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Glutathione and Malondialdehyde Levels in Late Pulmonary Complications of Sulfur Mustard Intoxication

Majid Shohrati · Mostafa Ghanei · Navvab Shamspour · Fatemeh Babaei · Majid Norozi Abadi · Mahvash Jafari · Amini Harandi Ali

Abstract It has been hypothesized that antioxidant and oxidant capacities may be related to the severity of obstructive lung impairment in patients with sulfur mustard (SM)-induced lung injuries. Our study was designed to measure the level of glutathione (GSH) and malondialdehyde (MDA) activities in patients intoxicated with SM and to evaluate the relationship between their activity and the severity of pulmonary dysfunction. A total of 250 patients with a history of exposure to a single high dose of SM gas and also 60 healthy nonsmoking individuals with no history of exposure to SM were selected. All patients underwent spirometry; based on its indices they were divided into two groups: mild ($n = 140$) and moderate-to-severe ($n = 110$) pulmonary dysfunction. Also, serum GSH and MDA concentration measurements were performed for all patients and controls. The mean GSH level in controls was 29.85 ± 3.26 µmol/ml, which was significantly higher than in patients with mild and moderate-to-severe pulmonary dysfunction (19.02 ± 2.36 and 17.89 ± 2.16 µmol/ml, respectively). Also, the mean MDA level in controls was 0.69 ± 0.09 µmol/ml, which was significantly lower than in patients with mild and moderate-to-severe pulmonary

dysfunction (0.74 ± 0.05 and 0.75 ± 0.05 µmol/ml, respectively). There was a weak linear correlation between GSH level and some of the pulmonary function indices. On the other hand, there was no significant relationship between the MDA level and pulmonary indices. Our study confirmed important alterations in the oxidative-antioxidative system in patients suffering from SM-induced lung injuries, as shown by a decreased serum level of GSH and an increased level of MDA. Individuals with moderate-to-severe SM-induced lung injuries show a greater tendency for a decreased level of GSH and an increased level of MDA than those with mild injuries; however, there is only minimal association between pulmonary function parameters and the serum level of MDA and GSH. These findings encourage us to examine therapeutic measures to correct such imbalances in future studies.

Introduction

Sulfur mustard (SM) is a vesicant-type chemical warfare agent introduced in World War I, which continues to be produced, stockpiled, and occasionally deployed by some countries and could be used by terrorists [1]. SM was used extensively by Iraq against both military and civilian populations during the Iran–Iraq conflict. Previous studies have shown that SM exposure in its chronic phase can lead to the development of airway hyperreactivity, chronic bronchitis, bronchiectasis, and lung fibrosis [2]. Some studies have shown that SM-induced pulmonary disease is a neutrophil- and/or lymphocyte-dominant disorder (e.g., neutrophil-dominated inflammatory process), and neutrophils and lymphocytes secret proteases which themselves

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generate reactive oxygen species [3]. Despite this fact, unfortunately, the pathophysiologic basis for these SM-induced complications is yet unclear [4].

Alterations in glutathione (GSH) and malondialdehyde (MDA) levels have been shown in various inflammatory conditions, including idiopathic pulmonary fibrosis (IPF), acute respiratory distress syndrome, cystic fibrosis, and human immunodeficiency virus [5]. To date, no studies have measured GSH and MDA levels in lung injuries due to SM. In this study we evaluated whether depletion of antioxidant capacity and overpresentation of lipid peroxidation products are related to the severity of obstructive lung impairment in patients with SM-induced lung injuries.

