

**Original Article****The Role of Oxidative Stress in Severity of Obstructive Pulmonary Complications in Sputum of Sulfur Mustard-Injured Patients***Javad Heydari<sup>1</sup>, Mahvash Jafari<sup>\*1</sup>, Saeed Khazaie<sup>1</sup>, Hassan Goosheh<sup>2</sup>, Mostafa Ghanei<sup>2</sup>, Ashraf Karbasi<sup>2</sup>**Received: 28.02.2017**Accepted: 05.04.2017***ABSTRACT**

**Background:** Sulfur mustard (SM) is a strong bifunctional alkylating agent that causes delayed complications in organs such as lung. Oxidative stress plays a pivotal role in the pathogenesis and progression of many pulmonary diseases. The aim of this study was to investigate the oxidative stress in sputum of SM exposed patients with mild, moderate and severe pulmonary dysfunction and assessing their relationship with pulmonary function.

**Methods:** In this cross-sectional study, oxidative stress biomarkers in sputum were examined on 26 patients with SM-induced bronchiolitis obliterans (9 mild, 14 moderate and 3 severe) and 12 matched healthy controls referred to Baqiyatallah Hospital, Tehran between October 2015 and April 2016.

**Results:** Sputum superoxide dismutase, catalase and glutathione S-transferase activities and malondialdehyde level in moderate and severe groups were significantly higher than in the control group ( $P=0.002$ ,  $P=0.004$ ,  $P=0.014$  and  $P=0.009$ , respectively). Glutathione (GSH) level in moderate (22.29%,  $P=0.025$ ) and severe (45.07%,  $P=0.004$ ) groups were significantly lower than the control. A decreased in GSH level in severe (41.7%) groups was observed as compared with the mild group. Pearson analysis revealed strong correlations between disease severity and oxidative stress biomarkers in sputum of patients with moderate and severe injuries.

**Conclusions:** Oxidative stress is involved in the pathogenesis of patients with moderate and severe pulmonary dysfunction following SM exposure. The presence of enhanced oxidative stress relates to the decline lung function and the progression of the disease. Sputum induction in SM-injured patients can be used to the assessment of the antioxidant status of bronchial secretions.

**Keywords:** Oxidative Stress, Severity of Disease, Sputum, Sulfur Mustard.

**IJT 2017 (5): 5-11****INTRODUCTION**

Sulfur mustard (SM; mustard gas) is a strong bifunctional alkylating agent that binds to a variety of cellular macromolecules of the living tissues. It has been used as a warfare agent against both military and civilian population during the Iran-Iraq War. Acute and late complications may occur following the SM exposure that the severity of damage largely depends on the dose and duration of exposure. The long-term complications of SM exposure are much serious than acute effects [1-3]. About 100,000 Iranians were exposed to chemical warfare agents, and 40,000 people suffer from pulmonary diseases such as asthma, chronic bronchiectasis and pulmonary fibrosis [3, 4]. Bronchiolitis obliterans (BO) is the main long-term respiratory pathology in chemical victims of SM, which is a complex

inflammatory disease with both airway and parenchymal lung injury [1, 5].

Reactive oxygen species (ROS) are involved in the pathogenesis and progression of many lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) [6, 7]. SM-induced pulmonary disease is associated with inflammation and neutrophil excess [8]. Neutrophils can accelerate the overproduction of ROS by NADPH oxidase, myeloperoxidase and xanthine oxidase activation [7, 9]. In addition, they secrete proteases, which in turn manufacture ROS [4]. Formation of ROS leads to oxidative stress and macromolecular damage, which triggers intricate signaling pathways and modulate gene expression, causing a series of SM-associated toxic responses [10]. Lung tissue is protected against oxidant challenges by a variety of antioxidant mechanisms including superoxide

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dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and large amounts of glutathione (GSH) [11]. SM-induced oxidative stress is associated with alteration of antioxidant enzyme activities, depletion of GSH and an increase of membrane lipid peroxidation in tissues, all of which can lead to inflammation and ultimately induction of cell death [9, 12-14].

Study of the molecular and cellular pathways activated in response to SM exposure is very important to a better understanding the pathophysiological basis of disease that leads to find new biomarkers and novel treatments [14]. The ability to neutralize oxidant species differs during the development of pulmonary diseases [13]. Very little is known about specific changes in the major antioxidant defense mechanisms in mild-to-severe BO-induced SM [15, 16].

