EVALUATION OF DELAYED TOXIC EFFECTS OF SULFUR MUSTARD POISONING IN SEVERELY INTOXICATED IRANIAN VETERANS: A CROSS-SECTIONAL STUDY

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ABSTRACT

Background

Sulfur mustard (SM) is a chemical warfare agent (CWA) that was widely used during the World War I and by the Iraqi forces against the Iranian soldiers and even innocent civilians between 1983 and 1988. Early toxic effects of SM in Iranian veterans have already been published, but late complications in different organs and the relationship between local and systemic toxicities have not been previously reported.

Objectives

The aim of this study was to investigate the delayed toxic effects of SM poisoning in severely intoxicated Iranian veterans and to evaluate the developing stages of late complications, as well as the possible correlations between different organ complications.

Methods

All SM-intoxicated veterans in the province of Khorasan, Iran who had severe clinical complications according to their previous follow-ups were studied. Respiratory, neuromuscular, dermatological, ophthalmological, hematological and immunological complications were evaluated through clinical and paraclinical investigations including cell blood counts (CBC), flow cytometry, serum immunoglobulins (Igs) and complements, arterial blood gas (ABG) analyses, pulmonary function tests (PFT), chest X-ray (CXR), high-resolution computed tomography (HRCT) of the chest, bronchoscopy, electromyography (EMG), nerve conduction velocity (NCV) and skin biopsy for both light and electron microscopy. Patients were graded according to their severity of complications in different

target organs. Correlation of different clinical complications with each other, as well as with the hematological and immunological complications, was evaluated, using the Spearman Rank Correlation Test. Control groups consisted of 35 and 12 healthy, age-matched subjects employed for the immunological and hematological studies, respectively. The Mann-Whitney U test was used for intergroup comparisons.

Results

Forty male patients (aged 43.8 ± 9.8 years) who had confirmed SM exposure between 16 to 20 years ago were studied. The most commonly affected organs were lungs (95%), peripheral nerves (77.5%), skin (75%) and eyes (65%). The most common respiratory complications were diagnosed as COPD (45%), bronchiectasis (37.5%), asthma (25%) and large airway narrowing (17.5%). NCV disturbances were more common in the sensory nerves compared with the motor nerves, and were more common in the lower extremities than in the upper extremities. Sensory tibial (70% to 75%) and sural (65% to 72.5%) nerves were the two most commonly affected ones. EMG abnormalities were found in 40% of the patients. Skin complications were recorded as hyperpigmentation (55%), erythematous popular rash (42.5%), dry skin (39%), multiple cherry angiomas (37.5%), atrophic scar (27.5%), hypopigmentation (25%), hair loss (10%) and hypertrophy (2.5%). Light microscopy revealed epidermal atrophy, hyperkeratosis, basal membrane hyperpigmentation and non-specific dermal fibrosis. Electron microscopy showed increased melanocytes and melanosomes within epidermis and increased collagen fibers and mononuclear inflammatory cells within dermis. Slit lamp examination of the eyes revealed pre-limbal hyperpigmentation (17.5%), vascular tortuosity (15%), corneal thinning (15%), corneal opacity (10%), corneal vascularization (7.5%) and corneal epithelial defect (5%). white blood cell (WBC), red blood cell (RBC), hematocrit (HCT), IgM, C3, and CD3+ lymphocytes were significantly higher in patients, but CD16+56 positive (N.K) cells were significantly lower in patients compared with the control group ($P<0.05$). Except for the significant correlation between the severity of respiratory complications and both Hb ($r=0.369$, $p=0.024$) and HCT ($r=0.470$, $p=0.003$), no other significant correlation was found between the severity of target organ complications and either of hematological or immunological parameters. A significant positive correlation was also observed between the severity of respiratory and eye complications ($r=0.322$, $p=0.043$).

Conclusion

Late complications of SM may be due to either its direct toxic effects on the respiratory tract, skin or eyes or the systemic effects such as the neuromuscular, hematological and immunological disorders. According to this study, no correlation was found between the severity of long-term local complications and the late systemic toxicities. Constant follow-up of these patients and further studies on a wider range of the SM veterans are required to provide them with more help and to better understand the delayed toxic effects and late complications of SM poisoning.

INTRODUCTION

The use of chemical weapons on the battlefield has been a long tradition. Modern chemical warfare, however, emerged in 1915 when the German army unleashed clouds of chlorine on French and Canadian troops in a field near Ypres, Belgium. The blistering agent,

sulfur mustard (SM) was used for the first time by the German army against the Allies in 1917. Thereafter it was employed extensively by both sides and was destined to cause over 400,000 casualties during World War I (Gilchrist, 1928; Prentiss, 1937). Its effectiveness made it a chemical weapon of choice from 1917 to the present day, as evidenced by its use by the Iraqi forces against the Iranian soldiers and even innocent civilians between 1983 and 1988 (United Nations Security Council, 1988). Despite several international conventions and treaties designed to prevent the use of these weapons of mass destruction, SM still remains a serious and realistic threat to the world's security and health (WHO, 2004).

SM is chemically named bis (dichloroethyl) sulfide. It is a colourless to amber oily liquid, freezing at 14ºC when pure and boiling at 217ºC with slow decomposition (Ballantyne et al., 1995). SM is absorbed by inhalation or through the skin. It may also be absorbed through the gastrointestinal tract following consumption of contaminated food or water (Sidell, 1998). After absorption, SM undergoes intermolecular cyclization to form an ethylene episulfonium ion intermediate (Gilman et al., 1946; Somani and Babu, 1989). This reactive cyclic intermediate, in the presence of water, reacts avidly with a number of biologically important molecules, including DNA, RNA, and protein, producing alkylation products (Smith and Dunn, 1991). SM can be bifunctional, in that some ion intermediates covalently bind adjacent strands of DNA, producing DNA cross-links. DNA alkylation and DNA cross-linking are known to be the major mechanisms for the pathogenesis of SM toxicity (Ball and Roberts, 1972; Walker, 1971; Wheeler, 1962).

It is observed that lungs, skin, and eyes are the three major targets affected by SM (Balali, 1984; Willems, 1989). The main acute respiratory toxic effects include acute tracheobronchitis, sever throat edema, acute pulmonary edema, and broncho-pneumonia (Balali and Navaeian, 1986; Balali et al., 1986; Sohrabpour, 1984). Ophthalmologic effects are mainly conjunctivitis, subconjunctival hemorrhage, blepharospasm, corneal edema and keratitis (Balali and Navaeian, 1986; Solberg et al., 1997). Dermatological features include erythema and edema, blister formation, denudation of skin, ulceration and necrosis (Davis and Aspera, 2001; Momeni et al., 1992; Momeni and Aminjavaheri, 1994).

As alkylating agents, SM is particularly toxic to rapidly proliferating cells, including lymphoid and bone marrow cells (Willems, 1989). Studies have revealed initial leukocytosis followed by leukopenia, thrombocytopenia and anemia within the first few days of exposure. Anemia would continue to exist if the patient survived and WBC of some patients strikingly dropped to less than 1000/cm3 (Balali et al., 1991; Sohrabpour, 1984, Tabarestani et al., 1990). The immune system and particularly lymphocytes have been reported to be highly susceptible to the toxic effects of SM. One of the most threatening effects of SM has been found to be lymphopenia and severe suppression of the immune system (Krumbhaar and Krumbhaar, 1919; Willems, 1989). Bone marrow biopsies have shown hypocellular marrow and cellular atrophy involving all elements (Balali et al., 1986; Eisenmenger et al., 1991).

There have recently been reports on late complications of SM poisoning including respiratory complications such as chronic bronchitis, bronchiectasis, asthma and interstitial fibrosis (Bagheri et al., 2003; Bijani and Moghadamnia, 2002; Emad and Rezaian, 1997; Ghanei, Fathi et al., 2004); dermatologic complications such as hyper- and hypopigmentation, atrophy, and dry skin (Balali, 1986; Khateri, 2003); ophthalmologic complications such as

chronic conjunctivitis, corneal vascularization, and delayed keratitis. (Javadi and Kazemi, 2000; Pleyer et al., 1999).