Materials and Method

Patients

For this case control study 250 patients from Sardasht in northwestern Iran were recruited. Participants were patients suffering from pulmonary disorders due to previous exposure to a single high dose of sulfur mustard gas during Iran-Iraq conflict in 1988. Inclusion criteria were as follows: documented exposure to SM based on the official certification issued by the Iranian Veterans Foundation; documented diagnosis of chronic pulmonary disease due to SM (histological evidence from previous biopsies); and no history of tuberculosis or resection of one or more lobes of the lung. Exclusion criteria were pneumonia and/or acute bronchitis; cigarette use or substance abuse; any illnesses such as heart failure, lung cancer, family history or prior diagnosis of asthma, pneumonia, and/or acute bronchitis; and use of any kind of antioxidant drugs. In addition, 60 healthy nonsmoking subjects from a suburb of Sardasht with no history of SM exposure and no signs or symptoms of bronchitis were randomly included as the control group. Written informed contest was obtained from all patients and controls and the study was approved by the ethics committee of the Chemical Injuries Research Center, Baqiyatallah University of Medical Sciences.

Methods

Each patient was visited by a general practitioner to assess his/her clinical condition. Clinical history concerning all respiratory symptoms (cough, dyspnea, and hemoptysis) and sputum production was recorded using a pre-established questionnaire. These symptoms (cough and dyspnea) were quantified by a scale of 1-5 where 1 denoted "no problem" and 4 or 5 denoted "worst condition." The questionnaire is given in Table 1. Sputum production and hemoptysis was reported as 0 for absent and 1 for present.

Table 1 Definition of scales that was used to quantify cough and dyspnea

Scale	Cough
1	I do not have a cough
2	I have cough, but it is not a serious problem
3	I have a cough, sometimes it disturbs my work
4	I have a disturbing cough, usually disturbs my work
5	I have a disturbing cough, always disturbs my work

Scale	Dyspnea
1	There is no dyspnea
2	Dyspnea only with extraordinary exercise
3	Dyspnea with ordinary exercise
4	Dyspnea with mild exercise
5	Dyspnea at rest

Before collecting blood samples, all patients underwent spirometry (Hi801 Chest M.I. Spirometer) during a screening visit. The spirometer was calibrated using the device provided by the manufacturer. To assess pulmonary function, the parameters vital capacity (VC), forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1), peak expiratory flow rate (PEFR), and maximal middle expiratory flow (MMEF) were measured. Based on post-bronchodilator FEV_1, patients were divided into two groups: mild ($FEV_1 \geq$ 80% predicted) ($n = 140$) and moderate-to-severe ($30\% \leq FEV_1 < 80\%$ predicted) ($n = 110$) pulmonary dysfunction [6].

All blood samples were taken after 10-12 h of fasting at morning (between 08:30 and 10:30 a.m.) for GSH and MDA assays. Samples were stored at $-70°C$ until laboratory analysis. GSH levels were determined using the Tietz method [7]. Cellular protein was precipitated by addition of 5% sulfosalicylic acid and removed by centrifugation at $2000g$ for 10 min. GSH was assayed in the supernatant as follows: To 100 μl of the protein-free cell lysate supernatant were added 800 μl of 3 mM NaHPO and 100 μl of 0.04% 5,5-dithiobis 2-nitrobenzoic acid (DTNB) in 0.1% sodium citrate. The absorbance of the solution was monitored at 412 nm for 5 min using a Genesys10 UV spectrophotometer. A standard curve of GSH was plotted and sensitivity of measurement was determined to be 1-100 μM. The plasma MDA level was measured using thiobarbituric acid (TBA) according to the method described by Satoh [8]. Briefly, plasma samples were mixed with trichloroacetic acid (TCA) (20%) and the precipitate was dispersed in H_2SO_4 (0.05 M). TBA (0.2% in sodium sulfate) was added and the mixture heated for 30 min in a boiling water bath. Lipid peroxide adducts were extracted by n-butanol and absorbance was measured at 532 nm.

Statistical Analysis

The Kruskal–Wallis test was used to examine the differences between the groups. Mann–Whitney U and χ^2 tests were also used for nonparametric variables; $P < 0.05$ was considered statistically significant.