Sputum induction is a non-invasive, easy, safe and reliable method and can be repeated within a relatively short period. This method is frequently used to show oxidative stress in many studies concerning pulmonary diseases [6, 17].

The aim of the present study was to investigate these effects on important biomarkers of oxidative stress including malondialdehyde (MDA) content as an important index of lipid peroxidation and antioxidant defense parameters such as GSH level, activities of SOD, CAT and GST in sputum of SM exposed patients and assessing their relationship with severity of disease and pulmonary function. Based on our knowledge, this is the first study on these effects in sputum.

## MATERIALS AND METHODS

### *Groups under Study*

The current study was a cross sectional among subjects referred to the Emergency Department of Baqiyatallah Hospital (the referral center for chemically injured patients), Tehran, Iran between October 2015 and April 2016.

### *Control Group*

The control group consisted from 12 healthy non-smokers with no history of respiratory or allergic disease and normal baseline spirometric parameters.

### *Case Group*

This study was performed on 26 patients with a history of SM exposure during the Iran-Iraq War who suffered from long term pulmonary complications. The diagnosis of bronchiolitis

obliterans was previously established with physical examination, pulmonary function test and high resolute computed tomography (HRCT).

Informed consent was obtained from all patients. The Ethics Committee of the Chemical Injuries Research Center of Baqiyatallah University of Medical Sciences approved the study.

A detailed questionnaire was completed by an investigator for each patient in order to obtain symptom information for typical and atypical symptoms. Patients with a history of smoking and occupational exposure to toxic agents, having dusty jobs and antioxidant therapy were excluded from the study.

### *Pulmonary Function Test*

Severities of obstructive lung disease using a standard spirometer (Hi801 Chest M.I. Spirometer) were classified based upon forced expiratory volume in 1 second (FEV1) values as mild (FEV1 60-80% predicted), moderate (40-59% predicted) and severe (less than 40% predicted) disease [6].

### *Sputum Induction and Processing*

Sputum induction was performed according to a method previously described [18]. The saliva contamination of sputum was removed by sampler, and the volume of the samples was measured by graduated cup. In the previous study [19], the authors demonstrated that dithiothreitol (DTT), the standard sputum homogenizing agent, significantly interferes with the enzymatic kinetic assay of biochemical parameters determination in this study. Therefore, DTT was skipped from the sputum homogenization procedure and sputum supernatant for these parameters analysis was obtained using phosphate buffer saline (PBS) as described below. Samples were homogenized with five volumes of chilled PBS and then centrifuged at 12000×g for 15 min at 4°C. The supernatants were separated and used for biochemical parameters assays.

### *Antioxidant Enzyme Activities*

**SOD activity:** The activity of SOD was determined [20], based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium by superoxide.

**CAT activity:** CAT activity in sputum supernatants was measured spectrophotometrically at 240 nm by calculating the rate of degradation of H<sub>2</sub>O<sub>2</sub> as the substrate of the enzyme [21].

**GST activity:** GST activity was assayed by monitoring the formation of the thioether product of the reaction between GSH and 1-chloro-2, 4-dinitrobenzene at 340 nm [22]. The enzyme activity was expressed as U/ml.

### Determination of GSH and MDA Levels

GSH level in was measured using the method of Tietz [23]. The end product of lipid peroxidation was estimated by measuring the level of MDA [24].

### Statistical Analysis

Statistical analysis of the data was conducted using SPSS statistical software version 22 (IBM Corporation, USA). Comparisons of means among groups were performed using analysis of variance (ANOVA) followed by post hoc analysis using Tukeytest. Analysis of the correlation between sputum oxidative stress biomarkers and FEV1 values was performed using Pearson correlation analysis. Results were expressed as Mean±SD and  $P<0.05$  was considered significant.

## RESULTS

Table 1 show that all participants were male and the mean age of the case and control groups was  $46.83 \pm 5.77$  and  $44.50 \pm 6.20$ yr, respectively. FEV1% in mild, moderate and severe groups was significantly lower than in the control ( $P=0.034$ ,  $P=0.000$  and  $P=0.000$ , respectively)

