

Comparative Pulmonary Protective Efficacy of Amifostine and its Analogue S-2(2-aminoethylamino)ethyl Phenyl Sulfide (DRDE-07) against Sulphur Mustard Induced Oxidative Stress and Inflammation in Female Mice

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ABSTRACT

Aim: The present study was undertaken to investigate the comparative pulmonary protective efficacy of Amifostine (S-2[3-aminoprophylamino] ethyl phosphorothioate) and its analogues DRDE-07 (S-2(2-aminoethylamino) ethyl phenyl sulfide) against sulfur mustard toxicity in mice. **Materials and Methods:** Twenty female mice were divided into four groups: Control, SM group animals were percutaneously exposed to 16.2 mg/kg. The third and fourth group of animals received amifostine and DRDE-07 (210 and 250 mg/kg respectively) through the oral route, 30 min before SM exposure. The clinical symptoms and body weight changes were observed daily and sacrificed on 7th day. Bronchoalveolar lavage fluid (BALF) and lung tissue were collected for biochemical and histopathological studies. The following biochemical endpoints were studied in BALF (total cell count, lactate dehydrogenase, protein content, β -glucuronidase activity, MMP-2, 9 activity and FSH) whereas reactive oxygen species (ROS), reduced glutathione (GSH), lipid peroxidation, superoxide dismutase, catalase and myeloperoxidase activity was measured in lung tissue. The above biochemical observations are also supported by histopathology studies. **Results:** Dermal exposure to SM significantly reduced body weight. The significant increase in BALF LDH leakage, protein content, cell number and MMPs activity in the SM exposed animals suggest disruption of endothelial barrier in the

lung ($p < 0.05$). Significant ROS generation ($p < 0.05$) was observed in lung tissue of SM group which results in a significant decrease in SOD GSH and CAT and an increase in MDA ($p < 0.05$). These alterations in BALF as well in lung tissue due to SM exposure was significantly prevented by the pretreatment of amifostine and DRDE-07 ($p < 0.05$). The histopathological observations also support the above results. The above results indicate that the preventive efficacy of DRDE-07 is better than amifostine. **Conclusion:** The percutaneous SM exposure-induced pulmonary damages were significantly protected by DRDE-07 than amifostine in mice.

Keywords: Amifostine, BALF, Chemical Warfare Agents, DRDE-07, Pulmonary injury, Oxidative stress, Sulfur mustard.

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INTRODUCTION

Sulfur mustard (SM, 2, 2'-dichlorodiethyl sulfide) is a vesicant chemical warfare agent which was widely used during World War-I, Iran-Iraq conflicts and recently by the Syrian terrorist groups.¹ Skin, eye and respiratory system are the major target organs to SM exposure and induce a small blister to severe pathological alteration and even death.² Even single exposure to mustard gas with respiratory injury was associated with increased risk of lung cancer in later life.³ Because of the lipophilicity nature, SM penetrates the human skin and distributed to the whole body within a short time, where they induced internal organs injury. Vijayaraghavan *et al.* (2001) demonstrated that SM is highly toxic through the dermal route than subcutaneous and oral route.⁴ Recently, Mohamed Batal *et al* also reported that percutaneous SM exposure induces time and dose-dependent DNA damage in internal organs and lung was the most affected organ.⁵ Oxidative stress and inflammations are attributed to the SM induced pulmonary toxicity in various experimental animals.⁶ The subcutaneous injection of half mustard induced oxidative stress through free radical generation and activation of the immune system through the infiltration of T cells and generation of inflammatory cytokines which are responsible for toxic effects in the lungs.^{7,8} Various strategies have

been adopted to counteract the SM toxicities which include administration of antioxidant, anti-inflammatory agents, protease inhibitors and radio protector.⁹ Treatment by antioxidant and anti-inflammatory agents have been reported for SM induced systemic injury include such as melatonin, pentoxifylline, N-acetyl cysteine, sodium thiosulphate.^{10,11} But none of them found to be effective in preventing the SM induced lethal toxicity.

