Original Article
The differences in apoptosis and pulmonary fibrosis between sulfur mustard-induced acute pulmonary injury via intraperitoneal injection and intratracheal instillation in rats

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Abstract: Objective: Sulfur mustard (SM) is a vesicant alkylating agent. There are still biochemical mechanisms underlying SM damage that are unknown. This study was to determine the differences of apoptosis and pulmonary fibrosis underlying SM-induced acute pulmonary injury via intraperitoneal injection and intratracheal instillation in rats. Methods: An acute pulmonary injury model was established in a preliminary experiment using intraperitoneal injection and intratracheal instillation of SM in rats, in order to determine the equal toxicity dose of SM (1LD50). Then, SM-induced changes in apoptosis and pulmonary fibrosis were observed by immunohistochemical staining, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, Masson staining and electron microscopic observation in intraperitoneal and tracheal SM groups that were injected intraperitoneally and instilled intratracheally with diluted SM [0.1 mL per rat (0.98 LD50 = 2 mg/kg)] and SM [0.1 mL per rat (0.96 LD50 = 8 mg/kg)] respectively, and an untreated control group. Results: In the alveolar septum, the positive expression ratio of apoptotic cells, Bax, caspase-3, and caspase-9 by TUNEL and Immunohistochemistry (streptavidin-perosidase method) in the intraperitoneal SM group was increased compared with the tracheal SM group (P < 0.05), but a significantly lower positive expression ratio of Bcl-2 was detected (P < 0.05). Electron microscopic observations confirmed that the type I and II alveolar epithelial cells in lungs exhibited apoptotic morphologic features, such as cracked, lost, and disordered microvilli of membranes, fuzzy mitochondrial cristae, and detached, dissociated ribosomes from the surface of the rough endoplasmic reticulum. A significantly higher positive expression ratio of MMP-2, MMP-9, TIMP-1, TIMP-2, collagen type I, collagen type III, TGF-β1, and Smad7 by immunohistochemical staining in the alveolar septum were detected in the intraperitoneal SM group compared with the tracheal SM group (P < 0.05). Conclusions: These data demonstrated increased apoptosis and pulmonary fibrosis via intraperitoneal injection under equal toxicity doses SM (1LD50) in rats.

Keywords: Sulfur mustard, acute pulmonary injury, apoptosis, pulmonary fibrosis, rat

Introduction

Sulfur mustard (bis [2-chloroethyl] sulfide {SM}) is a vesicant alkylating agent with strong cytotoxic, mutagenic, and carcinogenic properties. SM can cause skin irritation and blisters, respiratory tract lesions, bone marrow depression, and eye damage, with the epithelial tissues of these organs predominately affected [1, 2]. SM is still considered one of the most important chemical warfare agents [3]. Although the clinical evidence of acute SM injury is well-characterized by clinical documentation of the victims of the Iran-Iraq war [4, 5], the biochemical mechanisms underlying SM damage are still unknown and the antidote of SM-induced toxicity is still unavailable [6]. Acute respiratory lesions are the main cause of mortality, but there is little information available about the histopathology [7, 8].

The mechanism of SM-caused deleterious responses has been linked mainly to their alkyl-
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A SM nuclear lesion is believed to determine SM-induced cell death [11], which can be necrotic or apoptotic, depending on the concentration of the toxicant [12, 13]. SM has been shown to induce p53, reduce Bcl-2, and activate caspase 3 in vitro [14]. In vivo studies with rodent pulmonary tissue exposed to SM showed high levels of apoptosis-related genes overexpression [15]. High doses of inhaled SM may lead to signs and symptoms of systemic intoxication [16]. The respiratory tract is one of the main targets of SM and is the site of the most disabling lesions for exposed subjects [17, 18]. Pulmonary fibrosis (PF) is a well-known late complication of SM gas exposure in humans [19], however, the mechanisms driving the fibrotic evolution of SM-induced PF are still poorly understood.

To elucidate the biological and molecular mechanisms underlying apoptosis and PF, we investigated the special staining method and immunohistochemistry of pulmonary tissues in rats exposed to SM. We determined the changes in apoptosis and PF indices due to SM-induced acute pulmonary injury in rats via intraperitoneal injection and intratracheal instillation. The results were compared in the two groups.