Objectives

There are still several major gaps in the literature on the assessment of delayed toxic effects caused by exposure to SM. It has not been adequately studied whether or not there is an association between short-term and long-term effects and also between the local late complications and the delayed systemic toxic effects of SM. There is also very little information available on the probable correlation between late complications in different target organs and on the developing stages of each complication. Furthermore, there is paucity of literature describing delayed or long-term effects of SM on the immune system as well as the neuromuscular system.

The aim of this study was to investigate the delayed toxic effects and late complications of SM poisoning in severely intoxicated Iranian veterans to answer the above questions. Therefore a comprehensive cross-sectional study was undertaken between 2001 and 2003 on 40 Iranian veterans who had confirmed SM poisoning and suffered from its severe late complications.

MATERIALS AND METHODS

Patients and Study Design

There were 1845 chemically injured veterans in the province of Khorasan, Iran, according to the latest report from Khorasan Veteran Foundation in June 2001. An expert medical committee annually evaluates the severity of late complications in these patients and grades them as normal, mild, moderate and severe. The grading criteria, which was used by this committee, was summarized in Tables 1, 2, and 3. In cooperation with the Foundation we reviewed the patients' files and selected those who met the following criteria: (1) Confirmation of SM poisoning following exposure during the war (1983-1988) that was made by clinical manifestations and toxicological analysis (Hyndrickx et al., 1984). (2) Severe late complications in one or more of the target organs, including respiratory tract, skin, and eyes. Forty-seven male subjects fulfilled the above criteria. All these patients had their initial admissions in Imam Reza University Teaching Hospital between 1983 and 1988 and have had regular outpatient follow-ups since then. Of this group, 40 patients volunteered to participate in the study and signed the informed written consent.

After approval of the medical ethics committee of the university, a young doctor (the second author) reviewed the patients' previous files and summarized them in pre-designed forms. The patients were then hospitalized in groups of four or five in the Toxicology Ward of Imam Reza University Teaching Hospital and underwent a complete history and physical examination by the experienced consultants (pulmonologist, dermatologist, neurologist, and ophthalmologist). During four to five days of admission a uniform series of para-clinical investigations were carried out for each patient, as described below. Although some of these diagnostic investigations had been previously performed for the patients, we carried them out again in the uniform setting of our own study in order to avoid any unwanted bias. All clinical

and paraclinical findings were recorded in pre-designed forms and interpreted by the relevant specialists of our research team.

Hematological and Biochemical Studies

Peripheral venous blood and urine samples were taken for laboratory investigations, using standard procedures. HCT and CBC for RBC, hemoglobin (Hb), WBC, and platelet (PLT) counts were performed by autoanalyzer Technicon H1 (Bayer Medical Systems, NY, USA) in the Department of Hematology. Biochemical tests including fasting blood sugar, total cholesterol, triglyceride, urea, creatinine, uric acid, aspartate (AST) and alanine (ALT) transaminases were measured by autoanalyzer Technicon RA-1000 (Bayer Medical Systems, NY, USA) in the Department of Clinical Biochemistry.

Immunological Studies

Flow cytometric analyses were performed to determine the percentages of lymphocytes, monocytes, and neutrophils, as well as the CD3, CD4, CD8, CD19 and CD16+56 lymphocytes, using FACS Calibur cytometer (Becton Dickinson, San Jose, USA), equipped with CellQuest software. For the separation of lymphocytes, Ficol Hypac solution was used. All monoclonal antibodies (bicolours), calibration kit and other materials for flow cytometric analyses were obtained from Becton Dickinson, USA (Lainer et al., 1985). Serum immunoglobulin concentrations of IgA, IgG, IgM, and complement factors C3 and C4 were measured, using SRID quantification kits (Biogene; Mashhad, Iran). Serum IgE level was determined using ELISA (Radims; Roma, Italy). These investigations were performed in the Laboratory of Immunology of Imam Reza Hospital and in the Immunology Research center of Bu-Ali Research Institute under the supervision of the third author.

Pulmonary Diagnostic Tests

Pulmonary function tests (PFT) were measured according to the spirometric assessment standards of the American Thoracic Society (1987). Slow vital capacity (SVC), forced vital capacity (FVC), forced vital capacity in 1st second (FEV1), and the FEV1/FVC ratio (FEV1%) were recorded before and 5 minutes after two puffs of Salbutamol (100mg/puff), using a flow-sensing spirometer (FUDAC 50; FUKUDA Sangyo; Chiba, Japan). All these variables were expressed as percentages of predicted values. Arterial blood gas analyses (ABG) were also carried out with AVL 995 Blood Gas analyzer (AVL Biomedical Instruments, Graz, Austria). ABG and PFT results were interpreted by the chest physician of our research team, using the normal standards described by Andreoli (2001) and Boskabadi et al. (2002), respectively.

A chest roentgenogram and a high-resolution computed tomography (HRCT) of the chest were obtained in each patient, using a conventional chest stand (Siemens; Erlangen, Germany) and a computed tomography (CT) unit (High Speed; General Electric Medical Systems, Milwaukee, USA), respectively. Patients were scanned at fully suspended inspiration and none received contrast materials. The HRCT scans were performed with 1.0 to 1.5-mm sections taken at 10 mm intervals through the entire thorax and were reconstructed using a bone algorithm. Chest roentgenogram and CT scans were interpreted independently by a chest radiologist as well as the chest physician of our research team and assessed for the evidence of bronchiectasis and interstitial fibrosis.

Bronchoscopy was performed for 24 of 40 patients using a flexible fiberoptic bronchoscope (Olympus BF1T; Tokyo, Japan). Considering the risk of complications, the other 16 patients were excluded from this procedure. Supplemental oxygen and continuous cardiac monitoring were provided for all the patients throughout the procedure.

Chronic Obstructive Pulmonary Disease was defined when patients had a history of cough and sputum production for at least three months per year during the past two years and an irreversible obstructive pattern at spirometric testing. Patients in asthma group met the following criteria: (1) reversibility of FEV1, defined as >15% increase in the FEV1 after a standard dose of inhaled ?2-agonist and (2) typical history of attacks of dyspnea, wheezing, or both, and nocturnal cough either triggered by irritants, respiratory infections, or exercise (American Thoracic Society, 1987). Diagnosis of bronchiectasis and interstitial pulmonary fibrosis were made according to the typical radiologic and HRCT findings (Diederich et al., 1996; Armstrong et al., 2000). Simple chronic bronchitis was defined when patients had a history of cough and sputum production; but no obstructive PFT pattern was found on spirometry and there was no evidence of bronchiectasis on HRCT. Large airway narrowing was diagnosed according to the HRCT and bronchoscopy findings.

Dermal Histopathological Examinations

Skin examination was undertaken by the dermatologist of our research team. Skin biopsy was performed for only eight patients who had more distinct dermal lesions. The biopsy was performed by the dermatologist using 2% lidocaine as local anesthetic. For light microscopy, tissues were fixed in 10% phosphated-buffer (pH=7.0) formalin, dehydrated and embedded in paraffin. Serial sections of 3-?m thickness were made and stained with Hematoxylin and Eosin. Electron microscopy was also performed on skin specimens which were immediately fixed in 2.5% glutaraldehyde and 0.1 mol/L of phosphate buffer (pH=7.2). After fixation in 1% osmium tetroxide and 0.1 mol/L of phosphate buffer, the samples were dehydrated in graded series of alcohol (30 to 100%) and embedded in epoxyresin. Ultrathin (70 nm) sections were collected on 200-mesh copper grids for staining with 2% uranyl acetate and 5% lead citrate. Examinations were undertaken by the experienced pathologist and electron microscopic specialist of our research team, using LEO 910 transmission electron microscope (Zeiss, Oberkochen, Germany).