Results

With respect to age, weight, and height, the controls did not differ significantly from the patients (Table 2). The prevalence of all respiratory symptoms (cough, dyspnea, and hemoptysis) in those patients with moderate-to-severe pulmonary dysfunction was higher than in those with mild pulmonary dysfunction and controls [dyspnea ($P < 0.001$ for both groups), cough ($P < 0.01$ for both), and hemoptysis ($P < 0.05$ for both)]. There was no significant difference between patients with and without sputum production in dyspnea, cough, or hemoptysis.

The mean serum GSH level in healthy controls was 29.85 ± 3.26 μmol/ml, which was higher than that in patients with mild and moderate-to-severe pulmonary dysfunction (19.02 ± 2.36 and 17.89 ± 2.16 μmol/ml, respectively) ($P < 0.001$). In addition, the mean MDA level in the controls was 0.69 ± 0.09 μmol/ml, which was lower than that in those patients with mild and moderate-to-severe pulmonary dysfunction (0.74 ± 0.05 and 0.75 ± 0.05 μmol/ml,

Table 2 Demographic characteristics of mustard-exposed and control subjects

	Chemical ($N = 250$)	Control ($N = 60$)	Significance
Age	46.0 ± 10.9	43.14 ± 8.45	NS
Height	175.75 ± 86.56	173.03 ± 54.66	NS
Weight	71.49 ± 12.31	68.23 ± 15.85	NS

NS nonsignificant

Age, height, and weight are compared in groups with a t test. Data presented are mean $\pm$ standard deviation

Table 3 Correlation between of plasma GSH level and pulmonary function test variables for mustard-exposed patients

	Pearson correlation	Significance
VC	0.110	NS
FVC	0.138	$P < 0.05$
FEV$_1$	0.156	$P < 0.05$
PEF	0.129	$P < 0.05$
MMEF	0.165	$P < 0.01$

NS nonsignificant, VC vital capacity, FVC forced vital capacity, FEV$_1$ forced expiratory volume in 1 s, PEF peak expiratory flow, MMEF maximal middle expiratory flow

Table 4 Correlation between level of plasma malondialdehyde (MDA) and pulmonary function test variables for mustard-exposed patients

	Pearson correlation	Significance
VC	-0.030	NS
FVC	-0.073	NS
FEV$_1$	-0.122	NS
PEF	-0.108	NS
MMEF	-0.118	NS

NS nonsignificant, VC vital capacity, FVC forced vital capacity, FEV$_1$ forced expiratory volume in 1 s, PEF peak expiratory flow, MMEF maximal middle expiratory flow

respectively) ($P < 0.001$). There was no significant difference in GSH and MDA levels in the mildly affected group compared with the moderately to severely affected group.

There was a weak linear correlation between GSH level and some of the pulmonary function indices (Tables 3, 4). On the other hand, there was no significant relationship between the MDA level and pulmonary function indices (all $P > 0.05$).

Discussion

The most important findings of our study are the decreased level of GSH and the increased level of MDA in patients with SM intoxication compared with nonexposed individuals. In addition, those with moderate-to-severe SM-induced lung injuries showed nonsignificant tendencies for a decreased level of GSH and an increased level of MDA than patients with mild injuries.

Previously, Pant et al. [9] showed that in mice models, exposure to SM results in a significant loss of blood and hepatic and pulmonary GSH. These biochemical changes are accompanied by a number of histopathological alterations, the most prominent of which is congestion and degeneration in viscera and obliteration of chromatin material [9]. In fact, our study confirms the previous findings of Vijayaraghavan et al. [10, 11] and Kumar et al. [12] concerning SM-induced GSH depletion and increased MDA level in animal models and 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. Hence, these conclusions encourage us to examine the recommended methods of Vijayaraghavan et al. and also Kumar et al. in animal models (administration of GSH, vitamin E or flavonoids, and hydroxyethyl rutosides for individuals suffering from complications of SM intoxication) in future studies. Likewise, the coinstillation of

liposomes containing reducing agents (*N*-acetylcysteine [NAC] or GSH) significantly reduced acute SM-induced lung injury (even with delayed airway instillation) [13]. These conclusions have been confirmed in other studies [1].

