corp., Armonk, NY, USA). All values are expressed as mean ± standard deviation. Serum levels of SOD, CAT, and GSH-Px, as well as positive expression ratios of CuZn-SOD, Mn-SOD, PON-1, and ApoA1 proteins in the alveolar septum, were compared among the five groups by repeated measure ANONA at different times, respectively (F and *P* values). Study groups were compared with the control group using Student's t test. A value of $P < 0.05$ (two-tailed) indicates statistical significance.

Results

Changes in serum oxidative stress indices in rats with SM ($1LD_{50}$)-induced acute pulmonary injuries via intraperitoneal injection and intratracheal instillation

Serum levels of SOD, CAT, and GSH-Px in both the intraperitoneal and tracheal SM groups peaked at 24 hours. Afterward, they gradually decreased. Comparison of serum SOD, CAT, and GSH-Px levels among the five groups by repeated measure ANONA at different times points revealed: 1) Serum levels of SOD, CAT, and GSH-Px in the intraperitoneal and tracheal SM groups at each time point were significantly increased; 2) Compared with the other four groups, serum levels of SOD, CAT, and GSH-Px were significantly increased in the intraperitoneal SM group; and 3) In the intraperitoneal and tracheal SM groups, there was a trend for decreasing serum SOD, CAT, and GSH-Px levels over time that reached statistical significance (**Figures 1-3**).

Expression of CuZn-SOD, Mn-SOD, PON-1, and ApoA1 proteins in the alveolar septum of rats with SM ($1LD_{50}$)-induced acute pulmonary injury via intraperitoneal injection and intratracheal instillation at different time points

In intraperitoneal and tracheal SM groups, CuZn-SOD, Mn-SOD, PON-1, and ApoA1 protein expression in the alveolar septum was diffusely distributed at 6 hours. They were found to aggregate into clusters at 24 hours. This aggregation was also evident at 48 hours and at 72 hours. In contrast, in intraperitoneal propylene glycol, tracheal propylene glycol, and normal groups, CuZn-SOD, Mn-SOD, PON-1, and ApoA1 protein expression was scattered (**Figures**

