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RESEARCH ARTICLE

Pro-oxidant–antioxidant balance in Iranian veterans with sulfur mustard toxicity and different levels of pulmonary disorders

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Abstract

Objective: Sulfur mustard (SM) is a strong alkylating agent that primarily targets the skin, eye and lung. The current study evaluated the pro-oxidant–antioxidant balance (PAB) assay in human serum of SM-exposed patients. *Design and methods:* sera of 35 SM-exposed patients and 19 healthy volunteers were recruited. Both groups had nonsmoker and nonalcoholic people with no diseases such as diabetes, heart disease and other pulmonary diseases (COPD because of smoking, asthma and so on). All patients had *documented exposure to SM*. The PAB was measured. *Results:* SM-exposed patients with normal values for pulmonary function test and severe obstructive pulmonary disease demonstrated a significant increase in PAB value in compared with healthy volunteers (the PAB values in healthy volunteers, normal and severe patients were 48.74 ± 21.07 HK, 101.45 ± 32.68 HK and 120.23 ± 31.55 HK, respectively). However, the level of oxidation is not related to the severity of disease defined by spirometry findings. A significant negative correlation was established between the PAB value and FEV_1. *Conclusions:* The increased PAB value in chemical casualties showed that these patients are exposed to oxidative stress.

Keywords

Pro-oxidant–antioxidant balance, pulmonary disease, sulfur mustard

History

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Introduction

Sulfur mustard (SM, bis-2-[chloroethyl]sulfide) is a non-specific alkylating agent that primarily targets the skin, cornea and respiratory tract. SM is regarded as one of the most important agents of chemical warfare because of its simple and cheap chemical synthesis that makes it readily available for both terrorist and military use (Paromov et al., 2007). SM has remained the chemical weapon of choice in modern tactical warfare, as evidenced by its use during the Iran–Iraq conflict between 1983 and 1988 (United Nations Security Council, 1987). Early manifestations occur from 1 h to 48 after exposure to SM, including nausea, smarting of the eyes, lacrimation and photophobia from blepharospasm, rhinorrhea, sneezing, sore throat, erhthema develops on skin, blistering, aphonia and hoarseness (Balali-Mood & Hefazi, 2005). Late pulmonary complications of SM onset range from 6 months to more than 20 years after the exposure and include chronic bronchitis, airway hyper-reactivity, asthma and bronchiolitis obliterans. Symptoms can manifest as chronic cough, dyspnea, sputum production, chest pain and hemoptysis (Emad & Rezaian, 1997; Rowell et al., 2009).

SM alkylates a wide variety of biological molecules in target tissues including nucleic acids, proteins and lipids, as well as small molecular weight metabolites such as glutathione (GSH) leading to oxidative stress (OS) (Papirmeister et al. 1991; Wheeler, 1962). OS eventuates when the production of reactive oxygen species (ROS) exceeds the capacity of antioxidant responses. Many studies have divulged that OS plays a main role in the initiation and development of pulmonary diseases including chronic obstructive pulmonary disease (COPD), asthma, bronchiolitis obliterans and so on (Behr et al., 2000; Kirkham & Rahman, 2006; Repine et al., 1997). Investigations showed that during the progression of pulmonary diseases, antioxidant defense mechanisms are different (Comhair et al., 2000; Janssen et al., 1992).

The mechanisms underlying SM toxicity at the molecular level are not fully elucidated. The effects of topically applied

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SM on key antioxidant enzymes have been measured but with conflicting results. For example, Husain et al. (1996) found an decreased level of glutathione peroxidase in white blood cells, spleen and liver in patients intoxicated with SM compared to controls. However, Elsayed et al. (1992) found that SM increased the levels of glutathione peroxidase in comparison with control. Also, another study by Shohrati et al. (2010) reported elevated levels of malonaldehyde and reduced levels of GSH in plasma compared to control. These studies investigated a few oxidants or antioxidants.