Materials and methods

Ethical approval of the study protocol was obtained from the Animal Research Ethics Committee of Weifang Medical University, Weifang, China.

Reagents and instruments

Reagents were sourced from the following providers: SM was provided by Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences (Beijing, China). 1, 2-propylene glycol solution was provided by the Tianjin Zhiyuan Chemical Co. Ltd (Tianjin, China). Sheep serum was provided by the Beijing Zhongshan Jinqiao Biotech Co. Ltd (Beijing, China). Microwave buffer liquid self-made reagent was provided by the Beijing Chemical Plant (Beijing, China). Masson staining solution was provided by the Beijing Bioss Biotech Co. Ltd (Beijing, China). Protease K, apop A, and apop B were provided by Roche Biotechnology Company (Basel, Switzerland). Bcl-2 Associated X Protein (Bax), B cell lymphoma/leukemia-2 (Bcl-2), caspase-3, caspase-9,
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matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), tissue inhibitor of matrix metalloproteinases-1 (TIMP-1), tissue inhibitor of matrix metalloproteinases-2 (TIMP-2), collagen type I, collagen type III, transforming growth factor-beta 1 (TGF-β1), Mothers against decapentaplegic homolog 7 (Smad7) immunohistochemical kits were provided by the Beijing Bioss Biotech Co. Ltd (Beijing, China). Cold light source (AXEL-300 type; Olympus, Tuttlingen, Germany); Light microscope (BX51 type; Olympus, Tokyo, Japan); Hitachi H-7500 transmission electron microscope (Hitachi, Tokyo, Japan).

Experimental animals and groups

One hundred thirty-six Sprague-Dawley rats (male, 280-300 g, 15 weeks, specific pathogen-free, purchased from the Experiment-
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Figure 4. The positive expression ratio of apoptotic cells (A), Bax (B) and Bcl-2 (C) apoptotic proteins in the alveolar septum in rats. Compared with the tracheal SM group, intraperitoneal propylene glycol control group, tracheal propylene glycol control group, and normal control group, **P < 0.05; Compared with intraperitoneal propylene glycol control group, tracheal propylene glycol control group, and normal control group, *P < 0.05.

Figure 5. The expression of caspase-3 apoptotic proteins in the alveolar septum in rats. A-D: Show the positive expression (arrow) at 6, 24, 48, 72 h in intraperitoneal SM group; F-I: Show the positive expression (arrow) at 6, 24, 48, 72 h in tracheal SM group; E, J: Show the negative expression in the intraperitoneal and tracheal control group (caspase-3 staining, × 400, magnification, Bars, 20 μm).

Animal Center of the Chinese PLA Military Academy of Medical Sciences, certification No. 0015902, Beijing China) were randomized 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 (purity
96%) was diluted in propylene glycol for later use.

i. Establishment of the animal model via intratracheal instillation: Sub-tegumental atropine (0.05 mg/kg) was administered, and 30 min later ketamine hydrochloride (100 mg/kg) was used as an anesthetic. SM [0.1 mL per rat (0.98 LD50 = 2 mg/kg)] was instilled into the trachea of rats in the SM group, while propylene glycol (0.1 mL per rat) was instilled into the trachea of those in the propylene glycol control group. ii. Establishment of the animal model via intraperitoneal injection: Rats were anesthetized as described previously, then SM [0.1 mL per rat (0.96 LD50 = 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.

Observation indices

**Apoptosis staining:** Apoptotic cells were detected with in situ labeling of TUNEL as follows: (1) slice dewaxing–dehydrated in xylene for 1, 2 (overnight), and 3 h; (2) hydration-100%, 95%, and 80% for 2 min; (3) washed for 5 sec; (4) twice-washed with 1 x PBS for 5 sec; (5) after wiping dry, dripped protease K (diluted in PBS 1:10), then kept warm at 37°C for 10-20 min; (6) twice-washed in 1 x PBS for 5 sec; (7) dehydrated in 90% ethanol for 5 sec in the cold; (8) dripped 20 µl of slice reaction liquid (18 µl of apop A and 2 µl of apop B using an anterior preparation), kept warm at 37°C in a wet box for 1-2 h; (9) thrice-washed with Buffer I for 5 min; (10) HS sealing (diluted in Buffer I 1:100) at room temperature for 30-60 min; (11) SP-AP (diluted in Buffer I 1:500) at room temperature for 50 µl/slice; (12) twice-washed in Buffer I for 15 min; (13) twice-washed in Buffer III for 1 min; and (14) stained with NBT-BCIP. The cells in which the nucleus or cytoplasm had brown granules were referred to as apoptotic cells. The known positive sections were used as positive controls.

