

Downregulation of Super Oxide Dismutase Level in Protein Might Be Due to Sulfur Mustard Induced Toxicity in Lung

Leila Mirbagheri^{1,2}, Mehryar Habibi Roudkenar³, Abbas Ali Imani Fooladi⁴, Mostafa Ghanei¹, and Mohammad Reza Nourani¹

¹ Genomics Division, Systems Biology Institute. Chemical Injuries Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

² Biochemistry Department, Sciences and Technology Branch, Azad University, Tehran, Iran

³ Research Center, Iranian Blood Transfusion Organization, Tehran, Iran

⁴ Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

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ABSTRACT

Sulfur mustard (SM) has been identified as an important chemical weapon. During the Iran-Iraq war of 1980-88, the extensive usage of SM against Iranian civilians and military forces was proven. This agent has been shown to cause severe damage mainly in the skin, eyes, lungs, and respiratory tract in Iranian veterans. The most common disease is bronchiolitis obliterans (BO). SM increases the endogenous production of reactive oxygen species (ROS). Superoxide dismutases (SODs) are known as protective antioxidants against the harmful effects of ROS.

Twenty exposed SM individuals (43.2±6.4 years), and 10 normal controls (41.3±2.5 years) were enrolled in this study. Evaluation of SODs was performed by semiquantitative RT-PCR and immunohistochemistry.

Our results demonstrated that CuZnSOD and MnSOD mRNA were up-regulated 2.79±1.09 and 2.49±1.11 folds, respectively in SM-injured patients in comparison with control levels. In contrast, Immunohistochemistry results showed downregulation of CuZnSOD protein expression in SM injured patients.

Our results revealed that SODs may play an important role in cellular protection against oxidative stress due to mustard gas toxicity in airway wall of SM exposed patients.

Keywords: Gene expression; Lung; Sulfur Mustard; Super Oxide Dismutase

INTRODUCTION

Sulfur mustard (SM) has been identified as an

important chemical weapon and is classified as a harmful warfare agent. During the Iran-Iraq war of 1980-88, the extensive usage of SM by the Iraqi ex-regime against Iranian civilians and military forces was proven.¹

This agent has been shown to cause severe damage to exposed individuals, mainly in the skin, eyes, lungs,

Corresponding Author: Mohammad Reza Nourani, PhD;
Chemical Injuries Research Center, Baqiyatallah University of
Medical Sciences, Tehran, Iran. Tel: (+9821) 8821 1524, Fax:
(+9821) 8821 1523, E-mail: r.nourani@yahoo.com

and respiratory tract.²⁻⁵

To date, there are over 40,000 people in Iran diagnosed with different degrees of respiratory complications due to mustard gas exposure. There is no general consensus regarding the pulmonary pathophysiology of the patient population exposed to SM, but SM has been shown to induce respiratory diseases over long periods of time. A main late pulmonary complication of SM is bronchiolitis obliterans (BOs).^{3,6} Despite extensive research, the mechanisms by which SM causes injury are still incompletely understood. SM is an alkylating agent that interacts with nucleophilic functional groups such as the amino, carboxylic, and hydroxyl groups in DNA and proteins.^{7,8} SM is an activator of proteases, resulting in proteolysis of several vital intracellular enzymes and structural proteins.⁹ In addition to the alkylation of DNA and protease activation, SM causes the depletion of intracellular glutathione (GSH), which has been shown to be an important antioxidant in the lung.¹⁰ As another insult, disordered neutrophils and lymphocytes produce proteases, which in turn manufacture reactive oxygen species (ROS). Thus, free radicals recognized as inducers of oxidative injury in a number of molecules in the cell play a central role in the pathogenesis of lung diseases like bronchiolitis obliterans with oxidative stress.^{11,12}

Superoxide dismutases (SODs) are known as important protective antioxidants against the harmful effects of ROS.⁷ They serve as the primary defense of the human lung against free radicals (oxidative stress) produced as part of normal metabolism, and are also critical in protecting against the progression of oxidant-related lung damage.¹³ Exposure to mustard gas significantly inhibits the activity of SOD, glutathione peroxidase, and catalase.¹⁴ SODs are coded by different genes and their genetic variation plays a role in the pathogenesis of several free radical-associated disorders.¹⁵

At present, 3 distinct isoforms of SOD have been identified in mammals, and their genetic structure, cDNA, and proteins have been described. Two isoforms of SOD have Cu⁺⁺ and Zn⁺⁺ in their catalytic center and are localized to either intracellular cytoplasmic compartments CuZnSOD (SOD-1) or to extracellular elements ECSOD (SOD-3).^{16,17} MnSOD (SOD-2), the third isoform, has Mn as a cofactor and has been shown to localize to the mitochondria of aerobic cells.¹⁸