GSH and SM-Induced Injuries

SM toxicity appears to be mediated, at least in part, by generation of reactive oxygen species [14–17], and experimentally, oxidative stress plays a role in the pathogenesis and progression of SM-induced injuries [15]. On the other hand, GSH has been considered an important antioxidant in the lung with a protective effect against internal and external toxic agents [18]. The metabolite GSH fulfills many important and chemically complex roles in protecting cellular components from the deleterious effects of toxic species. GSH combines with hydroxyl radical as well as reactive electrophiles, including activated mustard analogs [19]. Intracellular levels of GSH have been shown to affect the sensitivity of cells to cell death-inducing stimuli as well as the mode of cell death. In fact, induction of cell death markedly accelerates as a result of GSH depletion [20]. Similarly, Colvin et al. [21] claimed that an elevated GSH level is associated with the resistance of cells to alkylating agents. They demonstrated that one mechanism of this resistance is inactivation of the alkylating agents by conjugation with GSH. This conjugation can be catalyzed by glutathione S-transferase. Although it has been confirmed that GSH-depleted cells are more sensitive to SM than normal cells, it has been emphasized that it is essential for the GSH also to be present extracellularly to protect cells from SM toxicity [22].

As mentioned earlier, GSH is the major nonprotein thiol that can protect cells from damage due to electrophilic alkylating agents by forming conjugates with the agent [23]. There is some confirmatory evidence supporting this claim: compounds that elevate or reduce intracellular levels of GSH may produce changes in cytotoxicity induced by SM, and augmentation of intracellular levels of GSH may provide partial protection against cytotoxicity of SM [23–26]. It is known that there is no medical countermeasure after SM exposure; however, Gross et al. [27] also confirmed that those compounds that can modulate GSH levels within the cell may help to reduce the cytotoxicity of SM when used as pretreatment. They have stated that GSH, a tripeptide that exists in high concentrations in cells, reacts with SM and is involved in SM detoxification. Moreover, they have noticed that pretreatment of human peripheral blood lymphocytes with L-oxothiazolidine-4-carboxylate (OTC), a "masked" cysteine precursor, increases intracellular GSH levels 25-50% over control values and leads to small decreases in cytotoxicity after SM exposure [27].

Although our study was the first of humans with respect to the effect of SM exposure and GSH, there are numerous similar reports in animal models. For example, total GSH content in the brain of mice treated with a single subcutaneous injection of the monofunctional SM was significantly lower than in the controls [28]. Elsayed et al. [28] also found decreased GSH and an almost threefold increase in susceptibility to lipid peroxidation. These changes are all consistent with oxidative stress. Similar to our study, Ray et al. [29] studied the mechanism of action of SM using in vitro the mouse neuroblastoma-rat glioma hybrid NG108-15 clonal cell line model and found that the cellular GSH level decreased 20% after 1 h and 34% after 6 h of SM exposure.

It should be emphasized that GSH depletion is not limited to SM intoxication; repeated exposure to other toxicants such as ozone [30] and tobacco (workers in tobacco factories) [31] is also associated with decreased GSH levels. Also, it has previously been demonstrated that the levels of GSH are reduced in chronic pulmonary disorders such as idiopathic pulmonary fibrosis (IPF) [32], acute respiratory distress syndrome [33], and cystic fibrosis [34]. Moreover, many reports have shown changes in GSH levels in asthmatic airways [35, 36]. On the other hand, GSH is present in increased concentrations in the epithelial lining fluid (ELF) of chronic smokers, a notable finding that does not occur in the ELF of acute smokers [37, 38]. As an explanation, in vitro studies have shown that exposure to oxidative stress causes a transient depletion of GSH, followed by prolonged elevation of intracellular GSH levels [39].

