Original Article

Sardasht-Iran Cohort Study of Chemical Warfare Victims: Design and Methods

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Background: **Insights into long-term clinical consequences of sulfur mustard have emerged from some investigations but less is known about the basic and molecular mechanisms of these complications. Sardasht-Iran Cohort Study is a comprehensive historical cohort study on Sardasht chemical victims' population which was designed to find out the long-term complications of sulfur mustard exposure and the basic mechanisms underlying clinical manifestations. This paper describes the design and methodology of Sardasht-Iran Cohort Study.**

Methods: **In Sardasht-Iran Cohort Study, 500 individuals including 372 subjects from Sardasht, as the exposed group, and 128 subjects from Rabat, as the unexposed age-matched control group were evaluated. The exposed group was divided into two groups based on the severity of clinical complications at the time of exposure. Different samples including blood, sputum, saliva, tear, urine, and semen were collected for immunologic, hematologic, biochemical, and other laboratory analysis. Data were gathered from medical records, clinical examinations, laboratory tests, and questionnaires for psychological and lifestyle situations.**

Conclusion: **The important distinctions setting this study apart from the previous ones are discussed. The Sardasht-Iran Cohort Study provides important information on various aspects of long- term consequences of sulfur mustard exposure.**

This database will provide a better position to suggest guidelines for the diagnosis, treatment, and prevention of delayed complications in the patients exposed to sulfur mustard.

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Keywords: Clinical complications • immune responses • inflammation • sulfur mustard • toxicology

Introduction

Sulfur mustard (SM) [bis-(2-chloroethyl) sulfide] is a powerful irritant, blistering, and alkylating agent that reacts readily with most biologic molecules and affects several organs immediately after exposure.[1–6] Once released into the air, SM can travel long distances by wind and contaminate people over a wide area.[7,8] The respiratory and gastrointestinal tracts, eyes, skin, bone marrow, and the central nervous system

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are the most important target organs affected by SM. The acute tissue injuries of these organs depend on the severity and duration of the exposure and mortality is usually low.[2–4,9] In the long term, the most common medical problems include lesions in the respiratory tract, eyes, and skin.[9–21] There is little information and very few studies aimed at identifying the cellular and molecular mechanisms involved in these complications in humans. Clinical evidence indicates the possible involvement of immune and inflammatory reactions in long-term SM complications.[13,17,18,22–26]

SM was extensively used by the Iraqi forces during the Iraq-Iran war (1980 – 1988).[9–31] To date, more than 100,000 Iranians, both military veterans and civilians, have suffered from the long-term effects of SM exposure.[9–10] Sardasht inhabitants have been victims of SM contamination.[11,12,27] Sardasht is a town in the north-west of Iran close to the Iraqi border. It is located geographically at the latitude of 36º 9′ 4.48″ and longitude of 45º 29′ 10.46″. On June 28, 1987, Iraqi air forces attacked Sardasht using seven 250-kg bombs of SM, four of which exploded in the densely-populated residential areas in the central part of the town.[9,11,27–31] Based on the official statistics, the bombardment caused SM exposure in 8025 of the 12,000 residents of the town at that time.[9,11,27–31] From the exposed population, at least 4500 people needed treatment; 3000 were exposed to mild doses of SM and were treated for acute effects on an outpatient basis, while the other 1500 were exposed to higher doses of SM, and developed moderate to severe acute medical complications requiring hospitalization at the time.[27] Most of these treated people, and also some who did not develop acute symptoms at the time of exposure, have clinical problems attributed to the exposure at the present time.

Sardasht-Iran Cohort Study (SICS) is a comprehensive historical cohort study on the basic mechanisms underlying clinical complications caused by SM in Sardasht residents 20 years after exposure. Initiated in June 2006 and still ongoing, this study evaluates the health status, long-term clinical complications, molecular patterns of systemic and local immune responses, inflammation, hematologic and biochemical parameters along with the lifestyle, and psychological situation of SM-exposed chemical victims compared with the unexposed (control) group. This paper describes the design and methodology used in the study.