Electrophysiologic Studies

Electromyography (EMG) and nerve conduction velocity (NCV) were undertaken by the experienced neurophysiologist of our research team, using MEDELEC MS6 electromyograph (MEDELEC, Oxon, U.K). EMG of the abductor digitorum, opponens pollicis, extensor digitorum communis, tibialis anterior, gastrocnemius, extensor and flexor digitorum brevis muscles of both extremities were performed. NCV of median, ulnar, tibial (medial plantar) and deep proneal motor nerves and relevant sensory nerves of both extremities were also carried out. The results were interpreted according to the normal standards described by Delisa et al. (1994).

Severity Grading of Complications

Severity of complications in respiratory tract, skin and eyes were graded as no specific complication, mild, moderate or severe. For grading the severity of skin and eye complication, we used the same criteria as employed by the Veteran Foundation (Table 1 and 2). For severity grading of respiratory complications, however, we made some modifications to the Foundation's criteria, because their grading system was only based on spirometric findings. We used a combination of PFT, ABG, and HRCT findings and judged the overall severity grading according to the diagnostic procedure which had the highest (most severe) grade in each patient (Table 4). Grading was not performed for the neuromuscular complications due to lack of a generally accepted grading system in this area.

Control Groups

A control group consisting of 35 healthy male subjects who had no history of exposure to SM was employed for flow cytometric studies. For comparison of serum immunoglobulins, complement factors, and hematological parameters 12 of these 35 controls participated.

Statistical Analyses

All data were expressed as mean (±SD) unless otherwise indicated. Type of data distribution was assessed by Kolmogrov-Smirnov Z test. The Mann-Whitney U test was then employed for intergroup comparisons. The Spearman Rank Correlation Test was used to correlate the severity of complications in different target organs with each other and with continuous variables such as hematological and immunological parameters. The "statistical package for social sciences" (SPSS) version 11.5 (SPSS Inc, Chicago, USA) was used throughout with the minimum level of significance set at $p=0.05$ for all the comparisons and correlations.

RESULTS

Forty male patients with the age range of 32 to 76 (43.8 ± 9.8) years were studied. The 35 controls were 26 to 55 (42.1 ± 8.8) years, and the 12 controls were 26 to 55 (41.3 ± 8.1) years. There was no significant difference between the age of patients and either of the 35 ($p = 0.479$) or the 12 ($p = 0.557$) controls. The interval between SM exposure and this investigation was 18.05 ± 1.55 years (range, 16 to 20 years). The most commonly affected organs were lungs (95%), peripheral nerves (77.5%), skin (75%) and eyes (65%), as shown in Figure 1. The symptoms recorded in this study were more common but less reliable in comparison with the signs.

Respiratory Complications

All patients complained of coughing and expectoration, 85% of dyspnea and 60% of haemoptesis. Main objective clinical findings were generalized wheezing (95%), crackles (50%) and stridor (10%). Mild (60?PaO2<85) and moderate (45?PaO2<60) hypoxemia were found in 57.5% and 37.5% of the patients, respectively. Arterial blood gases and pH results were summarized in Table 5.

Pulmonary function tests revealed a more obstructive pattern than restriction as shown in Figure 2. Of 23 cases with obstructive spirometric pattern, 10 patients revealed a reversible (>15% increase in FEV1 after bronchodilator) obstruction, while the other 13 patients had an

irreversible obstruction, including one fixed extra-thoracic pattern. Spirometric parameters of the patients before and after bronchodilator were summarized in Table 6.

Chest radiography revealed cystic or tubular bronchiectatic pattern (32.5%), hyperinflation (27.5%), increased bronchovascular markings (22.5%), evidence of pulmonary hypertension (15%), and interstitial pattern (2.5%). Bronchiectasis and interstitial fibrosis were confirmed by HRCT in 37.5% and 7.5%, respectively. Bronchiectatic lesions were most commonly observed in the right and left lower lobes (54 to 60%), followed by the right middle lobe (47%) and the singulate segment (33%). Right and left upper lobes were only involved in one patient. The 24 patients underwent bronchoscopy which revealed large airway narrowing in 7 patients. The most common site of narrowing was in the main bronchus of the right middle lobe (71% of all cases).

Based on the clinical findings and the para-clinical investigations, the most common respiratory complications were diagnosed in the order of COPD, bronchiectasis, asthma, large airway narrowing, simple chronic bronchitis and interstitial pulmonary fibrosis as shown in Figure 3. Severity grading was determined as 2 (5%) patients in grade 1, 11 (27.5%) in grade 2, 14 (35%) in grade 3, and 13 (32.5%) patients in grade 4. Since there were only two patients in grade 1 of respiratory complications, we did not consider this group in the correlation tests of the severity of respiratory complications with hematological and immunological parameters.

Neuromuscular Disturbances

Nerve conduction velocity results for the motor nerves in 40 patients were summarized in Table 7. The most common motor nerve disorders were found in left (37.5%) and right (35%) tibial followed by right (20%) and left (12.5%) proneal nerves. The NCV results for the sensory nerves in 40 patients were summarized in Table 8. The most common sensory nerve disorders were found in left tibial (75%) and right proneal (72.5%) nerves. On the whole, NCV disturbances were more common in the sensory nerves compared with the motor nerves, and more common in the lower extremities than in the upper extremities. Sensory and motor nerve disturbances in both upper and lower extremities were mostly symmetric. Electromyographic recording revealed normal pattern in 24 (60%) patients, incomplete interference with normal amplitude in six (15%) and incomplete interference with low amplitude in nine (22.5%) patients.

Skin Complications

Five (12.5%) patients had no complaints of skin. The most common symptoms in other patients were pruritus (66%) and burning sensation (21%). The signs were recorded in the order of hyperpigmentation, erythematous popular rash, dry skin, multiple cherry angiomas, atrophic scar, hypo-pigmentation, hair loss, eczema and lichenification and hypertrophy as shown in figure 4. Scars of the second degree burns which were classified as hyper and hypopigmentation, atrophy and hypertrophy were diagnosed in 70% of the patients. These complications were found on the genital areas (48%), back (48%), front thorax and abdomen (44%), lower extremities (44%), upper extremities (41%) and head and neck (15%). Severity grading was determined as 12 (30%) patients in grade 1, 10 (25%) in grade 2, 8 (20%) in grade 3, and 10 (25%) patients in grade 4.

Dermal Histopathological Findings

Light microscopy of the skin biopsy in eight patients revealed atrophic scar characterized by epidermal atrophy, hyperkeratosis, basal membrane hyperpigmentation, non-specific dermal fibrosis and mononuclear inflammatory infiltration within dermis. Electron microscopy also revealed increased melanocytes with increased number of melanosomes within epidermis and increased collagen fibers with some inflammatory cells within dermis.

Eye Examination

Only one patient did not complain of eye complications. The symptoms in patients were blurred vision (50%), itching (42.5%), burning sensation (37.5%), photophobia (30%), tearing (27.5%), reading difficulties (10%), red eye (10%), eye pain (2.5%) and foreign body sensation (2.5%). Visual acuity was above 9/10 in 28 patients, between 6 and 9 in eight patients and below 5 in only three patients. Schirmer test for dryness of the eyes revealed mild dryness (5-10 mm) in eight patients and severe dryness (<5mm) in four patients. Slit lamp examination of the conjunctiva was normal in eight patients. In the other patients, pinguecula (62.5%), ptyrigium (17.5%) and chronic conjunctivitis (17.5%) were diagnosed. Abnormal findings in the limbus were recorded as peri-limbal hyperpigmentation (17.5%), vascular tortuosity (15%) and limbal ischemia (12.5%). Slit lamp examination of the cornea was normal in 26 patients. In the other patients, subepithelial opacity (15%), corneal thinning (15%), severe opacity (10%), micro/macro pannus (7.5% each), corneal vascularization (7.5%) and corneal epithelial defect (5%) were recorded. Since the corneal abnormalities were more specific, they were shown in Figure 5. Intraoccular pressure and fundoscopy were normal in all patients. Severity grading was determined as 14 (35%) patients in grade 1, 15 (37.5%) in grade 2, 5 (12.5%) in grade 3, and 6 (15%) patients in grade 4.