Amifostine (WR-2721) is an analogue of cysteamine and selectively protect the normal tissues from the toxicities associated with chemotherapy and irradiation.¹² The cytoprotective properties of amifostine are attributed to the potent scavenging of drug or radiation-induced oxygen free radicals.¹¹ Since the SM toxicity mimics the radiation toxicity the prophylactic efficacy of amifostine was tested against SM both *in vitro*¹³ and *in vivo*.¹⁴ Few studies were also compared to the protective efficacy of amifostine and DRDE-07 on different animal models against SM and nitrogen mustard.^{15,16} Though the above studies suggested the better prophylactic efficacy of DRDE-07 than amifostine, on haematological, hepatic and renal biochemical parameters, there is no report on the pulmonary protection. The present study was aimed to investigate

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the comparative pulmonary protective efficacy of amifostine and DRDE-07 against SM induced toxicity in mice. In the present study, the focus was mainly on the SM induced biochemical changes in BALF and lung tissue and its modulation by pretreatment of amifostine and DRDE-07. The BALF and lung tissue oxidative stress and inflammatory biochemical endpoints were studied along with histopathological analysis.

MATERIALS AND METHODS

Chemicals

Amifostine and DRDE-07 S-2(2-aminoethylamino) ethyl Phenyl Sulfide were synthesized in Synthetic Chemistry division of DRDE and characterized by elemental analysis, IR, ¹HNMR and mass spectral analysis. SM was also synthesized in the declared facility of DRDE and was found to be 99 % pure by TLC. LDH kit was purchased from Tulip Coral Clinical System, India. Phosphate buffer saline, Reduced glutathione, Dithiobis 2-nitrobenzoic acid (DTNB), Ethylenediaminetetraacetic acid (EDTA), Trichloroacetic acid (TCA), PEG-300, Trizma, Sodium dodecyl sulfate (SDS), 1,1,3,3-Tetra ethoxy propane (TEP), Pthalaldehyde were purchased from Sigma-Aldrich Co. (St. Louis, MO). All other chemicals of extra pure grade were purchased from Merck (Mumbai, India).

Animals and treatment

Randomly bred female Swiss mice (23±2 g) were obtained from Establishment animal facilities. All animals were housed in polypropylene cages with free access to feed (Ashirwad laboratory, Chandigarh, India.) and tap water. Twenty four hours before SM topical exposure, hairs on the dorsal side of the animals were closely clipped using a pair of scissors. Twenty hair clipped animals were randomly distributed into following four groups containing 5 animals in each. LD₅₀ of SM was determined in a previous study by the moving average method¹⁷ using three to four groups, each group consisting of four animals.¹⁴

Group I: Control, only PEG-300 was applied on hair clipped back of animals.

Group II: SM was dissolved in the PEG-300 (final dose 16.2 mg/kg, which is equivalent to 2LD₅₀ through percutaneous route) and then uniformly applied on hair clipped back of animals.

Group III: Amifostine (210 mg/kg) was given orally, 30 min before SM application

Group IV: DRDE-07 (250 mg/kg) was given orally, 30 min before SM application

The above experiments were conducted in a fume cupboard with high-speed exhaust facilities. The clinical symptoms and body weight changes were observed for seven days. On day 7 the animals were sacrificed, BALF and lung tissue were collected for biochemical and histopathological studies. The experimental details are depicted as Figure 1. The above experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) and Control and Supervision of Experiments on Animals (CPCSEA) Ministry of Fisheries, Animal Husbandry and Dairying, Department of Animal Husbandry and Dairying, Government of India.

Collection of bronchoalveolar lavage fluid

At day 7, animals were sacrificed and BALF was collected as per the procedure described previously.¹⁸ Briefly, lungs were lavaged with ice-cold, sterile PBS (1.5ml) via a tracheal cannula secured with thread. This procedure was repeated 5 times using the same fluid and collected in 1.5 ml tube. There was no statistically significant difference in the recovery of bronchoalveolar lavage fluid (BALF) within groups. BALF was immediately centrifuged at 800 g for 15 min (4°C). Supernatants of BALF were frozen at -80°C until analysis. A portion of lung tissue was fixed in 10%

formalin buffer for histopathological studies and remaining was used for biochemical parameters indicative of oxidative stress and inflammation was analyzed by the following methods.