Patients and Methods

Objectives

The aims of this study were to study:

- The health status of SM-exposed people;
- The long-term clinical complications of SM exposure;
- The cellular and molecular patterns, particularly immunologic and inflammatory parameters related to SM long-term complications;
- The comparison and association between the early and late complications;
- The association between clinical complications, lifestyle, psychological situations, and cellular and molecular parameters;
- The impact ratio of each factor in long-term complications; and
- The potential biomarkers and noninvasive surrogate markers of SM-related clinical complications.

Study organization

The study was carried out with the collaboration of more than 100 investigators from 10 leading medical universities and research centers in Iran. The study investigators were organized into four coordinating groups including: 1) clinicians, 2) basic scientists and their affiliated laboratories, 3) biostatisticians and data analysists, and 4) support and executive personnel. Details of the centers, principal investigators, other executive, research and clinical personnel, and their affiliations are given in Appendix 1.

Study design

Sample size

Among the variables of the present research, cytokines and immunoglobulins have more variations. Since there was no evidence on serum cytokines of chemical victims, the sample size was calculated with immunoglobulin G (IgG) levels. According to Mahmoudi et al.,[26] the mean±SD of IgG in chemically -exposed veterans (n=40) and control group (n=35) was 1438.6±486.1 and 1140.0±244.2 (mg/mL), respectively. Thus, the 95% confidence interval (CI) for IgG difference ($\mu_2 - \mu_1$) is 127.6 to 469.6. In addition, a 95% CI for variance proportion (σ_1^2 / σ_2^2) is 2.1 to 7.4.

In the study by Mahmoudi et al.,[26] the variance

observed in the chemically-exposed group was more than that in the control group; so, the present study was designed to be unbalanced and thus the exposed group had more participants than the control group.

For a type I error (α) equal to 0.05, power of analysis of 0.95, type II error (β) of 0.05 and a variance proportion ($k = \sigma_1^2 / \sigma_2^2$) of 3, the lower band of *the CI* was chosen as the difference between two groups ($\Delta = \mu_2 - \mu_1$), i.e., 127.6. Then, two groups sample sizes, accordingly F_1 and F_2, were 351 and 117 in case and control groups.[32]

$$n_1 = (s_1^2 / k + s_2^2) \times (\varphi(1 - \frac{\alpha}{2}) + \varphi(1 - \beta))^2 / \Delta^2 \quad (F_1)$$

$$n_2 = (s_1^2 + k s_2^2) \times (\varphi(1 - \frac{\alpha}{2}) + \varphi(1 - \beta))^2 / \Delta^2 \quad (F_2)$$

Participants

The participants in this study were recruited from Sardasht (exposed) and Rabat (unexposed) residents. The exposed group was selected from the male individuals of Sardasht exposed to SM 20 years ago based on the documents in the medical records verified by the Medical Committee of the Foundation of Martyr and Veterans Affair. In the medical records, each victim has a medical file with a numeric code. The participants were selected by systematic random sampling method[33] from the chemical victims' list; since the ratio of sample size to records was 0.1, each sample was selected from every 10 records. The unexposed control group was enrolled from Rabat; these participants represented the exposed group from all aspects except exposure to SM. Rabat is a town located 15 kilometers away from Sardasht. Both towns have similar geographical situations and weather conditions. They have Kurdish populations with the same religion, culture, language, and nutritional habits. The control sample included males selected by systematic random sampling[33] from the list of latest demographic households' statistics at the local health center. They were matched with the exposed group by age considering the inclusion and exclusion criteria.

The inclusion and exclusion criteria for the study are summarized in Table 1. The age range covered by the study was 20 – 60 years. The minimum age of 20 years was chosen because this age includes individuals who were newborns at the time of exposure. The elderly persons over 60 also were excluded to avoid unintended results due to the decline of immune responses of elderly people. In order to avoid any drug interference, exposed participants taking systemic immunosuppressive drugs were excluded from the study.

After a few field trips to Sardasht and Rabat, the research team made a complete list of addresses and phone numbers of selected individuals. The subjects were then contacted by phone, and invited for a brief medical screening by clinicians to apply inclusion and exclusion criteria.