**Masson staining:** Dewatered paraffin sections were treated, and in turn rinsed with tap and distilled water. After Regaud hematoxylin staining for 5-10 min, the sections were rinsed with tap and distilled water. After using Ponceau red acid liquid for 5-10 min, the sections were briefly rinsed with 2% glacial acetic acid solution. The sections were immersed in 1% phosphomolybdic acid solution for 3-5 min without rinsing in water, directly stained with aniline blue liquid for 5 min and briefly rinsed with 0.2% glacial acetic acid solution, dipped in 95% alcohol and anhydrous alcohol, cleared in xylene, and fixed in neutral balata.

**Immunohistochemistry staining:** The expression of Bax, Bcl-2, caspase-3, caspase-9, MMP-2,
MMP-9, TIMP-1, TIMP-2, collagen type I, collagen type III, TGF-β1, and Smad7 were measured by immunohistochemical staining (streptavidin-peroxidase method). Tissue samples were embedded in paraffin, sectioned, and treated by conventional xylene dewaxing, followed by antigen repair. Rabbit anti-rat monoclonal antibodies against Bax, Bcl-2, caspase-3, caspase-9, MMP-2, MMP-9, TIMP-1, TIMP-2, collagen type I, collagen type III, TGF-β1, and Smad7 (20 μL/slice) were then added. Following incubation with secondary antibody, DABC coloration, and counterstaining with hematoxylin, the slides were sealed with conventional resin. For negative controls, PBS was used instead of the primary antibody. The known positive sections were used as positive controls.

**Electron microscopic observation**

A total of 1 mm³ of fresh specimens from all the groups were obtained. The specimens were fixed in 3% glutaraldehyde (Lebang Biotech Co. Ltd, Suzhou, China) for 24-48 h, dehydrated, embedded, and dried. The specimens were then made into ultrathin (70 nm) slices (UC7 ultrathin slicing machine; Leica). Slices were cleansed with water and soaked in a saturated aqueous solution of uranyl acetate (Zhenpu Biotech Co. Ltd, Shanghai, China). The slices were then cleaned with double distilled water and soaked in lead citrate solution (Tianyuan Co. Ltd, Yingkou, China). The ultrastructural changes of venous structure cells were observed using a Hitachi H-7500 transmission electron microscope (Hitachi, Tokyo, Japan).

**Microscopic image analysis:** Using Image Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA) pathological cell image analysis system to analyze the images of Bax, Bcl-2, caspase-3, caspase-9, MMP-2, MMP-9, TIMP-1, TIMP-2, collagen type I, collagen type III, TGF-β1, and Smad7 immunohistochemical staining of sections from each group, we selected the measurement parameters, and measured the abundance of strongly positive and positive cells. We selected a field of view for each interval of a high power field of vision (× 400) to be observed. At least five high-power fields of vision were examined from each slice, and the positive cell ratio of the alveolar septum (= positive cells/total cells in five high power fields × 100%) and the mean value were 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 the mean ± standard deviation. The positive expression ratio of apoptotic cells, apoptotic proteins, MMP-2, MMP-9, TIMP-1, TIMP-2, collagen type I, collagen type III, TGF-β1, and Smad7 proteins in the alveolar septum were compared in five groups by ANONA of repeated measures at different times, respectively (F and P values). Study groups were compared with the control...
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Results

Situation and positive expression ratio of apoptotic cells and Bax, Bcl-2 apoptotic proteins in rats

In the intraperitoneal and tracheal SM groups, the apoptotic cells and apoptotic proteins (TUNEL and Bax) in the alveolar septum were diffusely distributed at 6 and 24 h, but aggregated into clusters at 48 h, and coacervated at 72 h. The change in Bcl-2 apoptotic protein was opposite. In contrast, in the intraperitoneal propylene glycol, tracheal propylene glycol, and normal groups, the apoptotic cells and apoptotic proteins were scattered (Figures 1-3). The apoptotic cells and apoptotic proteins in the alveolar septum were compared in five groups by ANOVA of repeated measures at different times. i. The positive expression ratio of the apoptotic cells and apoptotic proteins in the alveolar septum in the intraperitoneal and tracheal SM groups at each time point were significantly increased. ii. Compared with the other four groups, the positive expression ratio of the apoptotic cells and apoptotic proteins in the alveolar septum were significantly increased in the intraperitoneal SM group. iii. In the intraperitoneal and tracheal SM groups, there was statistical significance in the changed trend of the positive expression ratio of the apoptotic cells and apoptotic proteins in the alveolar septum. TUNEL, Bax staining had an increasing trend, but Bcl-2 staining had a decreasing trend (Figure 4).