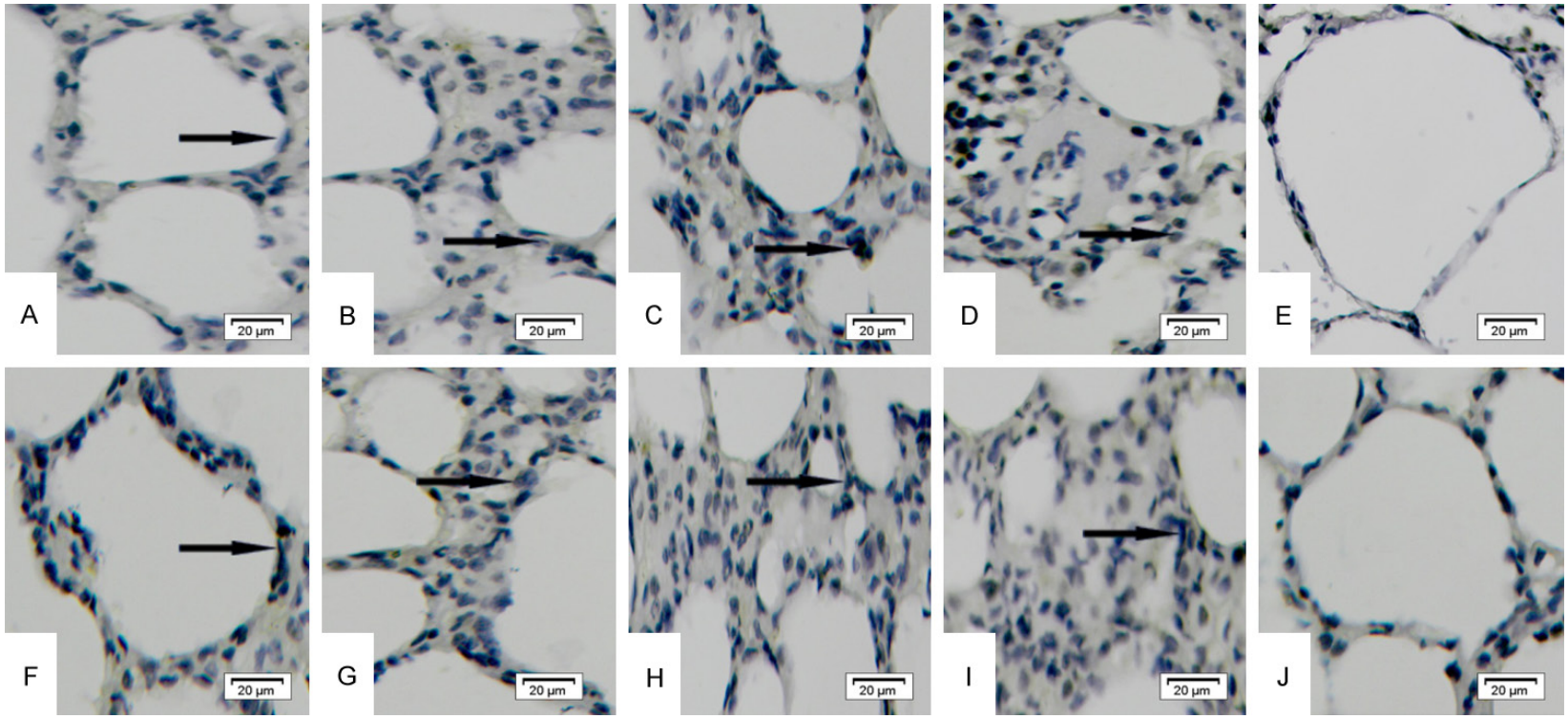

Figure 6. Expression of Mn-SOD proteins in the alveolar septum of rats with SM ($1LD_{50}$)-induced acute pulmonary injury via intraperitoneal injections and intratracheal instillation at different time points. See **Figure 1** for the rat treatment. (A-D) show the positive expression at 6, 24, 48, 72 hours intraperitoneal SM group; (F-I) show the positive expression at 6, 24, 48, 72 hours in tracheal SM group; (E) and (J) show the negative expression in normal group (The arrow indicates positive expression, × 400, magnification, Bars = 20 μm).

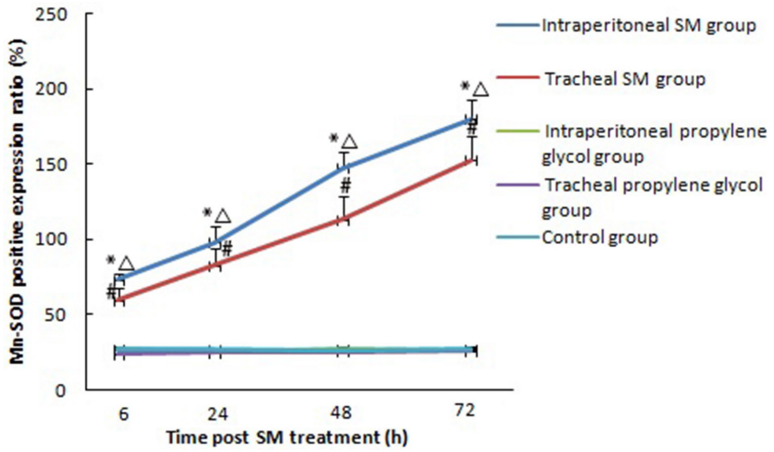

Figure 7. Positive expression ratios of Mn-SOD proteins in the alveolar septum of rats with SM ($1LD_{50}$)-induced acute pulmonary injury via intraperitoneal injections and intratracheal instillation at different time points. See **Figure 1** for the rat treatment. X ± s, n = 8. $^{*}P < 0.05$, compared with intraperitoneal propylene glycol control group. $^{\#}P < 0.05$, compared with tracheal propylene glycol control group. $^{\Delta}P < 0.05$, compared with tracheal SM group. Intraperitoneal propylene glycol control group, tracheal propylene glycol control group compared with corresponding normal control group.