Biochemical assays

Determination of vascular endothelial damage in lung

The total protein concentration in BALF was measured using the Pierce BCA protein assay kit (Thermo scientific Rockford, IL). The absorbance was measured at 562 nm, and Proteins were expressed in milligrams protein per millilitre. Bovine serum albumin was used as the standard. Lung epithelium damage was monitored by lactate dehydrogenase (LDH) released in the BALF according to manufacturer's instructions (Ecoline LDH kit from Merk specialities Pvt Ltd, Mumbai) and expressed in U/L. Non-protein sulphydryl (FSH), in BALF supernatant, was assayed using a slight modification of literature method.¹⁹ Briefly, 0.1 ml supernatant was mixed in 0.1ml of 10% (w/v) Trichloroacetic acid and centrifuged at 3000 g for 15 mins at 4°C condition. 0.1ml of the supernatant fraction was diluted with 0.9ml of phosphate buffer (0.1M pH 8.2) and further treated with 20µl of DTNB (10mM in methanol) then the concentration of FSH was calculated using absorbance at 412 nm.

Measurement of oxidative stress biomarkers in lung tissue

Oxidative stress biomarkers were assayed in lung tissue homogenate (10%, w/v) which was prepared in ice-cold 1.15% KCl, using a glass homogenizer. Subsequently, the homogenate was centrifuged at 10000 ×g for 30 min at 4°C and the supernatant was collected. Level of reactive oxygen species (ROS) was measured using the method of Gupta *et al.*²⁰ Lipid peroxidation was measured as thiobarbituric acid reactive substances by modified Okhawa method as described earlier.²¹ Briefly, 400 µl of tissue homogenate sample was mixed with 400 µl TCA (15%) for protein precipitation and then centrifuged at 2000g for 15 min at the 4°C. The supernatant was taken into a tube containing 40 µl of TBA (0.8%) and 160 µl of distilled water. Reaction mixture further heated for 1 hr at 95°C and then cooled. Finally, absorbance was measured and expressed in nmol/g of tissues. Tissue reduced glutathione concentrations were assayed using the Ellman method.²² Briefly, 200 µl homogenate supernatant was mixed with an equal volume of 10% TCA to precipitate protein. After centrifugation for 10 min, 0.8ml phosphate buffer solution (0.1M) was added to 200 µl of supernatant and finally, 50 µl of DTNB (10mM) added to the reaction mixture and absorbance was recorded at 412 nm. Superoxide dismutase (SOD) activity was determined by the method Marklund and Marklund.²³ Briefly, 20 µl of homogenate was mixed with 200 µl Tris-EDTA buffer (pH 8.2) then 50 µl of pyrogallol (0.2 Mm pH 6.5) was added. Immediately, the kinetics of this mixture was read at 420 nm for 1 min. SOD was expressed in unit/ml where one unit of SOD activity defined as the amount of enzyme required for 50 % inhibition of pyrogallol autooxidation. Catalase activity was determined using method of Beer and Seizer (1952).²⁴ Briefly, 20 µl of sample supernatant was mixed with 200 µl of phosphate buffer at pH 7.4 and finally, 50 µl of hydrogen peroxide (0.11Mm) added and absorbance was taken at 240 nm.

Biochemical analysis of inflammatory parameters in BALF

β-Glucuronidase activity in BALF was determined according to the method of Fishman *et al.*²⁵ Approximately 0.1ml of sodium acetate buffer (0.2M pH 4.5) mixed with 0.1ml of 6mM phenolphthalein mono-b-D-glucosiduronic acid as substrate then 0.05 ml of BALF supernatant added and incubated for 4 hr at 37°C. Further 0.75 ml of distilled water

and 0.5 ml of glycine NaOH buffer (pH 11.7 with 0.2% SDS) was added and centrifuged at 1500 rpm for 15 min. The absorbance of the reaction mixture measured at 540 nm to calculate the β -Glucuronidase activity. To determine the index for neutrophils sequestration in lung tissue, the activity of myeloperoxidase (MPO) was measured by the method of Hirano.²⁶ Briefly, 10% tissue homogenate was prepared in phosphate buffer (pH 6) containing 0.5% Hexadecyltrimethylammonium bromide (HTAB). Homogenate was centrifuged at 14000g for 30 mins. 20 μ l of supernatant was added to the 200 μ l of reaction buffer (0.5 M O-dianisidine, 5 Mm phosphate buffer and 0.0005% Hydrogen peroxide). The kinetic of the reaction was measured at 450 nm for 15 min using spectrophotometer (Biotek, USA).