Situation and positive expression ratio of caspase-3 and caspase-9 apoptotic proteins in rats

In the intraperitoneal and tracheal SM groups, the apoptotic proteins (caspase-3 and caspase-9) in the alveolar septum were diffusely distributed at 6 and 24 h, but aggregated into clusters at 48 h, and coacervated at 72 h. In contrast, in the intraperitoneal propylene glycol, tracheal propylene glycol, and normal groups, the apoptotic proteins were scattered (Figures 5, 6). The apoptotic proteins in the alveolar septum were compared in five groups by ANOVA of repeated measures at different times. i. The positive expression ratio of the apoptotic proteins in the alveolar septum in the intraperitoneal and tracheal SM groups at each time point was significantly increased. ii. Compared with the other four groups, the positive expression ratio of the apoptotic proteins in the
the alveolar septum were significantly increased in the intraperitoneal SM group. iii. In the intraperitoneal and tracheal SM groups, there was statistical significance in the changed trend of the positive expression ratio of the apoptotic proteins in the alveolar septum. caspase-3 and caspase-9 staining had an increasing trend (Figure 7).

**Ultrastructural changes in the alveolar epithelial cells in rats**

In both the intraperitoneal and tracheal SM groups at 72 h, the cell membrane of the type I alveolar epithelial cells was partially missing. There was a lack of definition of the mitochondrial cristae; specifically, the ribosomes that...
were attached to the rough endoplasmic reticulum (RER) had detached and were dissociated in the cytoplasm. The membranes of type II alveolar epithelial cells were intact, but the microvilli were cracked, lost, and disordered. The mitochondrial cristae were fuzzy, and the ribosomes were detached from the surface of the RER and dissociated. In the intraperitoneal and tracheal propylene glycol control groups were the same as the normal group (Figure 8).

**Situation of Masson staining in rats**

In both the intraperitoneal and tracheal SM groups, the minimum amount of green-staining collagen fibers in the alveolar septum were seen at 6 h, and a small amount of green-staining collagen fibers were noted at 24 and 48 h; green-staining collagen fibers were increased at 72 h. In contrast, in the intraperitoneal propylene glycol, tracheal propylene glycol, and

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**Figure 11.** The expression of MMP-9 proteins in the alveolar septum in rats. A-D: Show the positive expression (arrow) at 6, 24, 48, 72 h in intraperitoneal SM group; F-I: Show the positive expression (arrow) at 6, 24, 48, 72 h in tracheal SM group; E, J: Show the negative expression in the intraperitoneal and tracheal control group (MMP-9 staining, × 400, magnification, Bars, 20 μm).

**Figure 12.** The expression of TIMP-1 proteins in the alveolar septum in rats. A-D: Show the positive expression (arrow) at 6, 24, 48, 72 h in intraperitoneal SM group; F-I: Show the positive expression (arrow) at 6, 24, 48, 72 h in tracheal SM group; E, J: Show the negative expression in the intraperitoneal and tracheal control group (TIMP-1 staining, × 400, magnification, Bars, 20 μm).
normal groups, the minimum amount of green-staining collagen fibers in the alveolar septum were distributed (Figure 9).