Severity Grades of Complications in Different Target Organs and Their Correlations

Severity grades of respiratory, skin, and eye complications in 40 patients exposed to SM were summarized in Table 9. A significant positive correlation was observed between the severity of respiratory and eye complications ($r=0.322$, $p=0.043$) as shown in Figure 6. No correlation was found between the severity of skin complications and either the respiratory ($r=0.011$, $p=0.947$) or eye ($r=-0.068$, $p=0.679$) complications.

Biochemical Findings

Except in three patients who had high fasting blood sugar, and in five patients who had slightly elevated cholesterol and triglyceride levels, in all the other patients the result of biochemical analyses (total cholesterol, triglyceride, glucose, urea, creatinine, uric acid, ALT, and AST) were within the normal ranges. Urinalysis (U/A) results revealed microscopic hematuria in two patients and pyuria in three patients. No other abnormality was found.

Hematological Findings

Hematological parameters of 40 patients in comparison with 12 controls were summarized in Table 10. WBC, RBC and HCT were significantly higher in patients than in the control group. There were no significant differences between the two groups concerning Hb level and PLT counts.

Correlations between the hematological parameters and the severity of the organ complications

Correlation coefficients (r) and "p" values obtained from the correlation tests between the severity of complications in different target organs and the hematological parameters were shown in Table 11. While RBC and Hb had a significant linear positive correlation with the severity of respiratory complications (Figure 7), WBC, HCT and PLT counts did not show any significant correlations (Table 11). Correlation tests between the hematological parameters and the severity of skin and eye complications revealed a significant positive correlation between Hb level and severity of eye complications as shown in Figure 8. No other significant correlation was found (Table 11).

Serum Immunolobulins and Complements

Immunoglobulins and complements of 40 patients in comparison with 12 controls were summarized in Table 12. While serum IgA, IgE, and C4 did not show any significant difference between the two groups, IgM (p=0.000) and C3 (p=0.030) levels were significantly higher in patients than in the control group. Serum IgG was only noticeably higher (p=0.065) in patients than in the controls.

Correlation between serum immunoglobulins or complements and the severity gradings of organ complications

Correlation coefficients and "p" values obtained from the correlation test between the severity of complications in different target organs and serum immunoglobulins or complements were summarized in Table 13. A significant negative correlation was observed between C4 and the severity of skin complications as shown in Figure 9. Other serum immunoglobulins and complements had no significant correlations with the severity of complications in different target organs (Table 13).

Flow Cytometric Findings

Flow cytometric parameters of 40 patients in comparison with 35 controls were summarized in Table 14. The percentage of monocytes (p=0.013) and CD3+ lymphocytes (p=0.037) were significantly higher, and the percentage of CD16+56 positive (N.K) cells (p=0.006) was significantly lower in patients compared with the control group. Other flow cytometric parameters did not have any significant differences between the two groups (Table 14).

Correlation between the flow cytometric parameters and the severity gradings of organ complications

Correlation coefficients and "p" values obtained from correlation tests between the severity of complications in different target organs and flow cytometric parameters were summarized in Table 15. No significant correlation was found between flow cytometric parameters and the severity gradings of respiratory, skin, or eye complications.

DISCUSSION

Definitions of Delayed Toxic Effects and Late Complications

SM or mustard gas is distributed to the body following absorption. The term "delayed toxic effect" is defined as a lesion caused by an acute or sub-acute poisoning of a CWA such as SM, some months or years after initial exposure. The effect is essentially irreversible and may be residual injury or the unexpected onset of related symptoms or signs after a protracted period of months or years. It must be emphasized that a lesion of this kind results from a single dose or brief exposure and is not the same as one caused by chronic poisoning which requires further intake of the poison. Late complications of SM poisoning refer to all dysfunctions and abnormalities in different organs of the body that may occur years after initial exposure.

Target Organs

The first report of the delayed toxic effects of SM poisoning in 236 Iranian veterans (Balali, 1986) revealed the most common effects on the respiratory tracts (78%), CNS (45%), skin (41%) and eyes (36%). These effects were recorded between two and 28 months after exposure. The patients were all followed from the early phase of SM poisoning and thus included from mild to severe toxicity. The present report includes only severe cases of SM poisoning, and the investigations were undertaken 16 to 20 years after initial exposure. However, there were some similarities and some differences between the two studies. In a study by Khateri et al. (2002) on 34,000 Iranians exposed to SM, the most common complications were found on the lungs (42.5%), eyes (39.5%) and skin (24.5%). The difference can be due to the fact that their patients were mostly mildly SM-exposed veterans.

Respiratory Complications

The most commonly affected organ in this study was the respiratory tract as previously reported. However, more advanced respiratory complications such as COPD, bronchiectasis and interstitial fibrosis were found. Although this can be partly due to our patient selection that included only severe cases, there was clear evidence that respiratory conditions in the same patients were also deteriorating. For instance, a patient who had bronchiectasis confined to the lower lobes 12 years ago, now showed this complication in all lung lobes despite continuous medical treatment. This is most likely because of the immunodeficiency of the patients and higher incidence of respiratory infections.

It should also be noted that there is no sharp dividing line between different respiratory complications and a combination of several complications that were observed in some patients. This has lead to the use of various definitions such as chronic bronchitis, COPD, asthmatic bronchitis, and simple chronic bronchitis by different researchers. In this study we labeled cases as COPD only when diagnosis of bronchiectasis had been excluded. Simple chronic bronchitis was defined when patients had clinical history of chronic bronchitis but the criteria for neither of bronchiectasis or COPD was fulfilled.

Comparing PFT findings of this study with that of another study (Keshmiri et al., 1992) which was carried out mostly on these patients revealed invaluable information. They studied 43 CWA-intoxicated veterans in the province of Khorasan four to seven years after their initial exposure and reported obstructive, normal and restrictive spirometric pattern in

53%, 42% and 5% of the patients, respectively. This indicates that while obstructive pattern accounts for approximately 50% of all the subjects in both studies, the number of patients with a restrictive pattern has increased from 5% to 22.5% over this period. Apart from the15% of the patients who had a mixed pulmonary function pattern in our study, the number of patients with normal PFT results has also noticeably declined from 42% to 5% over the 12 year period. This indicates that acute effects do not necessarily remit on removal from exposure and in fact the complications increased over the years. Therefore there is probably a relationship between short-term and long-term respiratory complications.

In a study of 197 Iranian veterans with heavy exposure to SM (Emad and Rezaeian, 1997) the diversity of respiratory complications were reported in the order of chronic bronchitis (58.9%), pulmonary fibrosis (12.2%), asthma (10.6%), airway narrowing due to scaring or granulation tissue (9.6%) and bronchiectasis (8.6%). While bronchiectasis and asthma were less common than in our patients, pulmonary fibrosis was higher. This can be due to their application of carbon monoxide diffusion capacity for detection of pulmonary fibrosis.

Another limitation of this study was that we did not perform expiratory HRCT for all the patients. As a result, the frequency of bronchiolitis obliterans, which recent studies (Ghanei et al, 2004) have shown to be the most common late respiratory complication following SM exposure, was not determined in our patients.

Neuromuscular Disorders

One of the significant findings in this research was the high frequency of neuromuscular disturbances which have been underrepresented in the previous reports. There were two limitations, however, on evaluating the precise toxic effects of SM on the neuromuscular system. First, electromyography (EMG) is a procedure highly dependent on the patients' cooperation, and therefore the results can not be very reliable in these patients. Using single-fiber electromyography (SFEMG) instead of regular EMG can yield invaluable information, but of course requires a lot of time and patience from both the examiner and the subjects. We studied only one patient by this technique; the other patients did not agree to undergo due to its painful long procedure. There was no evidence of junctional neuromuscular disturbances in this patient. Secondly, although patients' intoxication with SM was certain due to initial confirmation of the diagnosis, exposures to the other chemical warfare agents, such as nerve agents, cannot be definitely excluded because of the lack of enough documentation and records in this regard.