Many methods have been developed to measure the total oxidants (for instance, total oxidant status assays; Erel, 2005) or antioxidants (e.g. the ferric reducing ability of plasma and oxygen radical absorbance capacity assays; Benzie & Strain, 1996; Cao et al., 1993), separately. Therefore, in these approaches for estimation of pro-oxidant/antioxidant balance (PAB), two separate tests should be performed. These methods are time-consuming and imprecise (Alamdari et al., 2007). However, we have previously developed a method which can measure the balance of oxidants and antioxidants using 3,3′,5,5′-tetramethylbenzidine (TMB) (Alamdari et al., 2007, 2008). PAB assay is developed in order to measure the pro-oxidant burden and the antioxidant capacity simultaneously in one assay (one enzymatic reaction and a chemical reaction) and give a redox index (Alamdari et al., 2007).

Growing evidence indicates that the production of pro-oxidants under pathophysiologic conditions is cause of tissue damage and cell apoptosis. There are a few reports on the long-term effect of SM on antioxidant system and OS in SM-intoxicated patients. Therefore, we tried to assess PAB in a group of SM-exposed subjects. This is the first time that the PAB is measured in chemical casualties.

Materials and methods

Chemicals

TMB powder (3,3′,5,5′-tetramethylbenzidine 2 HCl) is purchased from Sigma (Helsinki, Finland), peroxidase enzyme is purchased from Applichem (230 U/mg, A3791, 0005, Darmstadt, Germany), chloramine T trihydrate is purchased from Applichem(A4331), hydrogen peroxide (30%) is purchased from Merck. All the other reagents used were of reagent grade and were prepared in double distilled water.

Study population

A total of 54 subjects were included in the study. Of these 35 were diagnosed as having had previous exposure to SM and 19 were healthy volunteers. Nonsmoker and nonalcoholic healthy volunteers with no history of exposure to SM or other chemicals have been chosen. All samples were from male subjects. The patients were recruited from the outpatient clinic of the department of pulmonology of the Sasan Hospital (Tehran, Iran) from July 2012 to October 2013. The patients had no history of smoking or alcohol consumption and had been exposed to SM in Iran–Iraq war between 1983 and 1988 (nearly 30 years after exposure to SM). The patients' diagnosis was made on their existing documents in Ira–Iraq. Exclusion criteria were pneumonia, acute bronchitis, asthma, COPD (due to smoking) and the other pulmonary diseases, cigarette smoking, diabetes and heart disease.

The extent of tissue injury depends on the duration and intensity of exposure (Renshaw, 1946). The degree of airflow obstruction of patients exposed to SM was staged according to GOLD guidelines on the basis of FEV_1 and FEV_1/FVC (FEV_1: forced expiratory volume in one second, FVC: forced vital capacity) into five groups: normal ($FEV_1 > 80\%$ predicted), mild ($FEV_1 \geq 80\%$ predicted), moderate ($50\% \leq FEV_1 < 80\%$ predicted), severe ($30\% \leq FEV_1 < 50\%$ predicted) or very severe ($FEV_1 < 30\%$ predicted) and FEV_1/FVC (normal > 70, the other groups $< 70\%$) of the predicted value, respectively (Vestbo et al., 2013).

SM-exposed patients were classified according to the percentage of FEV_1 and FEV_1/FVC into normal and severe groups. Selected patients and healthy volunteers were matched for age and gender. Normal patient group had received no inhaled corticosteroid treatment for at least a month prior to enrollment. However, severe patients could not stop the treatment, because of severity of disease. They were receiving *fluticasone* 250–500 μg/salmeterol 25–50 μg per 12 h.

The characteristics of the patients and healthy volunteers are presented in Table 1. Informed consent was obtained from all patients. The study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences (SBMU).

Specimen of blood was drawn from every patient, the collected blood was immediately centrifuged at 1500*g* for 15 min and the serum samples were stored in aliquots at −80 °C until use.