Zymography analysis of matrix metalloproteases

The MMPs are zinc-dependent endopeptidases and can cleave the extracellular matrix components which contribute to the inflammatory cell recruitment, tissue remodelling in the lung.²⁷ Expression of MMPs in BALF was detected by zymography using gelatin as a substrate. Briefly, 10 μ g of BAL proteins were mixed with non-reducing sample buffer and subjected to electrophoresis on 10% SDS PAGE containing 0.1% gelatin as substrate. The gels were then incubated with gentle shaking at room temperature in a renaturing buffer for 40 min followed by incubation in developing buffer at 37°C overnight. The gels were stained with simple coomassie brilliant blue staining solution subsequently destained. Finally, the gel was washed in purified water at RT. Gelatinase (92 kDa) activity appeared as clear band on the gel.

Histopathological analysis of lung tissue

The lung tissues were harvested and fixed in 10% formaldehyde. The lung tissues were dehydrated with graded alcohol and embedded in paraffin, cut into 5 μ m thick sections (semi-automated microtome, Leica, Germany). After deparaffinization, tissue sections were stained with hematoxylin and eosin (H&E) reagent in automated stainer (Leica, Germany). Analysis of stained tissue sections was carried out by light microscopy.

Statistical analysis

All values are expressed as mean (\pm SEM). Difference between the mean values of normally distributed data was analyzed by using one-way ANOVA followed by Tukey multiple comparisons. The GraphPad Prism 5 software was used (GraphPad Software, CA) for performing analysis and Statistical significance was considered at values of $p < 0.05$.

RESULTS

Preventive effect of amifostine and DRDE-07 on BALF biochemical variables indicative of endothelial damage

It is now well established that SM exposure leads to alkylation of DNA, proteins, and other membrane components result in impairment of cellular functioning and cell death. Figure 2 represents a significant increase in BALF protein content (76.9 %), LDH activity (43.8%) while glutathione level decreased (37%) significantly ($p < 0.05$) on day 7 after percutaneous SM exposure as compared to control ($p < 0.05$). However, these alterations were significantly reversed by the pretreatment of amifostine and DRDE-07 ($p < 0.05$). Furthermore, DRDE-07 showed remarkable efficacy over the amifostine.

Preventive effect of amifostine and DRDE-07 on lung inflammation

Figure 3 shows the activity of myeloperoxidase, β -glucuronidase, matrix metalloproteases (MMP) and total cell numbers in the BALF. As shown in Figure 3A, the total cell number in BALF was significantly increased

in the SM group compared to control ($p < 0.05$). Pretreatment amifostine and DRDE-07 both significantly decreased the BALF cells counts in mice lung 7 days after SM exposure ($p < 0.05$). Figure 3B indicates dermal exposure to SM causes significant increases in β -Glucuronidase activity as compared to control ($p < 0.05$). On the other hand, pretreatment with amifostine reduced the activity and DRDE-07 significantly decreased ($p < 0.05$) β -Glucuronidase activity over the amifostine. Similarly, Figure 3C indicates a significantly higher level of MPO activity in the lung tissue of mice after topical SM exposure ($p < 0.05$). These increases were markedly reduced by amifostine treatment. However, MPO activities were significantly lower in DRDE-07 treated animals than those in the SM group at 7 days post-exposure ($p < 0.05$). Sulphur mustard induced both MMP-2 and MMP-9 and this may persist up to 7 days. Results showed that increased activity of MMP9 was seen in SM exposed group. MMPs activities were evidently reduced by DRDE-07 and amifostine treatment (Figure 3D).

Preventive effect of amifostine and DRDE-07 on lung oxidative stress

Figure 4 Indicates the increasing of oxidative markers in lung tissue of mice. Dermal exposure to SM significantly induced the increases in ROS (A) level followed by depleted GSH (C) level resulting in significantly increasing lipid peroxidation (B, $p < 0.05$). Concomitantly to this, decreased SOD and catalase activity also found to be inhibited in the groups exposed with SM (D and E respectively). On the other hand, the amifos-



Figure 1: Experimental protocol.