Having studied the medical records, chemical victims were categorized in two major subgroups based on the severity of the clinical problems at the time of exposure: 1) hospitalized: victims who had moderate to severe problems at the time of exposure and were hospitalized in major cities in Iran or sent aboard for treatment, and 2) non-hospitalized: patients who had subclinical or mild problems at the time of exposure and were treated for acute effects on an outpatient basis.

Based upon the severity of early clinical manifestations documented following SM exposure and the duration of hospitalization recorded in the medical documents, the hospitalized group (Group 1) could be further categorized into subgroups. In both exposed and control groups, those with certain clinical complications at the time of study, were divided

Table 1. Inclusion and exclusion criteria.

Inclusion
SM exposure in June 1987 based on medical records (for exposed group)
Age: 20 – 60 years
Informed consent
No systemic immunosuppressive medication
Exclusion
Age<20 and >60 years
Current treatment with systemic immunosuppressive drugs
History of systemic disease before exposure based on medical records
Suffering from an acute infectious disease at the time of sampling
Disinclination to continue participation

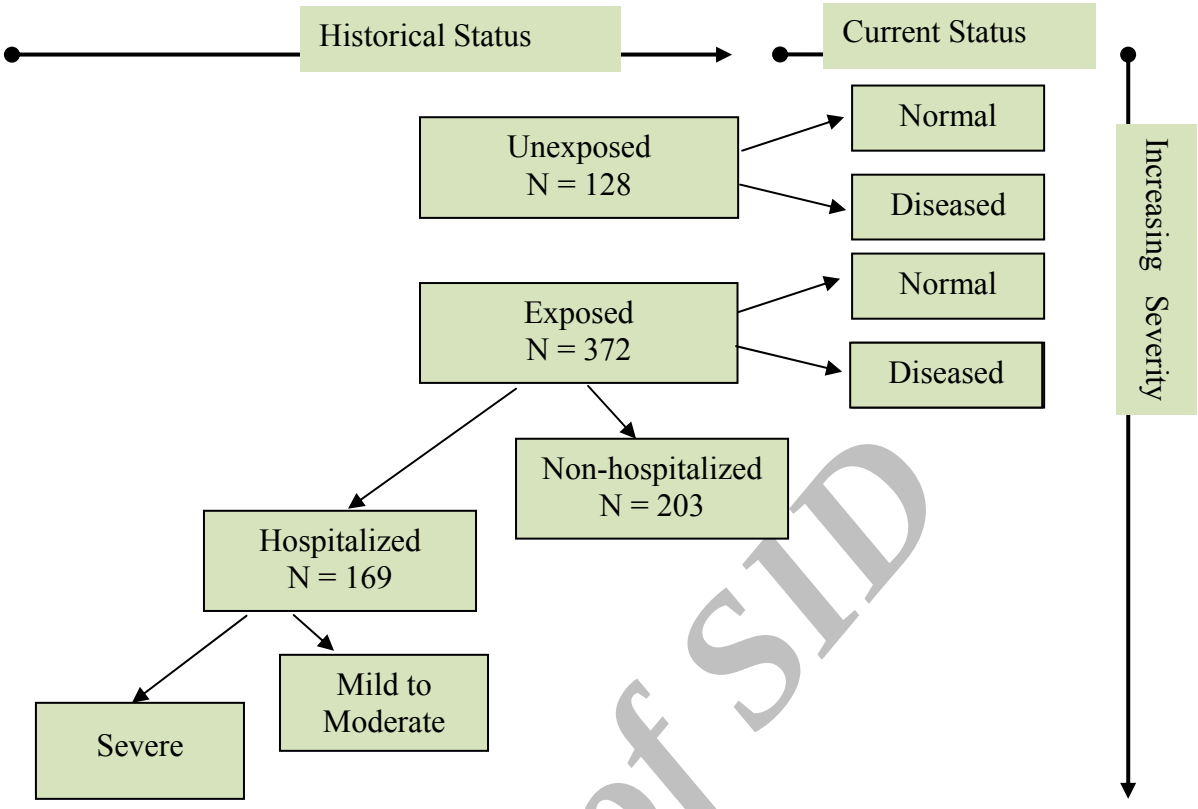

Figure 1. Flowchart illustrating study group classification.

into different categories based on results of clinical assessments (Figure 1).