Anthropometric and pulmonary function measurements

For all subjects, anthropometric parameters including weight, height and body mass index (BMI) were measured. BMI was calculated as weight (in kg) divided by height squared (in m^2). Lung function was measured by means of a spirometer.

PAB assay

PAB values were measured in serum samples. A modified PAB was applied based on a previously described method

Table 1. Demographic characteristics and pulmonary function test of normal and severe patients (because of SM) and healthy volunteers.

	Healthy volunteers	Normal patients	Severe patients
Number	19	18	17
Age (year)	47.84 ± 7.67	47.17 ± 4.44	47.88 ± 7.79
Height (cm)	174.26 ± 4.8	173.35 ± 8.13	171.35 ± 6.8
Weight (kg)	78.84 ± 9.29	79.47 ± 8.25	74.23 ± 15.86
BMI (kg/m^2)	25.96 ± 2.9	26.48 ± 2.64	25.29 ± 5.42
FEV_1 (% predicted)	95.84 ± 8.3	85.22 ± 14.23[b]	28.26 ± 10.2[a]
FVC (%)	89.26 ± 8.1	83.33 ± 13.09	40.46 ± 15.26[a]
FEV_1/FVC (%)	85.68 ± 3.44	82.94 ± 5.84	59.73 ± 16.17[a]
PAB (HK)	48.74 ± 21.07	101.45 ± 32.68[a]	120.23 ± 31.55[a]

Values are presented as mean ± SD. BMI, body mass index; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; PAB, pro-oxidant–antioxidant balance. Compared with the healthy volunteers:
[a] $p < 0.001$,
[b] $p < 0.05$.

(Alamdari et al., 2009). The standard solutions were prepared by mixing varying proportions (0–100%) of 250 μM hydrogen peroxide with 3 mM uric acid (in 10 mM NaOH). Sixty milligrams TMB powder was dissolved in 10 mL DMSO; for preparation of TMB cation, 400 μL of TMB/DMSO was added to 20 mL of acetate buffer [0.05 M buffer, pH 4.5] and then 70 μL of fresh chloramine T (100 mM) solution was added into this 20 mL, mixed well, incubated for 2 h at room temperature in a dark place; 25 U of peroxidase enzyme solution was added to 20 mL TMB cation, dispensed in 1 mL and stored at −20 °C; in order to prepare the TMB solution 200 μL of TMB/DMSO was added into 10 mL of acetate buffer (0.05 M buffer, pH 5.8); the working solution was prepared by mixing 1 mL TMB cation with 10 mL of TMB solution, incubated for 2 min at room temperature in a dark place and immediately used. Ten microliters of each sample, standard or blank, (distilled water) was mixed with 200 μL of working solution, in each well of a 96-well plate, which was then incubated in a dark place at 37 °C for 12 min; at the end of the incubation time, 100 μL of 2 M HCl was added to each well and measured in an ELISA reader at 450 nm with a reference wavelength of 620 or 570 nm. A standard curve was provided from the values relative to the standard samples. The values of the PAB are expressed in arbitrary HK units used by inventors of PAB method (Hamidi and Koliakos) (Alamdari et al., 2007), which represent the percentage of hydrogen peroxide in the standard solution. The values of the unknown samples were then calculated based on the values obtained from the above standard curve.

Statistical analysis

Results are expressed as mean ± SD. To assess normality distribution of the data in each of the research arms, the Shapiro–Wilk test was used. The one-way ANOVA was performed using the SPSS for Windows™, version 16 software (SPSS Inc., Chicago, IL). A p value <0.05 was considered statistically significant. Bi-variate correlations between different variables and PAB values were performed using Pearson correlation coefficient. Stepwise multiple linear regression analysis was used to determine which of lung capacities such as FEV_1, FVC and FEV_1/FVC could influence PAB values. Data for age, weight, height and BMI were not included in the analysis since the patients and healthy volunteers were matched for these parameters.