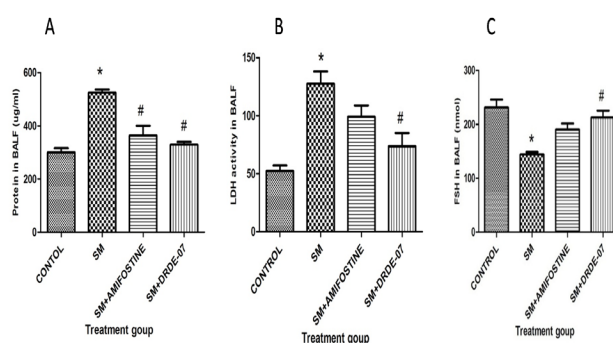


Figure 2: Comparative prophylactic efficacy of Amifostine and DRDE-07 against SM induced toxicity in BALF parameters. (A) Protein concentration. (B) LDH activity and (D) FSH level in BALF. Results are mean \pm SEM of 5 animals per group. * $p < 0.05$ compared with control group; # $p < 0.05$ compared with SM exposed group.

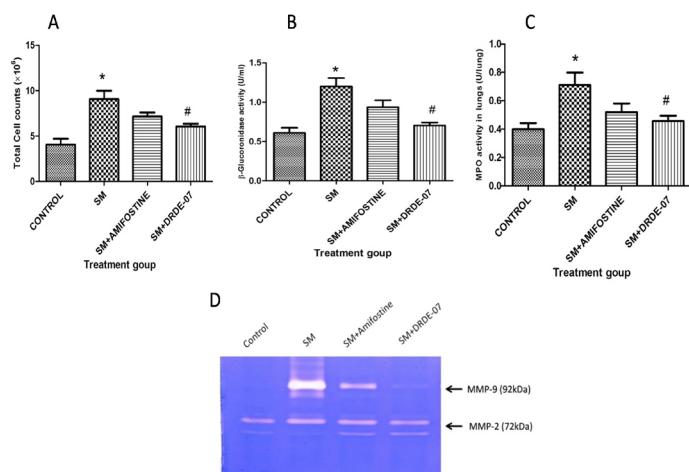


Figure 3: Comparative prophylactic efficacy of pretreatment with amifostine and DRDE-07 on SM induced change in inflammatory biomarkers (A) Total cell counts in BALF MPO (B) β -glucuronidase activity in BALF (C) activity in lung tissue and (D) Representative image of MMPs (Gelatinase) activity in BALF on Zymogram from 2-3 mice per group. Results are mean \pm SEM of 5 animals per group. * $p < 0.05$ compared with control group; # $p < 0.05$ compared with SM exposed group.

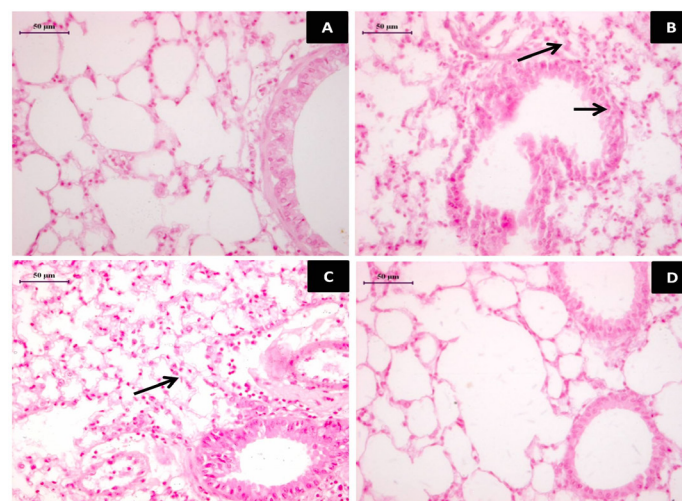


Figure 5: Histological changes after SM in lung tissue and the effect of amifostine and DRDE-07. One representative lung section from 2-3 mice/treatment groups is shown. A: Control group with normal alveoli without any inflammatory cells; B: SM group show bronchial epithelial cell damage and inflammatory cell recruitment (arrow) and thickening of alveolar septa, alveolar damage; C: SM+Amifostine on day 7; D: SM+DRDE-07 on day 7.