**Situation and positive expression ratio of MMP-2, MMP-9, TIMP-1, and TIMP-2 proteins in rats**

In both intraperitoneal and tracheal SM groups, the proteins (MMP-2, MMP-9, TIMP-1, and TIMP-2 staining) in the alveolar septum were diffusely distributed at 6 and 24 h, but aggregated into clusters at 48 h, and coacervated at 72 h. In contrast, in the intraperitoneal propylene glycol, tracheal propylene glycol, and normal groups, the proteins were scattered (Figures 10-13). Comparison of MMP-2, MMP-9, TIMP-1, and TIMP-2 proteins in the alveolar septum in five groups by ANONA of repeated measuring at different time. i. The positive expression ratio of MMP-2, MMP-9, TIMP-1, and TIMP-2 proteins in the alveolar septum in the intraperitoneal and tracheal SM groups at each time-point was significantly increased. ii. Compared with the other four groups, the positive expression ratio of MMP-2, MMP-9, TIMP-1, and TIMP-2 proteins in the alveolar septum were significantly increased in the intraperitoneal SM group. iii. In the intraperitoneal and tracheal SM groups, there is statistical significance in the changed trend of the positive expression ratio of MMP-2, MMP-9, TIMP-1, and TIMP-2 proteins in the alveolar septum.

**Situation and positive expression ratio of collagen type I, collagen type III, TGF-β1, and Smad7 proteins in rats**

In both intraperitoneal and tracheal SM groups, the proteins (collagen type I, collagen type III, TGF-β1, and Smad7 staining) in the alveolar septum were diffusely distributed at 6 and 24 h, but aggregated into clusters at 48 h, and coacervated at 72 h. In contrast, in the intraperitoneal propylene glycol, tracheal propylene glycol, and normal groups, the proteins were scattered (Figures 15-18). Comparison of collagen type I, collagen type III, TGF-β1, and Smad7 proteins in the alveolar septum in five groups by ANONA of repeated measuring at different time. i. The positive expression ratio of collagen type I, collagen type III, TGF-β1, and Smad7 proteins in the alveolar septum in the intraperitoneal and tracheal SM groups at each time-point was significantly increased. ii. Compared with the other four groups, the positive expression ratio of collagen type I, collagen type III, TGF-β1, and Smad7 proteins in the alveolar septum were significantly increased in the intraperitoneal SM group. iii. In the intraperitoneal and tracheal SM groups, there is statistical significance in the changed trend of the positive expression ratio of collagen type I, collagen type III, TGF-β1, and Smad7 proteins in
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Collagen type I, collagen type III, TGF-β1, and Smad7 staining had an increasing trend (Figure 19).

**Discussion**

In our study, the number of apoptotic cells, the positive expression ratio of Bax apoptotic protein, caspase-3 and caspase-9 apoptotic proteases in the alveolar septum had an increasing trend with time in the intraperitoneal and tracheal SM groups. Electron microscopy showed the mitochondrial cristae were fuzzy, and the ribosomes were detached from the surface of the rough endoplasmic reticulum and dissociated in the cytoplasm. The results of our study suggest that apoptosis is one of the mechanisms of SM-induced acute pulmonary injury via two routes and it is positively correlated with the exposure dose and time of SM, such as the inflammatory response [20]. At the same time, it was confirmed that the molecular mechanism underlying apoptosis is via the mitochondrial pathway. In vitro studies showed that SM induced apoptosis through the mitochondrial and death receptor pathways, and the correlation existed in the two pathways [21, 22]. The determination of the apoptotic protein indices revealed that the expression of the pro-apoptotic protein Bax was increased, and the expression of the anti-apoptotic protein Bcl-2 was decreased. Bax/Bcl-2 was increased and the change of in the ratio was consistent with the trend that TUNEL-staining apoptotic cells were gradually increased. Detection of the apoptotic protease indices showed that the promoter factor, caspase-9 and the effect factor, caspase-3 were positively expressed, suggesting that caspase cleavage and trigger action in the mitochondrial pathway were activated [23, 24]. The changes in apoptotic protease were consistent with the reports by Ray et al. [25] and Sourdeval et al. [26]. Electron microscopy showed that SM induced morphologic changes of lung epithelial cells mainly in the cell membrane, mitochondria, and rough endoplasmic reticulum. It is speculated that this kind of damage is related to the change in mitochondrial membrane permeability and the decrease in mitochondrial membrane potential and an imbalance of calcium ions [27, 28]. The results show that the mitochondria are the tiny organ which is easy to damage in cells and the main control point of the biological program [29]. Following mitochondrial damage, a variety of pro-apoptotic signaling pathways will be activated, which helps to accelerate self-destruction of the cells [30, 31]. The author believes that SM-induced
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Apoptosis is the result of many factors, the molecular and cellular mechanisms are very complex, and the network regulatory pathway has not been fully clarified. Our results showed that SM-induced acute lung injury via intraperitoneal injection and intratracheal instillation in rats caused abnormal regulation of apoptosis by an endogenous channel, and significantly increased apoptotic indices were observed following intraperitoneal injection compared with intratracheal instillation. The results suggest that the effects may be related to rapid absorption of the SM from the peritoneal cavity.