All tests were carried out free of charge and participants were at liberty to leave the project any time they wish. For the subjects with acute clinical signs, proper clinical modalities were undertaken as necessary.

Data collection

The outcome variables collected from four sources included:

Data in medical records

Medical records of the exposed individuals in the Foundation of Martyr and Veterans Affairs of Sardasht were studied by physicians. Important data such as the clinical manifestations at the time of exposure, date and duration of hospitalization, distance from the nearest site of chemical bomb explosion, all past and current medications, and other relevant information were collected.

Questionnaire variables

All the participants were evaluated regarding their socio-demographic information, medical and family history, medications, anthropometric measurements, resting blood pressure, heart rate, and any sign or symptom of acute infectious diseases. Standard questionnaires related to psychosocial factors, lifestyle (physical activity, stress management, and smoking habits), sleep quality, and healthcare services were administered by psychiatrists, clinical psychologists, health educators, and nurses. Name and purpose of questionnaires and some of their references are given in Table 2.[34–51] Other details of questionnaire design and validation will be reported in next papers.

Clinical evaluation

All participants were visited by clinicians and their respiratory functions were measured by spirometry under the supervision of a trained nurse. Respiratory signs and symptoms were evaluated by the experienced consultants of the research team. Special ophthalmic variables related to ocular problems including the patients' complaints were evaluated using slit-lamp biomicroscopy, and direct and indirect ophthalmoscoy; and then reports were filed by an ophthalmologist.

Using a predefined protocol, the patients' skin complications were documented by dermatologists. The severity of complications in

Table 2. Name and aims of the lifestyle and psychological questionnaires.

Name		Aim	Assessor or supervisor	Reference
DASS	Depression, Anxiety Stress Scale	To assess Depression, Anxiety and Stress	Clinical psychologist	34–38
SCL-90	Symptom Checklist 90	Assessment of psychiatric symptoms	Psychiatrist	39–41
PSQI	Pittsburg Sleep Quality Index	To assess sleep quality	Nurse	42–45
DSM-IV	Diagnostic and Statistical Manual of Post-traumatic Stress Disorders, 4th edition	To assess post-traumatic stress disorder	Psychiatrist	46
CS-R	Cope Scale Revised	To assess stress coping strategies	Clinical psychologist	47,48
GPAQ	Global Physical Activity Questionnaire	To assess physical activity	Health educator	49–52

respiratory tract, skin and eyes was graded as normal, mild, moderate, or severe based on the criteria verified by the Medical Committee of the Foundation of Martyr and Veterans Affair.[4,9] Clinical examination findings are analyzed for both primary and second-dary diagnoses among general ICD-9 medical condition categories.[52] In case of any physical or laboratory evidence of disease, such as cardiovascular or rheumatologic diseases, the specific diagnostic tests were carried out.

Laboratory procedures and specimen collection

Serum, urine, tear, saliva, and sputum samples were collected in aliquots and kept frozen at -70°C for the detection of cytokines, inflammatory and oxidative stress mediators, hematologic and biochemical factors, hormones, and other laboratory analyses. Semen samples were also collected for semen analysis. Phenotypic characterization of immune cells was done by flowcytometry on ethylene diamine tetraacetic acid (EDTA)-treated blood. Functional characterization of cytokine patterns was performed on heparinized venous blood. Blood cell culturing with and without mitogen, CBC, and total urine tests were carried out immediately after sampling. DNA and RNA extractions were carried out and saved. A DNA bank card was also issued for future genetic studies. Skin biopsy was performed for 50 patients who had moderate to severe dermal lesions. Some of the major laboratory variables are shown in Table 3.