Dermatological and Eye Complications

Out of the different skin complications found in our study, hyper- and hypopigmentation, eczema, hair loss and dry skin have already shown to have an association with SM exposure (Fekri and Janghorbani, 1992). Other abnormal findings such as erythematous popular rash and multiple cherry angiomas may be roughly estimated to have a higher frequency in our patients than in the normal population (Firooz et al, 1999). However, evaluation of a control group with the same sex and age is required before making this conclusion and also in order to find the exact relative risk for each complication.

Eye complications were less serious but were similar to those reported by Javadi and Kazemi, 2000.

Local versus Systemic Effects

Experimental studies have revealed that after cutaneous exposure to SM, a portion of the given dose passes rapidly from the skin into the bloodstream to elicit toxicity at distant sites. Under the most ideal circumstances, about 8 percent of a single dose will be absorbed through skin, and thus large doses of SM will yield systemic toxicity, including death (Cullumbine, 1947; Elsayed et al., 1992; Hambrook et al., 1993; Renshaw, 1947). However, it has not been adequately studied whether there is a relationship between local and systemic toxicities in the long run. According to our study, there was no correlation between severity of long-term local complications as a result of direct irritant effects on skin and mucosal membranes and the late systemic toxicities, such as the hematological and immunological disorders. Comparing the severity of local complications in the respiratory tract, skin, and eyes with each other, there was only a significant positive correlation between the severity of late complications of SM in the respiratory tract and in the eyes. This can be attributed to the common site of exposure (face) for both inhalation and eye injuries, and the fact that these two organs are protected simultaneously with a gas mask, whereas a protective suit is required to prevent skin exposure.

Hematological and Immunological Complications

Influence of SM on the immune system has been the subject of much research since 1919 (Heckton, 1921). Natural killer (NK) cells were reported to be impaired among the workers of SM factories in Japan (Yokogama, 1993). Other studies have indicated that this alkylating agent has short-term and long-term influence on antibody production both in animals and in humans (Gabrielsen and Good, 1967; Malaekeh et al., 1991; Sohrabpour, 1992; Zandieh, 1991). It also affects complement system factors of C3 and C4 (Hassan and Ebtekar, 2002).

Depression of cell-mediated immunity has been observed in Iranian veterans one, two, and three years after exposure (Zandieh et al., 1990). NK cells, which are known to be one of the most important components of the cellular immunity (Giorda et al., 1992), have been found to be significantly lower in patients with severe respiratory complications 10 years after exposure (Ghotbi and Hassan, 2002). Our results also indicated that the number of NK cells were significantly lower in patients compared with the control group. However, no correlation was found between NK cell decrement and the severity of clinical complications in any of the target organs. This can be due to the fact that patients selected for this study all belonged to the most severely affected group of the Veteran Foundation, even though we classified them again into four groups. Hence, to investigate the possible correlation between clinical and immunological complications, it is recommended that a complementary study on patients with milder complications be carried out and compared with the results of this study.

Studies have shown increased levels of IgG and IgM during the first few weeks and up to the sixth month post-exposure, especially in the severely affected group. Even eight years after exposure, the percentage of patients with increased IgM and IgG has been shown to be significantly higher than in controls (Hassan and Ebtekar, 2002). According to our study, the IgM level was still significantly higher 16-20 years after exposure and IgG was noticeably

(P=0.065) higher in patients compared with the control group. An increased IgM level, which was accompanied by a significant increase in leukocyte counts, can be a consequence of frequent, acute respiratory infections in these patients. This also indicates that marked hypogammaglobulinemia, leukopenia and loss of bone marrow reactivity, which were early dominant features in severe cases of mustard intoxication (Balali and Farhoodi, 1990; Eisenmenger et al., 1991, Malaekeh, 1991; Willems, 1989), become less important 16 to 20 years after initial exposure. On the other hand, decreased cellular immunity due to impairment of natural killer cells remains a major concern in late phases of intoxication and is probably the main cause of recurrent and opportunistic infections, septicemia, and higher incidence of malignancies in these patients (Hosseini et al., 1989; Keshmiri, 1989).

Carcinogenicity

It is clear that agents that change the genetic memory of cells (DNA) are prime candidates for causing cancer (Dabrowska et al., 1996). Recent work has specifically shown that SM alkylates the O-6 position of guanine in DNA, and this is primarily responsible for the mutagenic consequence of cellular exposure (Habraken and Ludlum, 1989). Previous studies have demonstrated that occupational exposure to SM is associated with respiratory cancer. (Easton et al., 1988; Kurozomi et al., 1977; Manning, 1981; Nishimoto et al., 1987; Watson, 1989). The battlefield experience, however, is more equivocal (Beebe, 1960; Dacre and Goldman, 1996; Kang and Bullman, 2000; Wada, 1968). Carcinoma of nasopharynx, bronchogenic carcinoma, adenocarcinoma of stomach, as well as acute myeloblastic and lymphoblastic leukemia has already been reported in Iranian veterans (Balali, 1992; Ghanei, 2002; Zakeripanah, 1991; Zojaji et al., 2004). Fortunately no malignancy in the respiratory tract or any other organs of the patients was found in our study. However, quantitative risk assessment cannot be developed from the data available in literature, and long-term follow-up and monitoring are required to discover the incidence of mutagenicity and carcinogenicity in these patients.

Teratogenicity and Reproductive Effects

Few studies are available regarding the reproductive effects of SM. Animal studies have shown that injection of SM in male rats results in damage to the testes, with inhibition of spermatogenesis. Nevertheless, the damage was usually transient with formation of mature sperm four weeks after exposure. Likewise, the fertility of female rats was not altered by administration of SM. No evidence of teratogenicity was found when female rats never exposed to mustard gas were mated with male rats that had been exposed to SM. Similarly, pregnant rats exposed to SM also did not display any fetal abnormalities (McNamara et al., 1975; Sasser et al., 1996).

Data addressing the reproductive effects of SM exposure in human models are lacking. While sperm count and motility have been previously reported to be significantly lower in 1428 SM-exposed patients compared with the control group (Balali, 1992), the infertility rate was just 6% which was even lower than the representative worldwide average of 10-15% (Santos, 1993). This conclusion was reinforced by the results obtained from a historical Cohort study which assessed fertility among 142 mustard-exposed residents of Sardasht (Ghanei, Rajaee et al., 2004). There were only two of our patients who complained of depressed growth of their children in comparison with their peers. However, evaluation of

teratogenic effects of SM poisoning requires a specific study on the children of all SM-exposed Iranian veterans.

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TABLES

Table 1. Severity Grading of Respiratory Complications in Patients with SM Poisoning according to the Veteran Foundation's Medical Committee

Diagnostic Category	Spirometric Findings*
No Specific Complication (Grade 1)	FVC>=80 or FEV1>=80
Mild Respiratory Complication (Grade 2)	80>FVC>=65 or 80>FEV1>=65
Moderate Respiratory Complication (Grade 3)	65>FVC>=50 or 65>FEV1>=50
Severe Respiratory Complication (Grade 4)	FVC<50 or FEV1<50

* Values represent percentage of predicted value

PFT: Pulmonary Function Test, FVC: Forced Vital Capacity, FEV1: Forced Expiratory Volume in the first second

Table 2. Severity Grading of Skin Complications in Patients with SM Poisoning according to the Veteran Foundation's Medical Committee

Diagnostic Category	Findings
No Specific Complication (Grade 1)	No objective signs ± symptoms
Mild Skin Complications (Grade 2)	(Hyper- & Hypo-pigmentation, atrophy, hypertrophy, poikiloderma, and dry skin) involving 1-18% of skin surface area
Moderate Skin Complications (Grade 3)	(Hyper- & Hypo-pigmentation, atrophy, hypertrophy, poikiloderma, and dry skin) involving 19-36% of skin surface area
Severe Skin Complications (Grade 4)	(Hyper- & Hypo-pigmentation, atrophy, hypertrophy, poikiloderma, and dry skin) involving more than 36% of skin surface area

Wallace's "rule of nines" was used to estimate percentage of burned skin surface area