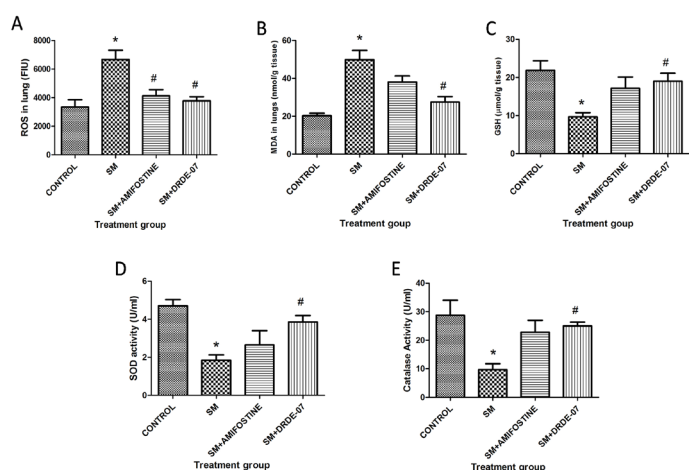


Figure 4: Effect of pretreatment with amifostine and DRDE-07 on oxidative stress markers in lung tissue (A) ROS level in lung tissue, (B) MDA level in lung tissue, (C) level of glutathione content, (D) SOD activity, (E) Catalase activity. Results are mean \pm SEM of 5 animals per group. * $p < 0.05$ compared with control group; # $p < 0.05$ compared with SM exposed group.

tine pretreatment decreases the above alteration while the DRDE-07 pretreatment significantly showed protection in all the altered oxidative biochemical variables compared to the SM exposed animals.

Histopathology of lungs

As shown in Figure 5, the control (A) group shows normal lung architecture without evidence of inflammation or alveolar disruption. While the accumulation of the inflammatory cells into the alveolar space and disruptions of alveolar walls were seen in SM exposed group. However, these pathological alterations were markedly reduced in DRDE-07 treated group. Results suggest that DRDE-07 may inhibit inflammation-mediated acute lung toxicity.

DISCUSSION

Despite a long history of military use, SM remains a threat for both militaries and civilians. Several pathways involved in SM toxicity but the

widely accepted mechanism is related to its potential alkylation ability to cellular components include proteins, RNA and DNA.²⁸ The primary target organs of SM are the eyes, respiratory tract, and skin. However, at high concentrations, SM is also capable of exerting systemic toxicity, leading to death.^{28,29} The previously published report has shown that cutaneous exposure of SM and its analogue not only limited to local injury but also caused a systemic effect.^{30,31} The systemic effect occurs mainly due to the overexpression of inflammatory cytokines, which leads to organ dysfunction resulting in severe body weight loss.^{31,32} In the present study also, a severe decrease in body weight and morphological change were observed which suggest this could be results of intestinal epithelial damage which leads to insufficiency of dietary nutrients. Previously, Batal *et al.* reported comparative internal organs toxicity after cutaneous exposure of SM and found that lung was the second most affected organ following cutaneous exposure of SM.⁵ Moreover, the SM DNA adducts were more persist in the lungs than the brain and other organs. Yun *et al.* also reported that the lung of the rat was the most affected tissue after cutaneous exposure of SM.³³ These all experimental studies support our investigation that demonstrated percutaneously exposure of SM causes significant lung injury, which was supported by biochemical and histopathological changes.

Increase in LDH activity and total protein the BALF has been used as an indicator of cytotoxicity by the variety of compounds including SM.^{34,35} Also in the present study, we have observed an increase in protein content and LDH activity in BALF reflected the lung endothelial damage caused by SM. In addition to that, Myeloperoxidase and β -glucuronidase are the major constituent of neutrophil cytoplasmic granules and its activity is a direct measure of the neutrophil presence and an indirect indicator of lung injury.³⁶ During the neutrophil respiratory burst, MPO produces hypochlorous acid from H_2O_2 and chloride ions. In our study prophylactic treatment with amifostine and DRDE-07 significantly reduced the MPO and β -glucuronidase activity. Besides, our result also supported by an earlier study of Fu *et al.* (2009), in which amifostine treatment reduced the lipopolysaccharide-induced MPO activity and neutrophil accumulation in the lung parenchyma cells.³⁷ Chakrabarti and Patel³⁸ demonstrated that the MMP-9 synthesis increased under a variety of physiological and pathological conditions which is responsible for epithelial cell detachment from the basement membrane. In