Data entry

Data were entered in database sheets designed for this purpose. All data were then rechecked. Missing data were replaced with *hot deck* single imputation method. In this method, a given subject's missing record was imputed by simply selecting from sample members with matching other records.[53]

Ethical approval

The research protocol of the study was approved by the Board of Research Ethics in the Janbazan Medical and Engineering Research Center (JMERC) and Shahed University. An informed written consent was obtained from all the subjects involved in the study.

Data analysis strategy

To achieve the study goals, data were considered from both clinical and statistical viewpoints. To show the relationship between the basic and clinical findings, association analysis (parametric and nonparametric correlation coefficient) were used. To compare basic and clinical variables in several groups from the retrospective or current status, nonparametric comparison tests such as Kruskal-wallis and Mann-Whitney were used.

One of the important goals of this study was to determine the variables predicting a group of objects and to distinguish between case and control groups. To achieve this purpose, data mining methods, processes by which one discovers the unknown information from large data sets, were used. These methods include association's analysis, clustering, factor analysis, classification tree, and special regression.

To extract important immunologic factors in the exposed group, factor analysis was used for data reduction, and then new factors were applied instead of variables in logistic regression.

In addition, a classification tree was employed to predict the membership of objects in the classes of a response variable (exposed and unexposed to SM) from their measurement on several predictor

Table 3. Laboratory variable lists.

Hematology, biochemistry, and hormones	Cytokines, chemokines, and inflammatory markers	Immunoglobulins and autoantibodies	Cell counts and CD markers
RBC	IL-1 α	IgM	WBC
HGB	IL-1 β	IgE	Polymorph %
HCT	IL-1Ra	IgA	Lymphocyte %
PLT	IL-2	IgG	Monocyte %
RDW	IL-4	IgG1	Eosinphil %
MCH	IL-6	IgG2	Basophil %
MCV	IL-8	IgG3	CD45+CD3+ cells
MCHC	IL-10	IgG4	CD3+CD4+ cells
MPV	IL-12		CD3+CD8+ cells
FBS	IL-17	Anti-DNA	CD45+CD19+ cells
BUN	IL-18	Antinuclear Ab	CD16/56+ cells
Creatinine	IL-18 BP		CD4+/CD25+ cells
Uric acid	IFNg		CD3+/HLA-DR+ cells
Cholesterol	TGF- β		CD45+/CD14+ cells
Triglyseride	TNF- α		CD4+/CD8+ ratio
Calcium	CX3L1		
Phosphorus	Fas-L		
Bilirubin total	MMP9		
Bilirubin direct	TIMP1		
ALT (SGOT)	TIMP2		
AST (SGPT)	TIMP2		
ALP	GM-CSF		
LDH	RANTEST		
Peripheral blood smear	CCL 2/MCP - 1		
FSH	sICAM - 1		
LH	sE-Selectin		
Prolactin	sL-Selectin		
Testosterone	sP-Selectin		
T3	CRP		
T4	RF		
TSH	Substance P		
PTH	LAG-3		
FT3,FT4	Nitric oxide		
Thyroglobulin	C3, C4 (complement)		
Semen analysis			

variables.

To make new clusters from immunologic variables, cluster analysis which is a data mining approach that partitions a large set of data objects into homogeneous groups was applied.

For the other goals of the current study, a risk scoring system was employed to determine the optimal cut-off point for detection groups (exposed and unexposed to SM) of new cases and also the receiver-operating characteristic curve (ROC curve) was calculated by plotting the sensitivity of the test versus the false-positive rate (1-specificity). The cut-off point was selected to maximize the true-positive rate while minimizing the false-positive rate. In this method, the risk equation was determined by the stepwise multiple logistic regression analysis.

Sample characteristics

There were no significant differences in terms of age, body mass index, marital status, and smoking habits between the control and exposed groups. Total sample size of the study, the sample size of each study group, their age distributions, and demographic information of the population under study are shown in Table 4.