Table 3. Severity Grading of Eye Complications in Patients with SM Poisoning according to the Veteran Foundation's Medical Committee

Diagnostic Category	Findings
No Specific Complication (Grade 1)	No objective signs ± Symptoms
Mild Eye Complications (Grade 2)	Injection ± Chemosis ± Subconjunctival hemorrhage ± Blepharitis ± Conjunctivitis
Moderate Eye Complications (Grade 3)	Grade 2 complications plus any of the following abnormalities: Involvement of the limbus: Vascular tortuosity, limbal ischemia, and perilimbal hyper-pigmentation; Mild Involvement of the cornea: micropannus and subepithelial opacity; Mild dry eye: Schirmer's test = 5-
Severe Eye Complications (Grade 4)	Grade 3 complications plus any of the following abnormalities: Severe involvement of the cornea: thinning, melting, epithelial defect, hyaline & lipid deposition, diffuse opacity, vascularization and macropannus; Severe dry eye: Schirmer's test < 5 m.m

Table 4. Our Severity Grading for Respiratory Complications in Patients with SM poisoning

Diagnostic Category	PFT Findings*	ABG Findings**	HRCT Findings
No Specific Complication (Grade 1)	FVC>=80 or FEV1>=80	PaO2>85	No evidence of bronchiectasis or interstitial fibrosis
Mild Respiratory Complication (Grade 2)	80>FVC>=65 or 80>FEV1>=65	60<PaO2<85	Bronchiectatic lesions or interstitial fibrosis involving one lung lobe
Moderate Respiratory Complication (Grade 3)	65>FVC>=50 or 65>FEV1>=50	45<PaO2<60	Bronchiectatic lesions or interstitial fibrosis involving two lung lobes
Severe Respiratory Complication (Grade 4)	50>FVC or 50>FEV1	PaO2<45	Bronchiectatic lesions or interstitial fibrosis involving three or more lung lobes

PFT: Pulmonary Function Test, FVC: Forced Vital Capacity, FEV1: Forced Expiratory Volume in the first second, ABG: Arterial Blood Gas, HRCT: High Resolution Computed Tomography

* Values represent percentage of predicted value

** Values represent mmHg

Note: Overall grading of respiratory complications was judged on the diagnostic criteria which had the highest (most severe) grade in each patient

Table 5 -Arterial Blood Gas Parameters in 40 Iranian Veterans with Late Complications of SM Poisoning

	PH	PaCO2 (mmHg)	HCO3 (mmol/l)	PaO2 (mmHg)	O2Sat (%)
Mean ± SD	7.39 ± 0.04	40.5 ± 3.85	24.4 ± 3.03	66.7 ± 9.69	91.1 ± 3.53
Normal Reference Ranges	7.35-7.45	35-45	22-26	85-100	95-98

Table 6. Pulmonary Function Test Results* in 40 Iranian Veterans with Late Complications of Sulfur Mustard Poisoning

	Before Bronchodilator (%Predict)				**After Bronchodilator (%Predict)**		
Parameter	SVC	FVC	FEV1	FEV1%	FVC	FEV1	FEV1%
Mean	60.9	55.2	45.2	73.6	59.2	47.5	76.9
SD	15.3	17.2	13.3	19.4	16.4	20.5	20.7

SVC: slow vital capacity
FVC: Forced vital capacity
FEV1: Forced vital capacity in the first second
FEV1%: the ratio FEV1/FVC
* Numbers are expressed as percentage of predicted value for the same sex, age, and height

Table 7- Nerve Conduction Velocity Disturbances of the Motor Nerves in 40 Iranian Veterans with late complications of sulfur mustard poisoning

	Median		Ulnar		Tibial		Proneal	
	R	L	R	L	R	L	R	L
Number of Partial Lesions	0	0	0	0	14	14	7	3
Number of Complete Lesions	0	0	0	0	0	1	1	2
Total Number of Lesions	0	0	0	0	14	15	8	5
Total Percentage of Lesions	0%	0%	0%	0%	35%	37.5%	20%	12.5%

Table 8- NCV Disturbances of the Sensory Nerves in 40 Iranian Veterans with Late Complications of Sulfur Mustard Poisoning

	Median		Ulnar		Tibial		Sural	
	R	L	R	L	R	L	R	L
Number of Partial Lesions	11	10	3	3	3	3	4	3
Number of Complete Lesions	4	1	2	4	25	27	25	23
Total Number of Lesions	15	11	5	7	28	30	29	26
Percentage of Total Lesions	37.5%	27.5%	12.5%	17.5%	70%	75%	72.5%	65%

Table 9. Severity Grades of the Target Organ Complications in 40 Iranian Veterans with SM Poisoning

	Number of patients with			
	No Specific Complications (Grade 1)	**Mild Complications (Grade 2)**	**Modearate complications (Grade 3)**	**Severe Complications (Grade 4)**
Respiratory Tract	2	11	14	13
Skin	12	10	8	10
Eye	14	15	5	6

Table 10. Haematological Parameters in 40 Iranian Veterans with Late Complications of Sulfur Mustard Poisoning

	Patients	**Control Group**	**P.Value**
WBC (1000/mm3)	7.24±1.90	5.79±1.16	**0.025***
RBC (million/ml)	5.46±0.45	5.19±0.28	**0.029***
HCT (%)	48.3±3.5	45.5±1.9	**0.042***
Hb (mg/dl)	15.9±0.7	15.6±0.7	0.223
PLT (1000/ml)	255±99	238±101	0.594

* Significant

Table 11. Correlation Coefficients (r) and p.values Obtained from the Correlation Tests between the Hematological Parameters and the Severity of Target Organ Complications

	WBC		RBC		Hb		HCT		Plt	
	r	p	r	p	r	p	r	p	r	p
Respiratory Complications	0.046	0.786	0.327	**0.045***	0.357	**0.028***	-0.008	0.962	-0.049	0.768
Skin Complications	-0.015	0.926	-0.049	0.763	0.094	0.563	0.081	0.617	0.009	0.956
Eye Complications	0.171	0.290	0.192	0.234	0.341	**0.031***	0.285	0.075	-0.192	0.236

* Significant

Table 12. Immunological Parameters in 40 Iranian Veterans with Late Complications of Sulfur Mustard Poisoning

	Patients	Control Group	P.Value
C3 (mg/dl)	109/8±30.1	90.9±14.8	**0.03***
C4 (mg/dl)	31.1±11.6	35.5±15.4	0.542
IgA (mg/dl)	302.6±142.1	233.1±59.3	0.154
IgG (mg/dl)	1438.6±486.1	1140.0±244.2	0.065
IgM (mg/dl)	235.3±84.4	136.8±58.3	**0.000****
IgE (IU)	92.4±112.1	86.5 ± 146.3	0.161

* Significant
** Highly Significant

Table 13. Correlation Coefficients (r) and p.values Obtained from the Correlation Tests between the Immunoglobulins or Complements and the Severity of Target Organ Complications

	C3		c4		IgA		IgG		IgM		IgE	
	r	p	r	p	r	p	r	p	r	p	r	p
Respiratory Complications	0.116	0.489	0.174	0.295	0.158	0.343	-0.048	0.776	0.073	0.661	-0.055	0.744
Skin Complications	-0.225	0.162	-0.440	**0.005***	0.075	0.646	-0.093	0.570	-0.051	0.755	-0.036	0.826
Eye Complications	0.011	0.945	-0.149	0.360	0.003	0.985	-0.156	0.337	0.154	0.342	-0.141	0.387

* Significant

Table 14. Flow Cytometric Parameters in 40 Iranian Veterans with Late Complications of Sulfur Mustard Poisoning

	Patients	Control Group	P.Value
%Lymph	31.5±8.4	30.5±8.0	0.651
%Mono	4.8±1.6	3.9±1.1	**0.013***
%Poly	63.8±8.7	65.4±8.7	0.372
%CD3	71.1±8.6	56.6±10.7	**0.037***
%CD4	57.7±8.3	57.8±8.1	0.983
%CD8	37.1±8.3	34.1±7.8	0.099
%CD19	11.9±5.9	13.6±6.2	0.187
%CD16+56	11.6±5.8	17.5±9.6	**0.006***