4-**7**). Positive expression ratios of CuZn-SOD, Mn-SOD, PON-1, and ApoA1 proteins in the alveolar septum were compared among the five groups by repeated measure ANONA at different time points, with results revealing that: 1) Positive expression ratios of CuZn-SOD, Mn-SOD, PON-1, and ApoA1 proteins in the alveolar septum in the intraperitoneal and tracheal SM groups at each time point were significantly increased; 2) Compared with the other four groups, positive expression ratios of CuZn-SOD, Mn-SOD, PON-1, and ApoA1 proteins in the alveolar septum were significantly increased in the intraperitoneal SM group; and 3) In the intraperitoneal and tracheal SM groups, there were increasing trends of positive expression ratios of CuZn-SOD, Mn-SOD, PON-1, and ApoA1 proteins in the alveolar septum over time, reaching statistical significance (**Figures 8**-**11**).

Discussion

SM is a lipophilic and damaged cell alkylating agent. SM depletes blood, hepatic, and pulmonary glutathione (GSH) and increases the oxidized form (GSSG). It also decreases anti-oxidase. A decrease in GSH content leads to the accumulation of naturally-produced ROS within cells, inducing lipids, proteins, and nucleic acid damage [18, 19]. ROS may alter remodeling of apoptosis, the extracellular matrix, the mitochondrial respiration system, maintenance of surfactant, cell proliferation, the anti-protease screen, effective alveolar repair responses, and immune modulation in the lungs. Following an increase in intracellular ROS, mitochondrial damage and dysfunction lead to apoptosis [16]. Balance of oxidation and anti-oxidation is a key link of the body in maintaining normal cellular physiological metabolism. Otherwise, the pathological conditions of imbalance can be transformed. Cellular antioxidant systems include antioxidant enzymes (SOD, CAT, GSH-Px, and GST) and antioxidants (GSH, and vitamins E, A, and C). SOD catalyzes dismutation of the superoxide anion into H_2O_2, and CAT and GSH-Px independently degrade H_2O_2 to water, inhibiting the formation of OH radical [20].

The current study showed that SM induced a gradual increase in serum SOD, CAT, and GSH-Px levels, reaching a peak at 24 hours, then exhibited a decreasing trend. Under SM ($1LD_{50}$) conditions, changes in serum zymogram in the intraperitoneal SM group were increased, compared with the tracheal SM group. Results showed that SM induced a transient increase in serum anti-oxidative enzymes in rats via two methods, indicating that SM can be absorbed into the blood via two routes, thus causing a systemic oxidative stress reaction. A short-term increase in serum SOD, CAT, and GSH-Px levels is a compensatory reaction of the body against an imbalance of oxidation and antioxidants. The compensatory response appears transiently. Decompensation of antioxidant enzymes and antioxidants will lead to the SM poisoning reaction. Results of this present study are consistent with results reported by Jafari et al. [20]. The findings may be related to enzyme elevation and protection against SM-induced acute pulmonary injury and are beneficial to produce adequate superoxide anion to antagonize the oxidative stress re-