MnSOD, CuZnSOD, and ECSOD are adequately present in healthy human lung.¹⁹ MnSOD is moderately expressed in respiratory epithelium, alveolar type II epithelial cells, alveolar macrophages, and interstitial fibroblasts in hypoxia-exposed rats.^{20,21} In the airway epithelium, CuZnSOD is highly expressed in ciliated epithelial cells.^{22,23} The most important superoxide scavengers in the cell cytosol and in mitochondria are MnSOD and CuZnSOD. ECSOD is not present in a sufficient concentration to act as a bulk scavenger of superoxide throughout the entire extracellular space²⁴. In any case, CuZnSODs are the dominant lung SODs.^{13,25}

Considering the production of ROS in lung injury induced by SM and the reduction of SOD in bronchial epithelium of respiratory lung diseases such as asthma, we investigated the effects of mustard gas at the mRNA and protein levels on CuZnSOD and MnSOD in the pathophysiological cytoprotection of airway walls in bronchial biopsies of patients who were exposed to SM in comparison to unexposed patients. These findings should be useful in developing a new treatment protocol with antioxidant medicine for chemical exposed patients.

MATERIALS AND METHODS

Study Design

In this study, 20 patients affected by SM and suffering from long term pulmonary complications were randomly enrolled, and 10 non-chemical participants were included as a control group. The chemical injured patient population comprised of individuals who had documented encounters to SM during 1980-1988 Iran-Iraq war. The exposure to SM in the SM-affected group was confirmed by documents in the Iranian military health services at the time of exposure and the beginning of pulmonary symptoms immediately after the contact without any symptom-free period. This study was approved by the Baqiyatallah University of Medical Sciences Ethics Committee. After the protocol of the experiment had been entirely explained to them, all the participants signed a written informed consent. The demographic data are shown in Table 1. Cases with positive histories of the other chronic pulmonary diseases, such as asthma, autoimmune diseases, lung cancer, diabetes mellitus, acute infective bronchitis, and pneumonia

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Table 1. Subjects' demographic characteristics

Subjects	n	Sex (M/F)	Age Range	Age (mean±SD)	p
SM-exposed	20	20/0	36-58	43.2±6.4	0.6
Control	10	10/0	39-44	41.3±2.5	

Table 2. Sequences and characteristics of PCR primers

Gene (Accession ID)		Primer sequence (5' to 3')	Annealing Tm	Product size
CuZnSOD (NM_000454)	Forward	AGGGCATCATCAATTTTCGAGC	57 °C	217 bp
	Reverse	ACATTGCCCAAGTCTCCAAC		
MnSOD (NM_000636)	Forward	GGAAGCCATCAAACGTGACT	57 °C	162 bp
	Reverse	CCTTGCAGTGGATCCTGATT		
β-actin (NM_001101)	Forward	TTCTACAATGAGCTGCGTGTGG	57 °C	119 bp

were excluded. Likewise, addicts, elders, smokers, organ transplant recipients, and patients with histories of occupational pulmonary contact with toxicants were also excluded from our study.

After inhalation of 2% aerosolized lidocaine and intravascular midazolam, bronchoscopy was performed with a flexible fiberoptic bronchoscope BFIT (Olympus, Japan) that passed through the airway to reach the segmental and sub-segmental carinae. Endobronchial biopsy samples were taken with a bronchoscopic forceps (Olympus, Japan). Throughout the bronchoscopy, supplementary oxygen was provided, and the oxygen saturation was checked constantly by the pulse oxymeter until the end of the procedure. Two biopsies were taken from each patient and separately placed in Tripure Isolation Reagent (Roche, Germany) and 4% formalin (Merck, Germany) instantaneously, followed by transfer to 30% sucrose. Specimens in Tripure were kept in -80°C until RNA extraction. Formalin samples were stored at 4°C for immunohistochemistry study.

RT-PCR of CuZnSOD & MnSOD Genes

RT-PCR was conducted as previously described in detail ⁶. In brief, total RNA was extracted with using Tripure Isolation Reagent (Roche, Germany) in accordance with the manufacturer's recommendations and stored in -80°C for the following procedures. The isolated RNAs were eluted in RNase-free water, and their quantity and quality were evaluated by Nano Drop electrophoresis (ND-1000, DE) and in 1% agarose gel (Cinnagen, Iran), respectively. A 500 ng aliquot of total RNA was reverse transcribed to create first-strand

complementary DNA by SuperScript III reverse transcriptase (Invitrogen, CA) according to the manufacturer's protocol and was treated by DNaseI (Invitrogen, Carlsbad, CA) and heat inactivation to remove any contamination with chromosomal DNA.