Discussion

Various studies have been performed on short- and long-term clinical manifestations of chemical victims,[3,4,9,11,12,15,27,15–21] but there are few studies discussing the basic mechanisms of long-term clinical manifestations of SM exposure.[22–26] Our knowledge regarding cellular and molecular mechanisms involved in these disorders is still incomplete. Long-term complications of SM can come from the different routes following exposure: paraclinical changes up to clinical and psychological complication aspects. A historical cohort study was designed to evaluate the clinical outcomes along with the basic mechanisms of the

Table 4. Demographic information of the study population.

Variables		Control	Exposed		*P* value
			Nonhospitalized	Hospitalized	
Sample size		128	203	169	
Age (mean ± SD)		41.7±9.8	43.4±10.8	44.4±10.7	0.103
Age group (year)	20 to 29	15(11.7%)*	24(11.8%)*	14(8.3%)*	0.422
	30 to 39	43(33.6%)	52(25.6%)	42(24.9%)	
	40 to 49	37(28.9%)	60(29.6%)	56(33.1%)	
	50 to 60	33(25.8%)	67(33.0%)	57(33.7%)	
Body mass index (Kg/m^2)	≤25	52(40.6%)	78(38.4%)	64(37.9%)	0.954
	25 – 30	54(42.2%)	94(46.3%)	78(46.1%)	
	≥30	22(17.2%)	31(15.3%)	27(16.0%)	
Marital status	Not married	10 (7.8%)	17 (8.4%)	12 (7.1%)	0.901
	Married	118 (92.2%)	186 (91.6%)	157 (92.9%)	
Smoking	Yes/Quitted	29 (22.7%)	49 (24.1%)	39 (23.1%)	0.946
	Never	99 (77.3%)	154 (75.9%)	130 (76.9%)	

*= Data presented as count (percentage).

outcomes along with the basic mechanisms of the long-term clinical complications and psychological and lifestyle situations. Since clinical evidence indicates the probable involvement of immunologic and inflammatory mechanisms,[22–26] molecular patterns of systemic and local immune and inflammatory responses were evaluated in this study. Psychological parameters, lifestyle, and socioeconomic situations were evaluated through questionnaires. In this way, the impact ratio of each risk factor was determined. This paper describes the design and methodology used in the study. Several important distinctions, setting this study apart from previous ones are:

- This was the first study that evaluated numerous factors including different clinical consequences, various basic and molecular mechanisms, lifestyle parameters, and psychological factors from a comprehensive viewpoint.
- In previous studies, the samples were selected based on their current problems. But in this study, the samples were selected based on the severity of signs and symptoms at the exposure time and followed-up for their current problems.
- The effect of many other confounding variables were reduced since chemical victims of this study came from the same geographical region, lived in similar socioeconomic conditions, and had similar demographic features.
- The exposed group in this study was exposed at the same time by the identical composition of SM whereas previous studies have been done on veterans exposed by different chemical warfare compositions at various times.
- Another characteristic of this study was the availability of the recorded data, extracted from the patient's medical records regarding clinical status at the time of exposure. This database provided good baseline information for our historical cohort study.
- The participation of a good matched control group was another distinction of this study. The control group participants in this study came from Rabat, a town close to Sardasht and the only difference between them was SM exposure. The population of both towns has similar ethnic, cultural, and geographical situations. They also have similar levels of other stressors, i.e., both towns were exposed to high intensity conventional warfare throughout the Iraq-Iran war, but only Sardasht was exposed to chemical weapons.
- One of the important aims of this study was to find out the potential biomarkers that are very important to the evaluation of clinical trials. The assessment of numerous types of biomarkers in this study makes it possible to achieve a surrogate marker for clinical endpoints.

The above-mentioned criteria of this research make it a unique and unprecedented study that covers the long-term complications of SM exposure, the comparison of the early and late complications, the health status of the people by

focusing on the molecular and immunologic parameters, and potential biomarkers and surrogate endpoints. We hope that the results of this study will provide important information on the basic mechanisms of the long-term clinical complications of SM exposure. Furthermore, we will be better positioned to suggest guidelines for the diagnosis, more effective treatment, and the prevention of the long-term complications following SM exposure. In addition, this research lays the groundwork for further research on the various aspects of SM-related problems.

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*Appendix 1

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