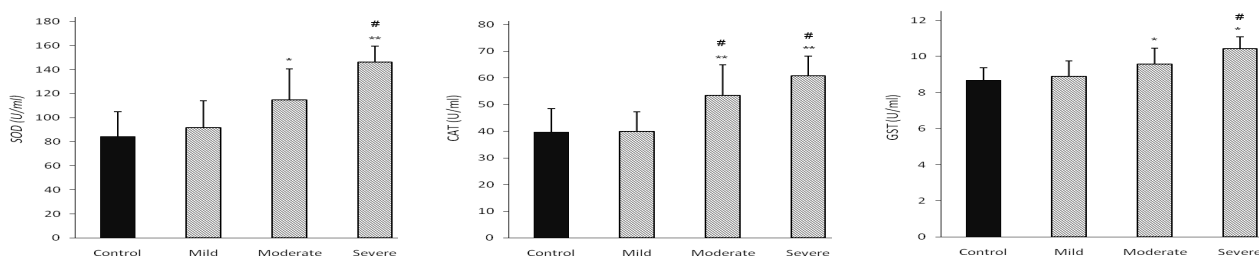
and mild ( $P<0.001$ ) groups. Spirometry data showed that 9 (36.6%) patients had mild, 14 (53.8%) moderate and 3 (11.5%) had severe respiratory impairment. HRCT findings revealed air-trapping in 1-25% in 18 (72%) cases and in 25-50% in 4 (16%) patients.

Sputum SOD, CAT and GST activities in moderate ( $P=0.012$ ,  $P=0.008$  and  $P=0.041$ , respectively) and severe ( $P=0.002$ ,  $P=0.004$  and  $P=0.014$ , respectively) groups were significantly higher than in the control. Significant increases in these enzyme activities in severe group were observed as compared with the mild values (59.39,  $P=0.006$ ); 52.4,  $P=0.012$  and 17.2%,  $P=0.036$ , respectively) (Fig. 1). GSH content was significantly decreased in moderate ( $P=0.025$ ) and severe ( $P=0.004$ ) groups by 22.29 and 45.07% of control, respectively. MDA level was significantly higher in moderate ( $P=0.024$ ) and severe ( $P=0.009$ ) groups by 18.41 and 32.01% of control, respectively. The significant decreased in GSH level (41.7%,  $P=0.019$ ) in severe group was observed as compared with the mild group (Fig. 2). According to Pearson correlation analysis, the FEV1 was positively correlated with GSH level, and negatively correlated with SOD, CAT and GST activities and MDA level in sputum of moderate-to-severe groups. There was no correlation between the FEV1 and oxidative stress biomarkers in mild group (Table 2).

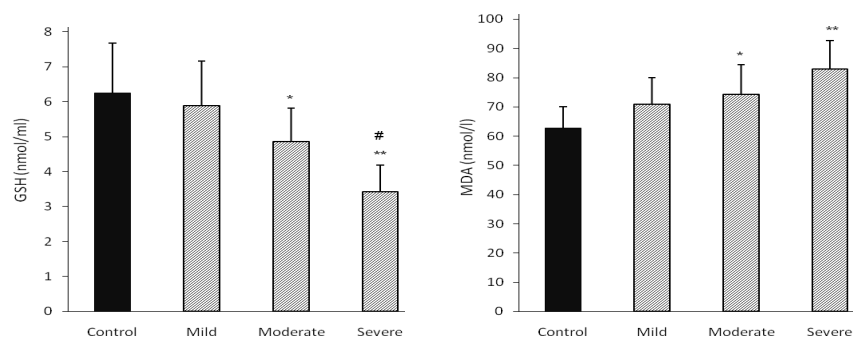
**Table 1.** Major demographic characteristics and respiratory disorders data in the control and patients with mild, moderate and severe pulmonary dysfunction following SM exposure.

Parameters	Control (n=12)	Mild (n=9)	Moderate (n=14)	Severe (n=3)
Age (yr)	44.50±6.20	48.50±5.60	43.00±6.80	49.00±4.90
Sex, M:F	1:0	1:0	1:0	1:0
FEV1 % predicted	85.50±3.99	78.00±7.00*	50.03±6.00***,#	29.00±9.00***,#
Dyspnea on ATS scale	0-I	I-II	II	III-IV
Air trapping intensity %	0	14±4	31±5	68±7

Values are expressed as mean±SD. \* $P<0.05$  and \*\*\* $P<0.001$  versus control, # $P<0.001$  versus mild group. FEV1: Forced expiratory volume in 1 second; ATS: American thoracic society.



**Figure 1.** Antioxidant enzyme activities in sputum of the control and patients with mild, moderate and severe pulmonary dysfunction following SM exposure. Values are expressed as mean±SD. \* $P<0.05$  and \*\* $P<0.01$  versus control, # $P<0.05$  versus mild group. SOD: Superoxide dismutase; CAT: Catalase; GST: glutathione S-transferase (GST).