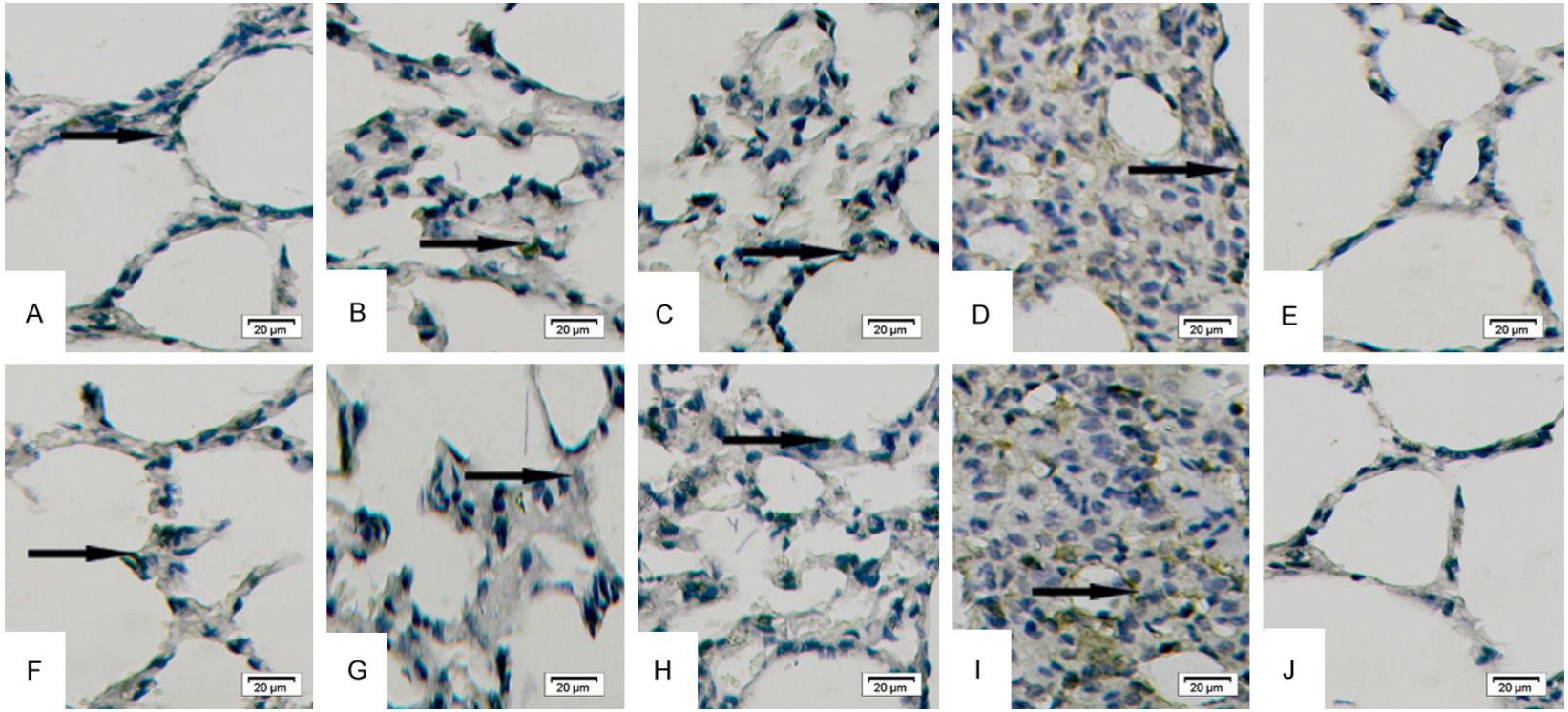

Figure 8. Expression of PON-1 proteins in the alveolar septum of rats with SM ($1LD_{50}$)-induced acute pulmonary injury via intraperitoneal injections and intratracheal instillation at different time points. See **Figure 1** for the rat treatment. (A-D) show the positive expression at 6, 24, 48, 72 hours intraperitoneal SM group; (F-I) show the positive expression at 6, 24, 48, 72 hours in tracheal SM group; (E) and (J) show the negative expression in normal group (The arrow indicates positive expression, × 400, magnification, Bars = 20 μm).