Results

Demographic characteristics and pulmonary function test

All three groups (healthy volunteers, normal SM and severe SM patients) were well-matched for age and gender. No significant differences in weight, height and BMI between the groups were found.

The healthy volunteers had significantly higher FEV_1 values compared to the normal patient group ($p < 0.05$) and severe patients ($p < 0.001$). Severe patients had also lower FVC than healthy volunteers ($p < 0.001$). However, no significant difference in FVC values was observed between healthy volunteers and normal patients. Finally, FEV_1/FVC values in severe patients were significantly lower compared to normal patients and healthy volunteers ($p < 0.001$) (Table 1).

PAB values among different groups

Mean PAB values in healthy volunteers were 48.74 ± 21.07 HK, being significantly lower than both patients groups exposed to SM (normal and severe) ($p < 0.001$). Moreover, in the patients who had stable pulmonary disease, normal group, PAB levels were 101.45 ± 32.68 HK, which was not significantly different between patients with severe group (PAB value = 120.23 ± 31.55 HK and $p > 0.05$) (Table 1).

Association between PAB values and pulmonary function test

The Shapiro–Wilk test demonstrated normal distribution of the data. Stepwise multiple linear regression showed that only FEV_1 ($\beta = -0.811$, $p < 0.001$) was found to have a significant effect on PAB values and FVC and FEV_1/FVC were excluded from regression equation. The regression model yielded the following equation for the prediction of serum PAB values [PAB values $= -0.811 \times (FEV_1) + 146.825$].

Discussion

In the present study, we showed that OS in patients previously exposed to SM was significantly higher in comparison to healthy volunteers with no history of SM exposure using PAB assay. Furthermore, our results showed that the mean of PAB value in severe patients was higher than normal ones. The use of inhaled corticosteroids in the severe-disease group might have confounded the results. Although the direction of this confounding effect is against detection of significant difference between the study groups, we found significantly elevated OS in both SM exposed groups (normal and severe) compared with the healthy volunteers. The lack of significant difference between the severe and normal SM-exposed subjects could be due to the relatively small population size because the trend in PAB changes favors heightened OS in the severe group ($p = 0.059$).

Our results are consistent with other studies, in which SM produce oxidative components. Several studies reported that pro-inflammation cytokines, namely interleukin-6 (IL-6), IL-4, IL-13 induce OS as well as tumor necrosis factor-alpha (TNF-α) which could cause nuclear factor-κ-binding protein (NFκB) activation and ROS production (Dong et al., 2007; Li et al., 2000; Stentz et al., 2004; Wassmann et al., 2004; Won et al., 2013). In addition, it has been shown that SM induces secretions of IL-1, IL-6, IL-8 and TNF-α in comparison to control human skin cells (Arroyo et al., 1999, 2000, 2001; Lardot et al., 1999). Arroyo et al. (2003) have reported decreased levels of IL-6 and IL-8 secretion in human skin cells exposed to SM after treatment with 1-α, 25-dihydroxyvitamin D_3 (1-α, $25(OH)_2D_3$). In addition, they found that SM reduced cell proliferation in human skin cells. However, 1-α, $25(OH)_2D_3$ increased cell proliferation in these cells after exposure to SM.

Seagrave et al. (2010) examined responses of differentiated primary airway epithelial cell cultures exposed directly to SM vapor. They demonstrated that sub-cytotoxic levels of SM

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vapor can induce inflammatory (an increase that was statistically significant was observed for IL-8) and proteolytic (elevated matrix metalloproteinases-13 levels) responses. Also, the levels of heme oxygenase 1, a marker for OS, were assessed. However, cultures did not show significant changes in heme oxygenase 1 levels.