* Significant

Table 15. Correlation Coefficients (r) and p.values Obtained from the Correlation Tests between the Flow Cytometric Parameters and the Severity of Target Organ Complications

	%Lymph		%Mono		%Poly		%CD3		%CD4		%CD8		%CD19		%CD16+56	
	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p
Respiratory Complications	-0.089	0.596	-0.036	0.828	0.078	0.642	-0.049	0.768	-0.093	0.590	-0.055	0.743	-0.209	0.214	0.230	0.164
Skin Complications	0.135	0.406	0.164	0.312	-0.172	0.289	0.082	0.615	0.266	0.097	-0.181	0.263	0.081	0.621	-0.173	0.285
Eye Complications	-0.061	0.708	0.095	0.561	0.075	0.647	-0.024	0.885	-0.006	0.973	-0.024	0.883	0.043	0.794	-0.015	0.929

FIGURES

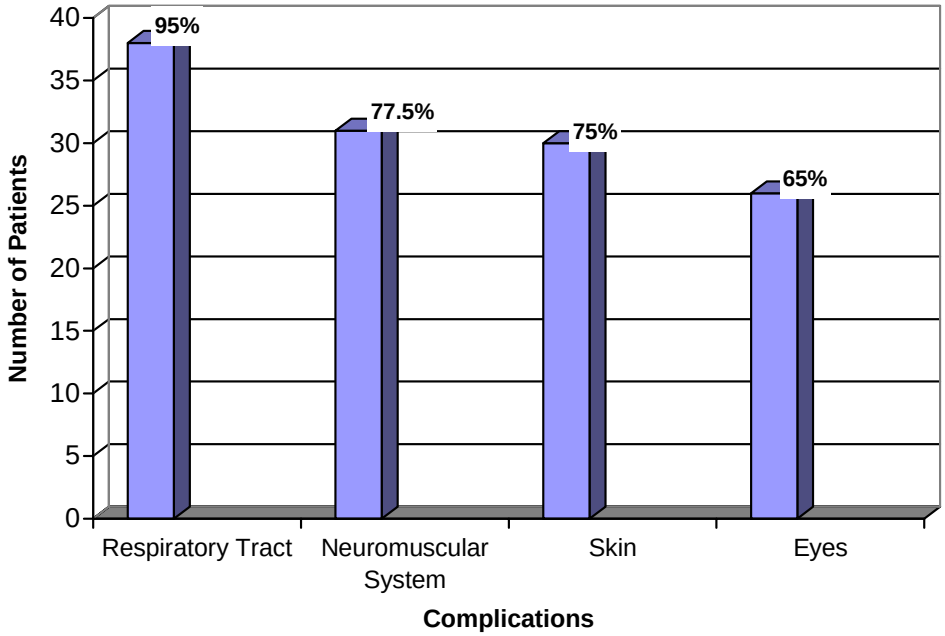

Figure 1. Frequency of Common Complications of Sulfur Mustard Poisoning in Different Organs of 40 Iranian Veterans

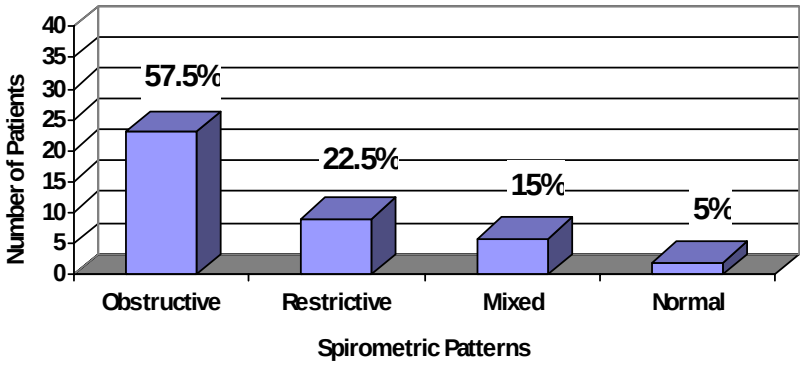

Figure 2. Pulmonary Function Test Results in 40 Iranian Veterans with Late Complications of Sulfur Mustard Poisoning

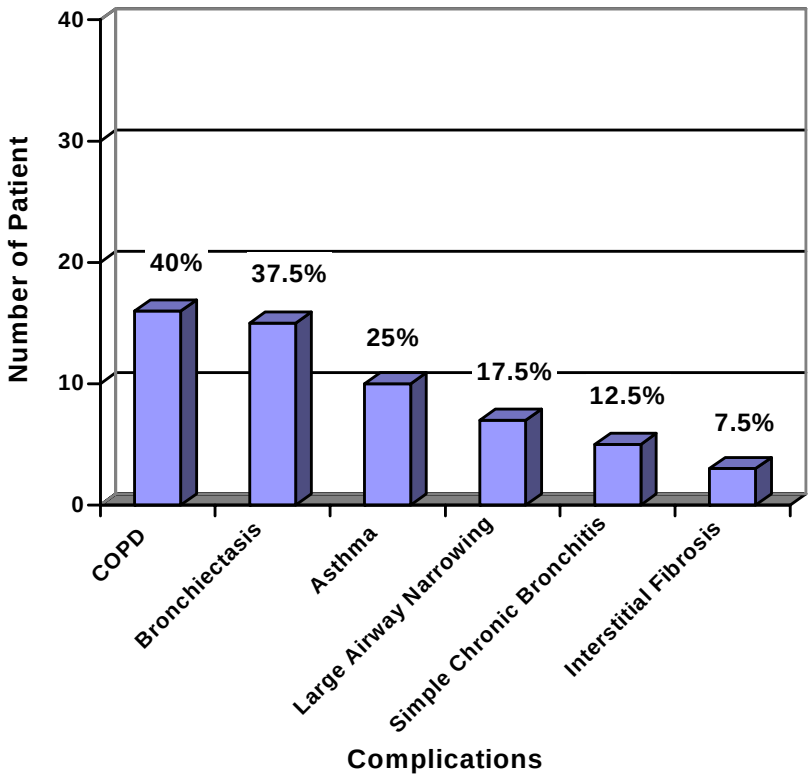

Figure 3. Respiratory Complications in 40 Iranian Veterans with Sulfur Mustard Poisoning

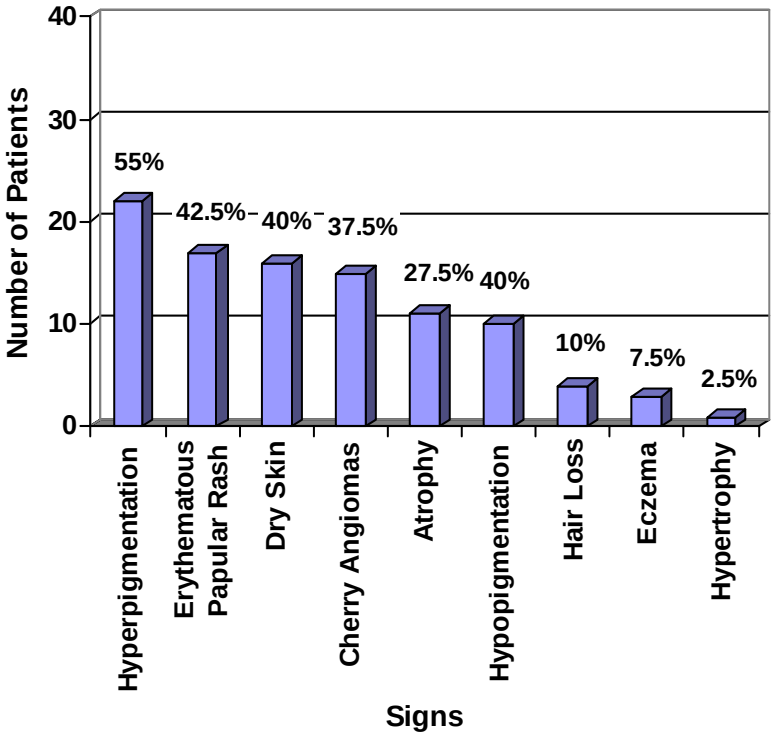

Figure 4. Skin Complications in 40 Iranian Veterans with Sulfur Mustard Poisoning