addition to that, Calvet *et al.* (1999) also reported that SM caused increased expression of proteolytic MMPs in lung tissue.³⁹ Our results also exhibited a significant increase in gelatinolytic activities particularly MMP-9 in BALF, compared to control animals suggest that these gelatinases are secreted by the inflammatory cells such as macrophages and PMNs. This was further supported by the increasing number of cells in BALF (Figure 3). In the present study, the significant inhibition of MMP-9 by DRDE-07 than amifostine suggest that these compounds may be acting as a protease inhibitor against SM. Previous studies reported that histological changes as well as pathogenic response to SM exposure mainly attributed to the inflammatory cell infiltration and increased number of activated macrophages in BALF.^{40,41} Furthermore, by the use of electron paramagnetic resonance (EPR) and spin trapping techniques, Anderson *et al.* demonstrate that SM induces ascorbyl radicals in rat lung and this free radical formation was associated with an increase in lipid peroxidation.⁴² Reduced glutathione plays a pivotal role in protecting the lung surface from oxidative attack caused by various factor.⁴³ In our study also depletion of GSH was observed after SM exposure. However, pretreatment with amifostine and DRDE-07 demonstrated a significant restoration of GSH and reduction in lipid peroxidation resulting in pulmonary protection against SM toxicity. Various studies on protection mechanism of amifostine have been suggested such as amifostine has superoxide anions and peroxide radical scavenging properties and also likely augmentation of GSH level by providing thiol (SH) pool.⁴⁴ While the protection offered by the DRDE-07 may likely be due to the presence of functional amino group and sulfide group might have help in scavenging SM or its metabolites, thereby reducing SM toxicity in tissues. The better protective efficacy of DRDE-07 over the amifostine may also be attributed due to its analgesic and anti-inflammatory properties.⁴⁵ Oxidative stress induced by reactive oxygen species (ROS) and as the result antioxidants depletion is now considered as the major mechanism of SM toxicity on lung injuries.⁴⁶ In our study also we have observed an increase in lung oxidative stress after dermal exposure of SM. The pretreatment of animals with amifostine and DRDE-07 decreased the above altered oxidative biomarkers. SM is a strong mutagenic agent which binds to DNA and leads to cascades of pathological events.²⁸ As reported earlier, the protection offered by the amifostine may also be expected due to its binding and detoxify capacity of alkylating agents.⁴⁷ Generation of ROS resulting in the reduction of antioxidant enzyme SOD suggests that a decrease in selective elimination of superoxide radical in dismutation reaction. The reduction of SOD activity is also caused by the high level of cellular peroxides which directly alter its activity.⁴⁸ Results from the present investigation show that DRDE-07 not only improves the glutathione reserve but also resolves the inflammatory changes in animals exposed to SM. Mechanism of protection by DRDE-07 could be due to elevation of GSH and detoxification of SM by up-regulation of cyto-P450 and suppression of inflammatory cytokines. A report also suggested that DRDE-07 has promising protective efficacy against SM-induced mutagenicity.⁴⁹ Hence, in this way, it may protect DNA alkylation and NAD depletion and further overcome the toxicity of SM. Although exactly mechanism is not known, further molecular studies are needed to clarify this lacuna.

CONCLUSION

The percutaneous SM exposure induces oxidative stress and inflammation by depleting the GSH level, ROS generation by the inflammatory cells and secretion of proteases which leads to the structural and functional changes in the lung tissue architecture. In the present study, we have shown that pretreatment of animals with amifostine and its analogues (DRDE-07) significantly reduced the above biochemical alterations. Furthermore, DRDE-07 exhibited more pronounced protection efficacy than amifostine.

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CONFLICT OF INTEREST

The authors declare no competing interest.

ABBREVIATIONS

SM: Sulfur mustard; **BALF:** Broncho Alveolar lavage fluid; **DRDE-07:** S-2(2-aminoethylamino) ethyl phenyl sulfide); **MMPs:** Matrix Metalloproteases; **MPO:** myeloperoxidase; **LDH:** Lactate Dehydrogenase; **ROS:** Reactive Oxygen Species; **TBA:** Thiobarbituric Acid; **SOD:** Superoxide Dismutase; **CAT:** Catalase; **SDS-PAGE:** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; **PMNs:** Polymorphonuclear; **GSH:** Reduced glutathione; **Min:** Minutes.

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