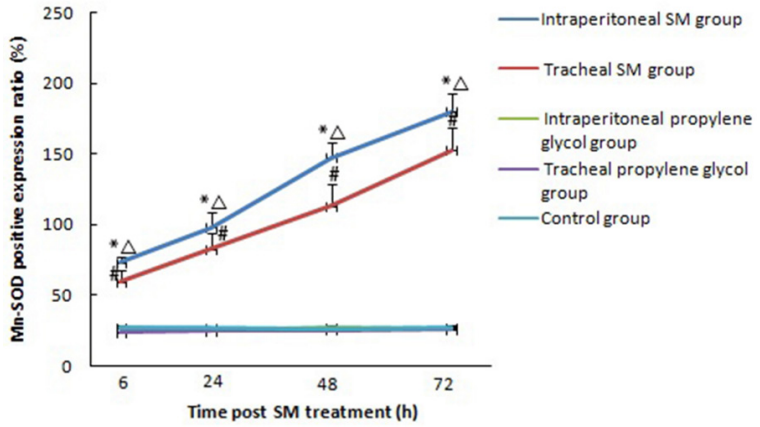

Figure 9. Positive expression ratios of PON-1 proteins in the alveolar septum of rats with SM ($1LD_{50}$)-induced acute pulmonary injury via intraperitoneal injections and intratracheal instillation at different time points. See **Figure 1** for the rat treatment. X ± s, n = 8. $^{*}P < 0.05$, compared with intraperitoneal propylene glycol control group. $^{\#}P < 0.05$, compared with tracheal propylene glycol control group. $^{\Delta}P < 0.05$, compared with tracheal SM group. Intraperitoneal propylene glycol control group, tracheal propylene glycol control group compared with corresponding normal control group.

sponse in alveoli and blood. As reported in the literature, antioxidant enzymes and antioxidants were significantly reduced in patients with SM-induced chronic pulmonary injuries, indicating a serious imbalance of the body between ROS production and the cellular antioxidant defense system [17, 21]. Therefore, it is believed that supplementation of exogenous antioxidant enzymes and antioxidants at the appropriate time may be one of the key steps in preventing the development of SM-induced pulmonary injuries [22, 23].

It has been demonstrated that SM-induced oxidative stress responses (cytochrome C release and ROS accumulation) is through ROS and reactive nitrogen species signaling pathways, resulting in cell damage [24]. PON-1 is a multi-functional antioxidant enzyme which hydrolyzes a variety of organic compounds, including organophosphates, aryl esters, lactones, and specific oxidized lipids [25]. SOD forms a family containing three isoforms [cytosolic (Cu-Zn), mitochondrial (Mn), and extracellular (Cu-Zn) enzymes]. Mn-SOD is moderately expressed in respiratory epithelium, alveolar type II epithelial cells, alveolar macrophages, and interstitial fibroblasts in hypoxia-exposed rats. In the airway epithelium, CuZn-SOD is highly expressed in ciliated epithelial cells [26, 27].

This study also demonstrated that SM induced a higher expression of CuZn-SOD, Mn-SOD, PON-1, and ApoA1 proteins in alveolar septa. Positive expression ratios of CuZn-SOD, Mn-SOD, PON-1, and ApoA1 proteins in the intraperitoneal SM group increased significantly, positively correlating with exposure time. Results suggest that pulmonary oxidative stress responses were linearly increased in the acute pulmonary injury phase, according to the regularity of SM-induced pulmonary injury (injury gradually increasing with time). This was different from the transient elevation of serum enzyme spectrum (an excessive increase), which indicated that the oxidative stress response of SM to the target organ was present under continuous conditions. Many studies have shown that, in the acute phase of SM-induced pulmonary injury, CuZn-SOD, Mn-SOD, PON-1, and ApoA1 protein markers in bronchoalveolar lavage fluid and lung tissue are elevated and mRNA expression and protein content are increased [26-28]. During the chronic period, however, the expression and content of the markers was significantly reduced [25, 29, 30]. These results suggest that elevation in antioxidant markers during the acute phase of SM-induced pulmonary injuries indicate the body's antioxidant ability, an important part of normal physiological metabolism [27]. PON-1 and ApoA1 proteins in the chronic phase may be associated with SM-induced COPD or bronchiolitis [29].