Semiquantitative RT-PCRs of the CuZnSOD and MnSOD genes were accomplished by using equivalent amounts of synthesized cDNAs in a final reaction volume of 25 µl. Recombinant Taq DNA polymerase and all other PCR reagents were purchased from Cinnagen (Tehran-Iran), and the reactions were run in a master cycler thermal cycler (Eppendorf, Germany). Specific primer sets for the CuZnSOD and MnSOD genes and β-actin, as a housekeeping gene, were designed with the aid of primer3 software (<http://frodo.wi.mit.edu/>) and obtained from Bioneer Company (South Korea). Characteristics of all primer sets were shown in Table 2.

The RT-PCR was carried out under the following conditions for all genes: primary denaturation at 94°C for 5 min followed by 30 PCR cycles consisting of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, extension at 72°C for 60 sec, and 5 min terminal extension at 72°C. PCR products were electrophoretically separated in a 2% agarose gel (Cinnagen, Iran) and stained with ethidium bromide (Cinnagen, Iran). Lastly, bands were visualized under UV light in a gel documentation device (Bio-Rad Laboratories, CA).

Following semiquantitative RT-PCR, quantitative real-time RT-PCR was performed in a Rotor-Gene RG 3000 (Corbett Research, Australia). Amplification was done in triplicate for each sample using SYBR Green

Premix (Takara, Japan) according to the manufacturer's protocol. The PCR conditions were as follows: initial heat hold at 94°C for 1 min followed by 40 amplification cycles including denaturation at 94°C for 20 sec, annealing at 57°C for 30 sec, and extension at 72°C for 30 sec. β -actin expression was used to normalize cycle threshold (Ct) values and gave a control for relative quantitative evaluation of the transcripts abundance.

Immunohistochemistry

We have already described the immunohistochemistry procedure in detail elsewhere.²⁶ Briefly, 8 airway biopsy samples from SM-exposed patients and 8 specimens from unexposed control subjects were checked. All samples were fixed in 4% formalin (Merck, Germany) and then placed in phosphate buffered saline (PBS) (Takara, Japan) containing 30% sucrose (Wako, Japan). After 15 μ m thick, water-embedded sections were prepared with a cryostat (Histo-line, Italy), they were incubated for 12 hours at 4°C with primary antibody at a dilution of 1:200 in PBS. This primary antibody was a rabbit monoclonal antibody raised against human CuZnSOD (Abcam, UK). For immunostaining after the incubation, sections were incubated with 1:200 diluted biotinylated anti-rabbit secondary antibody (Santa Cruz Biotechnology, CA). The prepared sections were finally visualized with the aid of rabbit ABC Staining System (Santa Cruz Biotechnology, CA) using 3, 3'-Diaminobenzidine (DAB) as the color substrate.

Statistics

Data are shown as mean \pm SD of fold-changes of CuZnSOD and MnSOD gene expressions. SPSS

software version 15.0 (SPSS, IL) was utilized for statistical analyses. For assessment of differences in the expression of the mentioned genes between the SM-injured group and unexposed controls, student's t-test was used, and $P < 0.05$ was considered as statistically significant.

RESULTS

Thirty subjects participated in the current study. They included 20 SM-exposed patients and 10 unexposed persons as a control group. The average ages of SM-injured patients and unexposed controls were 43.2 ± 6.4 and 41.3 ± 2.5 , respectively, which was not significantly different (0.6) (Table 1).

First, semiquantitative RT-PCR was performed to clarify whether there were any differences in CuZnSOD and MnSOD gene expressions within the control group subjects. Our results showed that no significant differences existed among the control cases in terms of SOD gene expression (Data not shown). The next step was to study the CuZnSOD and MnSOD gene expressions in the SM-exposed patients. Our results demonstrated that CuZnSOD and MnSOD mRNA were up-regulated in SM-injured patients (Figure 1).

The expression of SOD genes were also quantitatively assessed by real-time RT-PCR. In harmony with the semiquantitative RT-PCR results, the expression of CuZnSOD at the mRNA level increased 2.79 ± 1.09 fold in SM-exposed patients in comparison with control cases; moreover, the mRNA level of MnSOD in SM-exposed patients also increased 2.49 ± 1.11 fold in comparison with control levels (Table 3).