**Figure 2.** Glutathione (GSH) and malondialdehyde (MDA) levels in sputum of the control and patients with mild, moderate and severe pulmonary dysfunction following SM exposure. Values are expressed as mean±SD. \* $P<0.05$  and \*\* $P<0.01$  versus control, # $P<0.05$  versus mild group.

**Table 2.** Pearson correlation analysis between lung function, measured as FEV1, and oxidative stress biomarkers in sputum of patients with mild, moderate and severe pulmonary dysfunction following SM exposure.

Parameters	FEV1-Mild		FEV1-Moderate to Severe		FEV1-Mild to Severe	
	FEV1-Mild	<i>P</i> -value	FEV1-Moderate to Severe	<i>P</i> -value	FEV1-Mild to Severe	<i>p</i> -value
SOD	-0.643	0.061	-0.745	0.001	-0.756	<0.001
CAT	-0.590	0.095	-0.535	0.027	-0.737	<0.001
GST	-0.634	0.066	-0.587	0.013	-0.645	<0.001
GSH	0.620	0.075	0.837	<0.001	0.777	<0.001
MDA	-0.588	0.096	-0.710	0.001	-0.586	0.002

FEV1: Forced expiratory volume in 1 second; SOD: Superoxide dismutase; CAT: Catalase; GST: glutathione S-transferase (GST), GSH: Glutathione; MDA: Malondialdehyde.

## DISCUSSION

Oxidative stress plays a pivotal role in the development of pulmonary diseases following SM exposure [8, 16]. The major enzymatic antioxidants of the lungs are SOD and CAT that serve as the primary defense of the human lung against oxidant stress. SOD catalyzes the conversion of superoxide radical to hydrogen peroxide ( $H_2O_2$ ) and CAT converts  $H_2O_2$  to oxygen and water. In addition, GST as a supporting antioxidant enzyme plays an important role in protecting tissue from lipid peroxidation and oxidative stress through inactivation of secondary metabolites such as hydroperoxides by conjugation with the GSH [8, 12, 25].

In this study, sputum SOD, CAT and GST activities in moderate and severe groups were significantly higher than in the control and mild groups. The increased these antioxidant enzymes activities are probably a response to neutralize the impact of increased ROS generation in lung, which provides mainly protection against SM-induced tissue oxidative injury. These results suggest the pivotal role of SOD, CAT and GST for cell adaptation to oxidative stress [12, 25]. The

mechanism of their increasing activities is not quite clear. The expression of the genes such as nuclear factor-kappa B (NF- $\kappa$ B) may stimulate by SM, which leads to increase the expression of these enzymes in lung tissue of patients with OB [26]. The mRNA expressions of Mn-SOD and Cu Zn-SOD were significantly increased in the lungs of SM-exposed patients [27]. SOD, CAT and GST activities may be increased or decreased in various pulmonary diseases including asthma and COPD [6, 11, 17, 25]. Jafari and Ghanei reported a significant increase in SOD and CAT activities in plasma, erythrocytes, and bronchoalveolar lavage fluid (BAL) and GST activity in BAL of patients with SM-induced lung injuries [8]. Plasma CAT activity in the moderate-to-severe group of SM-injured patients was higher than in the healthy control group, while SOD activity was remained unchanged [15].

GSH is an important antioxidant in the human airways with a protective effect against free radicals and peroxides. It serves as a substrate for several enzymes in different metabolic pathways including GST and in maintenance of other antioxidants, such as ascorbate and  $\alpha$ -

tocopherol [16, 8, 12]. The GSH concentration in the lung exceeds its plasmatic concentration by more than 100 fold illustrating high capacity of this system in the lung [7]. In the current study, sputum GSH level in moderate (22.29%) and severe (45.07%) groups was significantly lower than the control. In addition, patients with moderate and severe injuries showed tendencies for a decreased level of GSH than patients with mild injuries. The decline in GSH level could be due to increased utilization of GSH in protecting SH-containing proteins from ROS, which leads to oxidative stress in lung cells [8, 28]. These findings are in agreement with those reported SM-induced GSH depletion in animal and human models, which suggests that oxidant-antioxidant imbalance can be considered one of the main underlying abnormalities in SM-induced injuries, correction of which may have beneficial clinical impact for these patients [12, 8, 16]. However, studies in the sputum of patients with pulmonary diseases provided conflicting results. In patients with asthma, studies reported normal [29] or elevated [30] GSH levels. Both total and oxidized GSH are increased in the sputum of patients with moderate to-severe COPD [19]. By contrast, SOD, GSH, and glutathione peroxidase (GPx) levels in the sputum were lower as compared with the healthy smoker and nonsmoker subjects [6].