In the study of Ham et al. (2012), the effect of SM on human neutrophils from venous blood was evaluated. They found that SM increased intracellular calcium through transient receptor potential melastatin, a major calcium channel in human neutrophils, and calcium-dependent p38 MAPK (mitogen-activated protein kinase) phosphorylation. Moreover, SM can induce p38 MAPK phosphorylation in keratinocyte (Rebholz et al., 2008). Afterwards, phosphorylated p38 MAPK induces NFκB p65 phosphorylation and the release of TNF-α, IL-6 and IL-8 (Ham et al., 2012). It has been also reported that MAPKs increase the release of ROS from neutrophils (Johnson et al., 1999). Also, the activation and nuclear translocation of the cytoplasmic transcription factor NFκB can be caused by TNF-α, likely by modifying the oxidation status of the cell through ROS (Anderson et al., 1994). Another study demonstrated that SM upregulated the gene expression of IL-15, IL-18r, IL-4, TNF-α, and TNF-α super family receptors (inflammatory genes) in mice liver. However, it down-regulated anti-inflammatory genes IL-10 and six genes related to DNA repair proteins (Anand et al., 2009). Furthermore, Pohank et al. (2011) demonstrated that OS is an important pathway of SM toxicity.

The delayed effects of SM on inflammatory responses were investigated. For example, Attaran et al. (2010) demonstrated that serum levels of IL-6 and highly sensitive C-reactive protein (hsCRP) were significantly elevated in the SM-exposed group (approximately 20 years after exposure to SM) in comparison to controls. Also, the levels of CRP in patients with SM poisoning are correlated inversely with FEV_1. Studies indicated that a significant positive correlation was established between the hsCRP levels and OS (Kotani & Taniguchi, 2012; Park et al., 2013). Another study for evaluating delayed effects of SM is assessment of some antioxidants (such as glutathione-*S*-transferase family, neutrophil gelatinase-associated lipocalin 2, metallothioneins, heme oxygenases and superoxide dismutase) expressions in these patients. They showed that levels of these antioxidants were upregulated in the patients who were *exposed to SM approximately 20 years ago* compared to normal samples (Nourani et al., 2010). Some studies indicated that antioxidant enzymes can elevate as a result of OS (Black et al., 2010). Hence, previous studies have demonstrated long-term effect of SM exposure increased OS that it is consistent with our results in the current study.

Perhaps, the patients intoxicated with SM increase OS via inflammatory molecules (CRP and some ILs). Knowledge of the mechanisms of SM-induced OS can play a critical role in the process of devising new therapeutic. However, the PAB assay cannot determine a mechanism for producing OS. Owing to the paucity of data regarding the PAB in SM-exposed subjects, the results of the present study could be useful to justify the value of antioxidant therapy in subjects who are suffering from chronic pulmonary complications of SM. Future studies are needed to explore the potential mechanisms involved SM-induced OS.

The limitation of this study is lack of a patient group with severe lung disease from nonchemical origins to compare with SM exposed. Future studies are recommended to include subjects with respiratory disease/lung injury with nonchemical origin in order to allow a more reliable assessment on the pathologic effects of SM on the respiratory system.

Conclusion

In summary, our results showed that patients with lung lesion because of exposure to SM have higher levels of OS in comparison to healthy volunteers. The level of oxidation is not related to the severity of disease defined by spirometry findings. It seems that OS plays a major role in pathogenesis of diseases. However, larger population is needed to confirm these results. As mentioned above, most investigations assessed short-term (from several hours up to several days) effect of SM on various cells and animals in which a significant increase in inflammatory cytokines and enzymatic antioxidants was observed, whereas in the current study, we evaluated long-term effect of SM on human (nearly 30 years postexposure). *Long-term consequence* of exposure to SM includes increase in inflammatory molecules and enzymatic antioxidants. Likely, at first cytotoxic levels of SM can directly induce OS, but many years after exposure the levels of OS were elevated through the indirectly effects of inflammatory indicators such as ILs and CRP because of lung diseases.

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Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of this article.

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