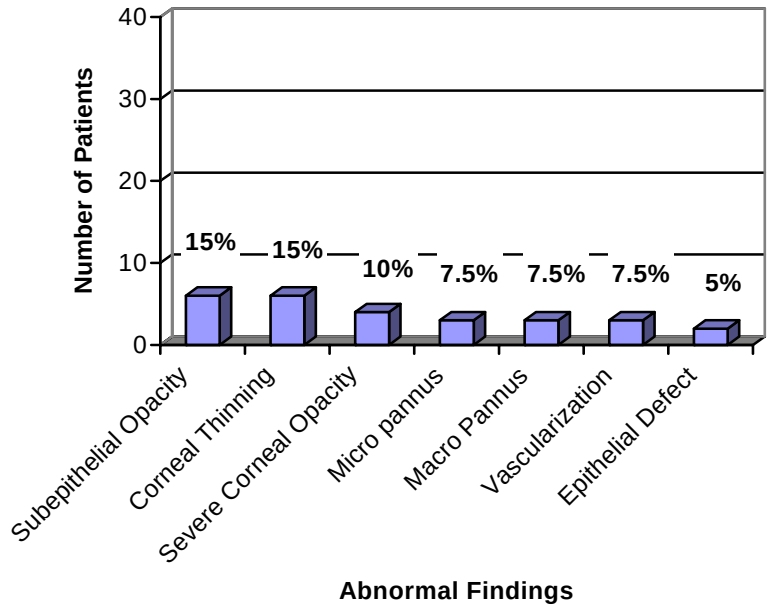

Figure 5. Corneal Abnormal Findings in 40 Iranian Veterans with Late Complications of Sulfur Mustard Poisoning

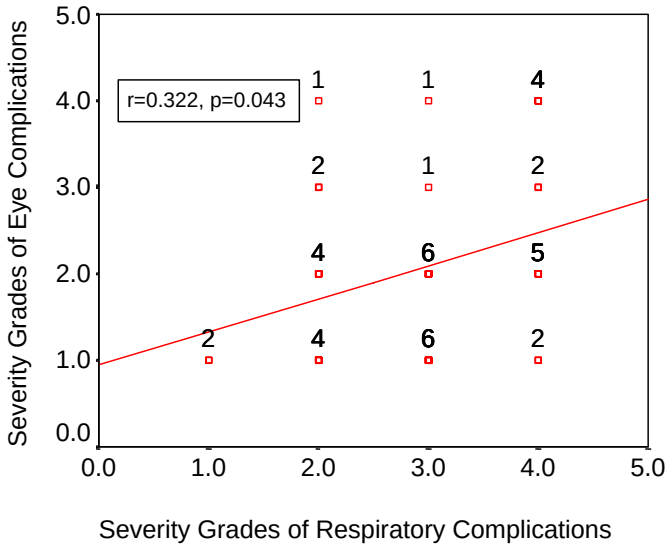

Figure 6. Correlation between Severity of Respiratory and Eye Complications in 40 Iranian Veterans with SM Poisoning

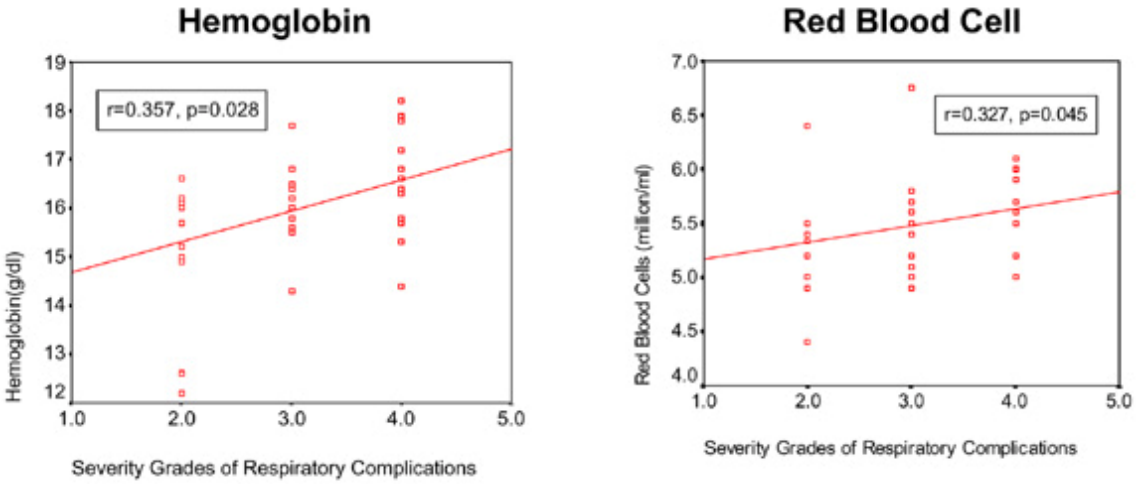

Figure 7. Significant Positive Correlations between the Hematological Parameters and the Severity of Respiratory Complications in 40 Iranian Veterans with SM Poisoning

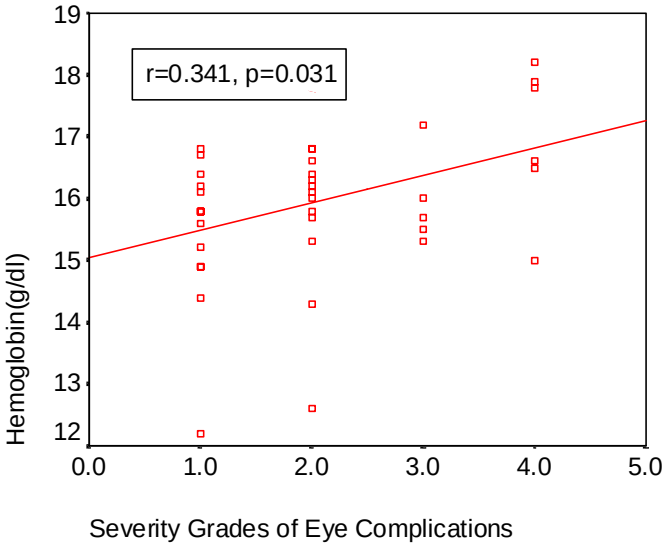

Figure 8. Correlation between the Hemoglobin Level and the Severity of Eye Complications in 40 Iranian Veterans with SM Poisoning

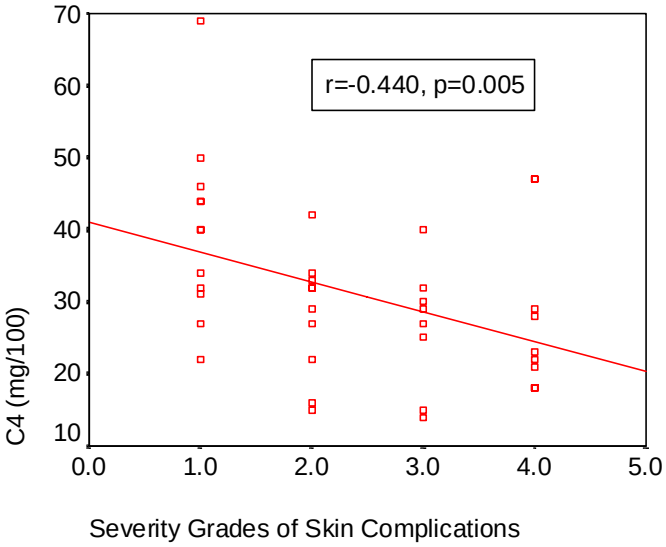

Figure 9. Correlation between C4 Level and the Severity of Skin Complications in 4o Iranian Veterans with SM Poisoning

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