MDA as a marker of lipid peroxidation in laboratory diagnostics may be seen as the most important decomposition product of clinically relevant polyunsaturated fatty acids [16, 17]. Our results demonstrate that sputum MDA level was significantly higher in moderate and severe groups by 18.41 and 32.01% of control, respectively. An increase in MDA levels could be a direct reflection of an oxidative injury of the lung of these patients. "In fact, measurement of MDA in sputum discriminated between patients and controls with greater accuracy than in plasma, where MDA level was no marked change" [8, 16]. "The detection of imbalance between oxidant and antioxidant statuses might be more difficult due to the number of confounding factors (comorbidities, nutrition, etc.)" [17]. MDA levels in the sputum and exhaled breath condensate and plasma of patients with different pulmonary diseases have been reported to be in the range of controls [31] or elevated compared to healthy controls [6]. Similarly, the MDA level was increased in BAL

of patients with SM-induced pulmonary injury [8, 32].

Disease severity as measured by FEV1 was positively correlated with GSH level, and negatively correlated with antioxidant enzyme activities and lipid peroxidation products in sputum of patients with moderate and severe injuries, but not in patients with mild injuries, suggesting that the presence of enhanced oxidative stress relates to the progression of the disease [33]. Similarly, numerous studies have reported a relationship between oxidative stress biomarkers and the FEV1 in erythrocytes and lung and sputum in smokers and COPD patients [34, 35]. However, there was no significant correlation between plasma antioxidant system status and spirometric data in subjects with COPD [11]. The discrepancies between the studies may be due to the differences in study populations [17]. There was only minimal association between pulmonary function parameters and the serum levels of MDA and GSH in patients intoxicated with SM [16]. "Therefore, these authors suggested that antioxidant capacity in plasma would be unlikely related to measurements of airway obstruction, due to variability in the consumption of dietary antioxidants, which may result in different oxidative stresses, depending on conditions" [16, 11].

SM-induced oxidative stress is thought to be a primary event triggering inflammation [16, 32]. In our study, oxidant-antioxidant imbalance due to changes in GSH and MDA levels in moderate and severe groups may lead to progressive inflammation. The increased proinflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and transforming growth factor- $\beta$  (TGF- $\beta$ ) are rapidly generated in lung in early response to oxidative stress. TNF- $\alpha$  activates NF- $\kappa$ B and activator protein-1 (AP-1), transcription factors regulating expression of inflammatory genes including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), and this may be important in its pathogenic actions. It is also known to upregulate expression of matrix metalloproteinases (MMPs), which degrade extracellular matrix and have been implicated in lung toxicity [32]. In addition, TGF- $\beta$  may increase oxidative stress via the downregulation of  $\gamma$ -glutamylcysteine synthetase RNA and the decreased GSH synthesis in lung cells [33]. Many

of these inflammatory pathways can be blocked with the use of antioxidants [14].

“SM injury to the respiratory system has been related to apoptotic cell death that SM induces apoptosis in lung-derived cells and that the effector caspase-3 is activated in a dose- and time-dependent manner after SM injury” [12,15]. In the present study, patients with moderate and severe injuries induce oxidative stress through the generation of ROS, depletion of GSH and increased lipid peroxidation, which may lead to increase of intracellular  $Ca^{2+}$  level, DNA damage, the release of cytochrome c from mitochondria to cytosol and ultimately cell death [12, 8]. The depletion of GSH induces apoptosis in cells by interacting with proapoptotic and antiapoptotic signaling pathways and activates several transcription factors, such as AP-1 and NF- $\kappa$ B [36].

## CONCLUSION

Our observations suggest the presence of oxidative stress due to the depleted GSH content and increased MDA level in sputum of SM exposed patients with moderate and severe pulmonary dysfunction, which may lead to progressive inflammation and apoptosis or/and necrosis. There was a correlation between enhanced oxidative stress levels and the progression of the disease. Sputum induction in SM-injured patients can be used to the assessment of the antioxidant status of bronchial secretions. However, the clinical utility of oxidative stress biomarkers measurements in the sputum should be investigated further to clarify its role in monitoring and to predict disease outcomes.

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