It was speculated that the oxidative stress mechanisms of SM-induced pulmonary injuries may be related to intracellular GSH and thioredoxin depletion, a change in the mitochondrial membrane, decreased mitochondrial electron transfer chain activity, accumulation of intracellular ROS, reduced antioxidant enzymes, a disturbance in oxidation-related channel regulation and the internal environment with oxidation maintaining an imbalance of oxidation and antioxidants, damaged cells membrane lipids, protein, and DNA, eventually leading to pulmonary edema, pulmonary surfactant injury,

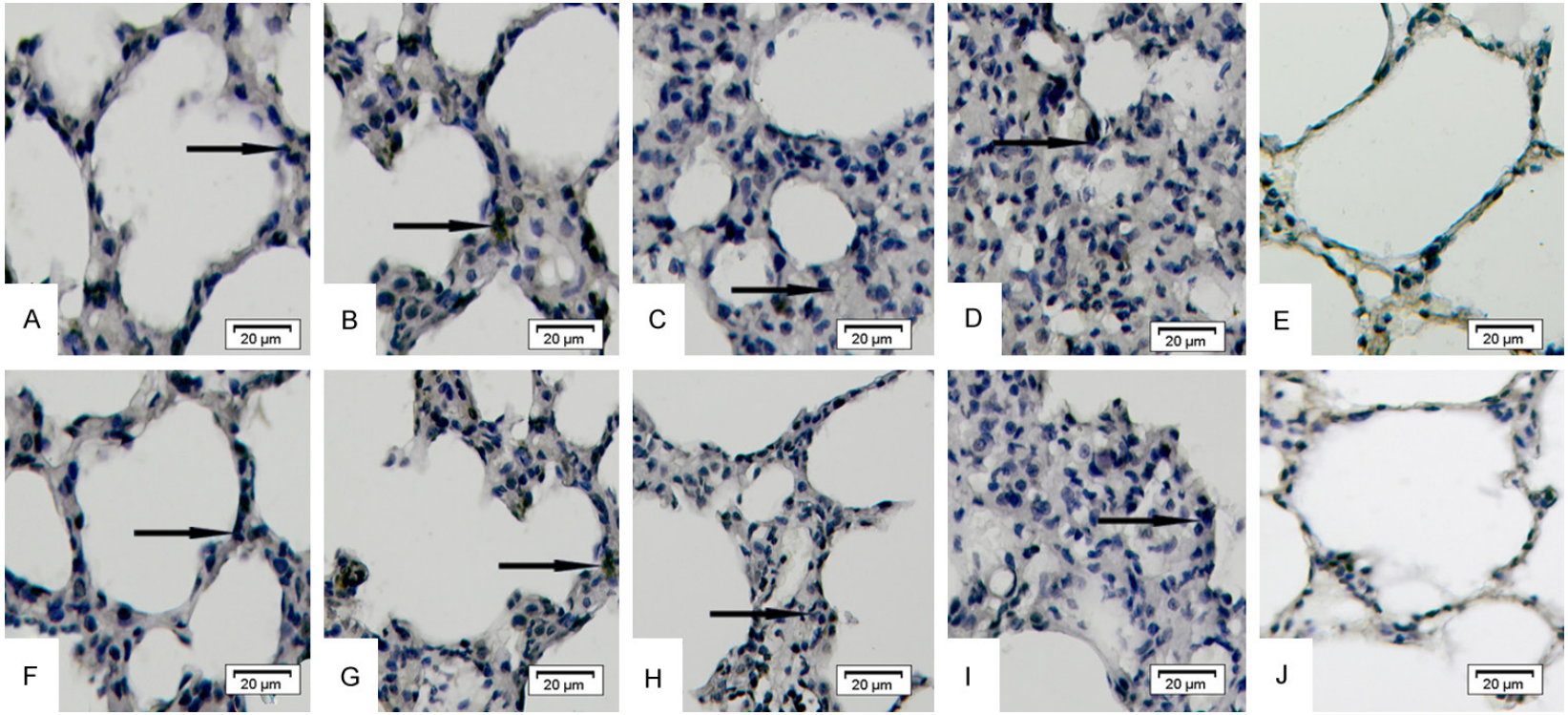

Figure 10. Expression of ApoA1 proteins in the alveolar septum of rats with SM ($1LD_{50}$)-induced acute pulmonary injury via intraperitoneal injections and intratracheal instillation at different time points. See **Figure 1** for the rat treatment. (A-D) show the positive expression at 6, 24, 48, 72 hours intraperitoneal SM group; (F-I) show the positive expression at 6, 24, 48, 72 hours in tracheal SM group; (E) and (J) show the negative expression in normal group (The arrow indicates positive expression, × 400, magnification, Bars = 20 μm).

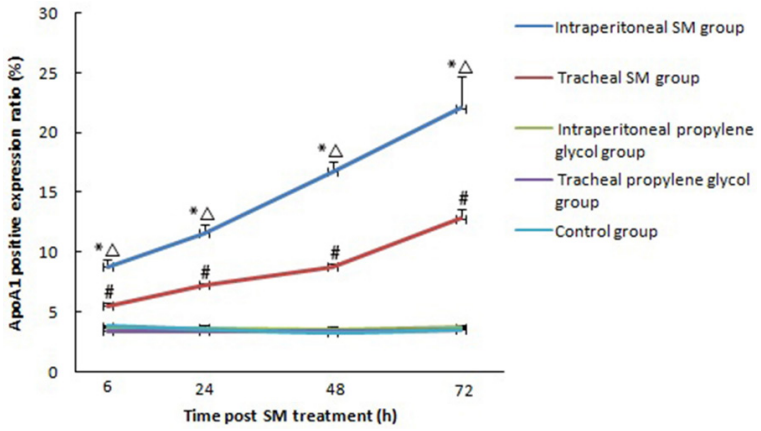

Figure 11. Positive expression ratios of ApoA1 proteins in the alveolar septum of rats with SM ($1LD_{50}$)-induced acute pulmonary injury via intraperitoneal injections and intratracheal instillation at different time points. See **Figure 1** for the rat treatment. X ± s, n = 8. $^{*}P < 0.05$, compared with intraperitoneal propylene glycol control group. $^{\#}P < 0.05$, compared with tracheal propylene glycol control group. $^{\Delta}P < 0.05$, compared with tracheal SM group. Intraperitoneal propylene glycol control group, tracheal propylene glycol control group compared with corresponding normal control group.

emphysema, and "SM lung" [31, 32]. Under SM ($1LD_{50}$), there are also significantly higher serum inflammatory factor levels and protein-related expression levels in the alveolar septa of rats injected intraperitoneally with SM, compared with rats administered SM by intratracheal instillation. Results suggest that differences in oxidative stress indices at the molecular level associated with SM ($1LD_{50}$)-induced pulmonary injury are dependent on the route of exposure.

Conclusion

The cellular and molecular mechanisms of SM-induced pulmonary injury are complex and poorly understood, but oxidative stress plays a key role in the pulmonary injury process. Antioxidant enzymes and antioxidants play an important role in preventing or changing the peroxidation process. Reciprocal causation between oxidative stress and inflammation is a starting factor of SM-induced pulmonary injury on the cascade effects, which determines the complications and consequences of SM-induced pulmonary injuries. Interestingly, in the late stage of SM-induced pulmonary injury, it was not possible to achieve the expected effects, even though the corresponding antioxidant enzymes and antioxidants were administered [33]. Results reveal that SM-induced pulmonary injuries are through complex mechanisms, especially the irreversibility of DNA oxidation, which determines the uncertainty of SM prevention and treatment [34].

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Disclosure of conflict of interest

None.

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