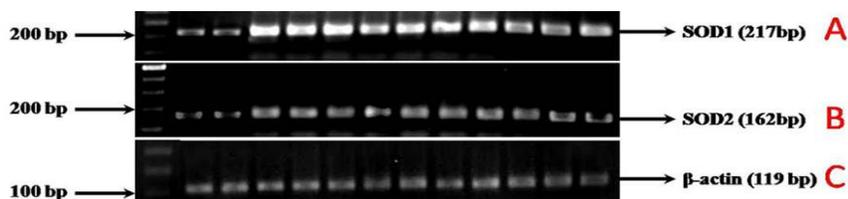


Figure 1. Up-regulation of SOD1 (CuZnSOD) and SOD2 (MnSOD) gene expression in airways of SM-injured patients. CuZnSOD & MnSOD gene expressions were measured by semiquantitative RT-PCR. This panel shows gel bands in order as PCR amplification products of CuZnSOD (217bp), MnSOD (162bp) and β -actin (119 bp) transcripts. (A) A marked increase in the CuZnSOD gene expression levels of SM-exposed patients (lanes, 4-13) was recognized in comparison with the expression levels of unexposed ones (lanes, 2 and 3). (B) A noticeable increase in the MnSOD gene expression levels of SM-exposed patients (lanes, 4-13) was recognized in comparison with the expression levels of unexposed ones (lanes, 2 and 3). (C) B-actin was used as internal control. 1st line is as , 100 bp DNA ladder.

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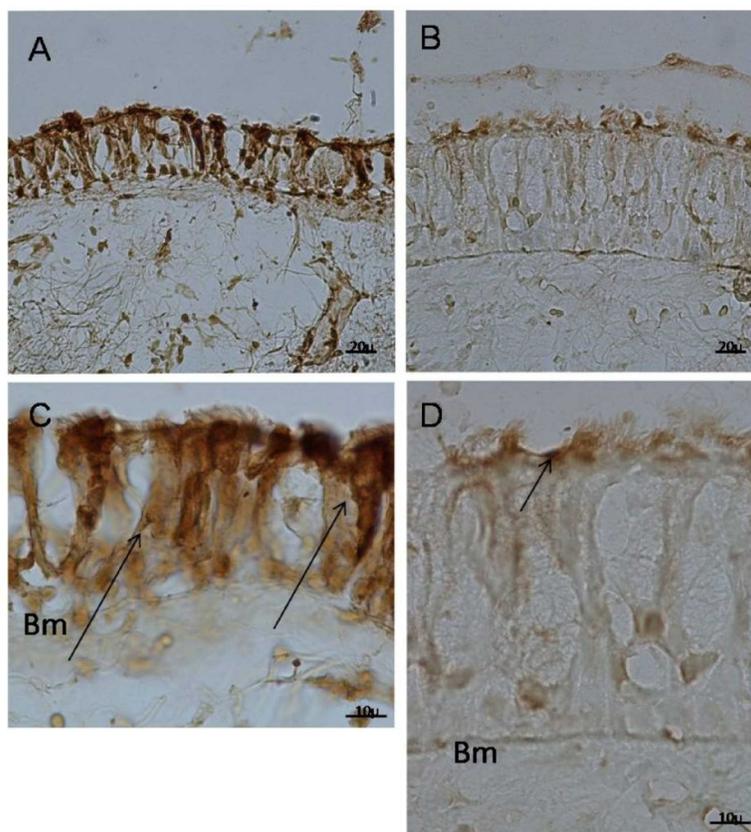


Figure 2. Immunohistochemical localization of CuZnSOD in the bronchial epithelium of human airway. (A) A section from nonexposed human airway wall immunostained for CuZnSOD. CuZnSOD protein is strongly expressed in the luminal border and basal epithelial cells. (C) Higher magnification corresponding to (A) showed strong immunoreaction for CuZnSOD in the airway epithelial cells (Arrows). (B and D) Very weak immunoreactivity is seen in the human airway epithelium exposed to SM. (D) Very weak expression was seen in luminal border (arrow). Note the thickness of SM exposed bronchial epithelium, which is significantly increased in comparison to unexposed tissue. (Bm=Basement membrane). A, B = X400 , C, D =X1000

For localization and evaluation of CuZnSOD protein expression in bronchial biopsy samples of the two groups, an immunohistochemistry study was carried out. It was immediately observed that the thicknesses of the bronchial epithelium layer in SM-injured patients was approximately two times that of control samples (Figure 2: C, D). A strong CuZnSOD immunoreactivity was seen in normal control airway

epithelium cells, especially in the basal layer and brush border cells of the epithelium (Figure 3 A, C). In contrast, in SM-injured ones, a weak expression of CuZnSOD protein was seen in the bronchial epithelial cells of luminal side of brush border cells, indicating a very weak CuZnSOD protein expression in these samples (Figure-2 B, D).