Our study also showed that green-staining collagen fibers in the alveolar septum were significantly increased 72 h after SM exposure via intraperitoneal injection or intratracheal instillation. In the intraperitoneal and tracheal SM groups, MMP-2, MMP-9, TIMP-1, TIMP-2, colla-
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Figure 17. The expression of TGF-β1 proteins in the alveolar septum in rats. A-D: Show the positive expression (arrow) at 6, 24, 48, 72 h in intraperitoneal SM group; F-I: Show the positive expression (arrow) at 6, 24, 48, 72 h in tracheal SM group; E, J: Show the negative expression in the intraperitoneal and tracheal control group (TGF-β1 staining, × 400, magnification, Bars, 20 μm).

Figure 18. The expression of Smad7 proteins in the alveolar septum in rats. A-D: Show the positive expression (arrow) at 6, 24, 48, 72 h in intraperitoneal SM group; F-I: Show the positive expression (arrow) at 6, 24, 48, 72 h in tracheal SM group; E, J: Show the negative expression in the intraperitoneal and tracheal control group (Smad7 staining, × 400, magnification, Bars, 20 μm).

gen type I, collagen type III, TGF-β1, and Smad7 proteins positive expression ratio had an increasing trend. The results suggested that in the early stage of acute pulmonary injury induced by high-dose SM, the pulmonary fibrosis indices could be changed, and increased with time of injury; the intraperitoneal SM group was more apparent. There is a limited amount of MMP-2 and MMP-9 expression in normal lung tissue, but in the process of the SM-induced pulmonary inflammatory response, fibroblasts, bronchial epithelial cells, type II alveolar epithelial cells, and some inflammatory cells (such as macrophages and neutrophils) can produce MMP-2 and MMP-9, injure the alveolar basement membrane, and initiate pulmonary fibrosis. As physiologic inhibitors of MMP-2 and MMP-9, TIMP-1 and TIMP-2 form a covalent bond with MMP-2 and MMP-9 to form a proenzyme complex, then specifically inhibit the
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Activity of MMP-2 and MMP-9 [32]. Our study suggests that the expression of MMP-9 and MMP-2 proteins in the alveolar septum are increased, which indicates that the lung tissue injury was mainly based on an inflammatory response. The expression of TIMP-2 and TIMP-1 proteins was increased, which could be interpreted as a kind of transient physiologic compensation. Our results also revealed a possible mechanism of “migration and transmigration,” thus MMP-9 can promote the transformation of monocytes into myofibroblasts, which is beneficial to the migration of myofibroblasts from vessels to tissues in the location of an injury. At the same time, MMP-9 is also involved in the transmigration of neutrophils across the basement membrane [33, 34]. Pulmonary vascular endothelial cells and alveolar type II epithelial cells are the major target cells in pulmonary fibrosis; stromal cells, such as macrophages and mast cells are involved. Fibroblasts and myofibroblasts cells are the main effector cells, and TGF-β1 functions as a molecular “switch” [35]. Our study showed that the expression of TGF-β1, collagen type I, and collagen type III in alveolar septum are increased, and suggested that the pulmonary fibrosis indices on a molecular level may be changed in the early stage of SM pulmonary injury. Increased expression of Smad7 protein can trigger a negative feedback mechanism of TGF-β1 [36, 37]. Excessive expression of TGF-β1 is a kind of pathological reaction (damage or inflammation). Smad7 can inhibit the signal transmission of TGF-β1 and the activity of Smad2 and Smad3 through competitive binding of type I receptor [38, 39]. During the stage of the inflammatory response in SM pulmonary injury, the injury and inflammation can trigger higher expression of MMP-2 and MMP-9 secondary to the compensatory higher expression of TIMP-1 and TIMP-2. The higher expression of TGF-β1, collagen type I, and collagen type III secondary to the compensatory higher expression of Smad7. The existence of these pathologic and physiologic reactions can promote the rebalancing of the disorder in the molecular signaling pathway. Using a equal toxicity dose of SM (1LD50) in rats, increasing pulmonary fibrosis indices were observed following intraperitoneal injection compared with tracheal injection. The results suggest that SM via intraperitoneal injection more easily induces pulmonary fibrosis. Regarding the choice of the dose via the intraperitoneal injection and intratrachea instillation, studies have shown that the pulmonary