MDA and SM-Induced Injuries

MDA, which arises from the breakdown of lipid peroxyl radicals, is one of the indicators of oxidative stress. MDA is also important in that it can cause further oxidative injury by oxidizing protein molecules; thus, it is both an indicator and an effector of oxidative stress. In contrast, GSH functions only as a "sacrificial" antioxidant [40]. Our findings show increased MDA levels in patients with SM-induced lung injuries. Also, in previous studies [41], SM injection caused oxidative stress, reflected by dramatically increased levels of the lipid peroxidation end product, MDA. Moreover, Ucar et al. [42], using the prototypic nitrogen mustard (mechlorethamine/HN2), showed increased MDA levels in lung tissue of animal models. It seems that increased MDA levels indicate increased lipid peroxidation which may be due to excessive production of free radicals after exposure to SM [42]. These changes in lipid peroxide (LP) content were also observed in other respiratory diseases, including the aggravating phase of bronchitis induced by SO_2 exposure in rats [43]. Moreover, it has been confirmed that morphological changes of the lung, a decrease of PaO_2, and an increase of $PaCO_2$ of blood are all dependent on an

increase of LP on the airway surface. Hence, it has been concluded that LP may be involved in the development of bronchitis [43]. Similarly, the MDA and lipid peroxidation product levels are increased in the lungs of smokers without pulmonary disease [5] and also in the sputum and exhaled breath condensate [44] of patients with different pulmonary diseases. For example, compared to the normal population, the serum level of MDA was significantly higher in patients with acute pneumonia, chronic bronchitis complicated by lung infection, and bronchopneumonia. Hence, it was concluded that the determination of the serum MDA level may be helpful in the diagnosis and treatment of pneumonia [45].

MDA and GSH Levels in Relation to Pulmonary Function Indices

Based on previous reports, inflammation in SM-induced lung abnormalities generates increased amounts of reactive oxygen species (ROS) [35, 46] that correlate with disease severity. Accordingly, the GSH system is expected to be altered in the inflamed lung condition of SM-induced injury. However, our study showed that the GSH level has a minimal correlation with lung function indices. In fact, although the severity of airway obstruction in patients with chronic obstructive pulmonary disease is associated with GSH level [47], a similar association between GSH level and airway obstruction in SM-intoxicated patients is weak. Similarly, relationships between antioxidative enzymatic systems and lung function impairment have been seen in previous reports on antioxidative enzymes in erythrocytes but not in plasma [48]. Therefore, it has been suggested that antioxidant capacity in plasma would unlikely be related to measurements of airway obstruction. There seems to be, however, less intraindividual variability in antioxidative enzymes measured in erythrocytes [25].

Antioxidant enzymes are crucial for the protection of the airway against oxidative stress. The GSH level is changed in the oxidant-rich environment of pulmonary inflammation, which leads to enhanced production of oxygen free radicals by inflammatory cells [48, 49]. According to our findings, inflammatory pathways initiated by sulfur mustard may lead to a lower GSH level, perhaps through increasing oxidative stress.

Finally, oxidant-antioxidant imbalance due to variable changes in GSH level in sulfur mustard patients suffering from pulmonary inflammation may lead to progressive inflammation.

Conclusion

Our study confirmed important alterations in the oxidative-antioxidative system in patients suffering from SM-induced lung injuries, as shown by a decreased serum level of GSH and an increased level of MDA. Individuals with moderate-to-severe SM-induced lung injuries show a greater tendency for a decreased level of GSH and an increased level of MDA than those with mild injuries. There was also a nonsignificant difference in GSH between patients with mild obstructive disease and those with mild and moderate disease. MDA values were essentially identical in these two groups. However, there is only minimal association between pulmonary function parameters and the serum level of MDA and GSH. These findings encourage us to examine therapeutic measures to correct such imbalances in future studies.

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