Table 3. CuZnSOD & MnSOD expression Fold-changes in SM-exposed patients in comparison with controls (* Statistical significance: $p < 0.05$)

Gene	Range (SM-exposed/ Control)	Fold-change of gene expression (Mean \pm SD)	P
CuZnSOD	1.5-5.5	2.79 \pm 1.09	0.006*
MnSOD	1.5-6.0	2.49 \pm 1.11	0.018*

DISCUSSION

Increased oxidative stress is a significant part of the pathogenesis of obstructive lung diseases such as asthma, chronic obstructive pulmonary disease and lung diseases induced by SM. Normal lung tissue is protected against oxidant challenges by a variety of antioxidant mechanisms.²⁷ Superoxide dismutases (SODs) are antioxidant enzymes that convert superoxide radicals to hydrogen peroxide and provide a defense mechanism against oxidative stress. Iran has been the victim of chemical warfare use against its population and thousands of Iranians are still suffering from long-term complications of SM gas exposure.²⁸

There have been no reports of the expression changes in SOD and their interaction with SM-induced pulmonary complications of the delayed form. Therefore, the goal of our study was to examine the expression pattern of SODs in SM-induced lung biopsies of the exposed patients. Expressions of SODs in the endobronchial biopsies of patients and controls were analyzed by semiquantitative RT-PCR and immunohistochemistry. Our results demonstrated the mRNA expressions of MnSOD and CuZnSOD were significantly upregulated. 2.49 ± 1.11 and 2.79 ± 1.09 fold, respectively, in the lungs of SM-exposed patients compared to the levels in the unexposed control group.

In a recent study, 2-chloroethyl ethyl sulfide (CEES, Cl-CH₂CH₂-SCH₂CH₃) was administered intratracheally to guinea pigs and it was reported that an upregulation (3.5-fold) in CuZnSOD gene expression was evident from the northern blot analysis.²⁹ This is in line with another observation that exposure of rats to hyperoxia leads to a transient increase in the mRNA of MnSOD in lung homogenates.^{30,31}

Our immunohistochemistry results revealed that, in contrast to the up-regulation of CuZnSOD mRNA expression in airway biopsy samples of SM-injured patients, the protein level was lower than that in the control subjects. Several immunoreactive cells were found in the bronchial epithelium of our control cases, and immunoreactivity was more present in luminal border (LB) and basal cells (BC). Another recent study has demonstrated that mustard gas exposure does not result in any significant differences in the level of SOD-1 and SOD-2 proteins, but decreases the overall activity of SOD.²⁹ This finding is in agreement with our data showing lower expression of CuZnSOD in SM-

injured patients in comparison to a control group.

In most human studies, SOD activity decreases in the bronchial epithelium (50%), in the cells of bronchoalveolar lavage (25%), and in bronchial brushings (nearly 50%) in patients with asthma compared with control subjects.²⁵

Airway wall immunohistochemical staining of CuZnSOD in the bronchial epithelium pulmonary vasculature of ozone-exposed rats has also shown lower reactivity than alveolar macrophages from control lungs.³²

In contrast to our study, Kinnula et al evaluated the expression of CuZnSOD in human normal lung and observed positively stained pleural endothelial cells and basal cells from the bronchial epithelium. We found only very weak expression of CuZnSOD in the luminal side of the bronchial epithelium.¹⁹

We have also already shown an inconsistency between the mRNA and protein expressions of NGAL, heme oxygenases and methalothionin in the bronchial epithelium of SM-exposed patients in comparison with unexposed cases.^{3,6,36} Taken together, these reports lead us to hypothesize that this discrepancy between the mRNA and protein expressions of CuZnSOD may be caused by translational efficiency and/or post-translational regulation.³³ Therefore, the lack of CuZnSOD protein is one important reason for the increase in oxidative stress and progression of disease in SM-exposed patients.

We suggest a new hypothesis that therapeutic antioxidants such as SOD mimetics might provide new approaches to attenuating oxidant-associated lung injury in patients.

In support of this notion, we have previously observed that a 4-month administration of N-acetylcysteine in patients exposed to SM could significantly improve FEV1/FVC over placebo.³⁴ Additionally, it was shown that treatment with scavengers of hydroxyl radicals such as DMSO could prevent acute lung injury in an experimental animal model of thermal injury.³⁵ Based on the studies presented here, we suggest that administration of SODs may ameliorate the injuries induced by SM and the oxidative stresses in these patients. However, further and complementary studies are required to clarify the reasons for the discrepancy between mRNA and protein level of CuZnSOD in SM-exposed patients. Our results highlight the importance of SOD action for relief of stress challenges in this diseases.

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