Figure 19. The positive expression ratio of collagen type I (A), collagen type III (B), TGF-β1 (C) and Smad7 (D) proteins in the alveolar septum in rats. Compared with the tracheal SM group, intraperitoneal propylene glycol control group, tracheal propylene glycol control group, and normal control group, **P < 0.05; Compared with intraperitoneal propylene glycol control group, tracheal propylene glycol control group, and normal control group, *P < 0.05.
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injury induced by SM via intraperitoneal injection was more serious than by subcutaneous injection or the oral route [40]. When rats were injected intraperitoneally with a dose of SM > 10 mg/kg, the rats died [41].

Conclusions

We chose the equal toxicity dose of SM (1LD_{50}) to establish an acute pulmonary injury model via intraperitoneal injection or intratracheal instillation. The apoptosis and pulmonary fibrosis indices in SM pulmonary injury had differences, indicating that the absorption of SM in the blood may play a dominant role. Our study suggested that pulmonary injury induced apoptosis and pulmonary fibrosis via intratracheal instillation under the equal toxicity dose of SM was less compared with intraperitoneal injection. The results of this study provide valuable parameters for the targeted intervention of SM-induced pulmonary injury.

The preliminary experiment of SM exposure in rats via intraperitoneal or tracheal injection - the results of calculation in LD_{50} (Horn’s method)

1. Via intraperitoneal injection: SM (0.96 LD_{50} = 8 mg/kg)

Based on body weight SD rats (male) 50 were randomly divided into 5 groups, each group of ten rats, fasting 12 h, but cannot need water deprivation. A series of concentrations in SM solution were formulated, according to 2.0, 5.0, 8.0, 9.0, 11.0 mg/kg dose, each rat was received via single intraperitoneal injection, and normal saline was given in the control group. After administration, the toxic symptoms and death number of rats in different concentration gradient were observed in detail, and then LD_{50} of SM and confidence limit were calculated via intraperitoneal injection.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Logarithmic dose X</th>
<th>Number of death</th>
<th>Mortality rate (p)</th>
<th>p^2</th>
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<td>0</td>
<td>0</td>
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The number of death were recorded in each dose group in rats, and mortality rate was calculated (p), according to Horn’s method, the value of LD_{50} were calculated.

LD_{50} = log^{-1}[X_m - i (\Sigma p - 0.5)] = 8.31 mg/kg

LD_{50} 95% confidence interval is [7.76-8.71] mg/kg.

0.96 LD_{50} = 8 mg/kg

2. Via tracheal injection: SM (0.98 LD_{50} = 2 mg/kg)

Based on body weight SD rats (male) 50 were randomly divided into 5 groups, each group of ten rats, fasting 12 h, but cannot need water deprivation. A series of concentrations in SM solution were formulated, according to 1.0, 1.5, 2.0, 2.5, 3.0 mg/kg dose, each rat was received via single tracheal injection, and normal saline was given in the control group. After administration, the toxic symptoms and death number of rats in different concentration gradient were observed in detail, and then LD_{50} of SM and confidence limit were calculated via tracheal injection.

<table>
<thead>
<tr>
<th>Group</th>
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<th>Logarithmic dose X</th>
<th>Number of death</th>
<th>Mortality rate (p)</th>
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<td>5</td>
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</tr>
<tr>
<td>4</td>
<td>2.5</td>
<td>0.39794</td>
<td>6</td>
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</tr>
<tr>
<td>5</td>
<td>3</td>
<td>0.477121</td>
<td>10</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Control group</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The number of death were recorded in each dose group in rats, and mortality rate was calculated (p), according to Horn’s method, the value of LD_{50} were calculated.

LD_{50} = log^{-1}[X_m - i (\Sigma p - 0.5)] = 2.04 mg/kg

LD_{50} 95% confidence interval is [1.862-2.29] mg/kg.

0.98 LD_{50} = 2 mg/kg

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Disclosure of conflict of interest

None.

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References


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