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ORIGINAL ARTICLE

Deoxyribonucleic acid damage in Iranian veterans 25 years after wartime exposure to sulfur mustard

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Background: More than 100,000 Iranian veterans and civilians still suffer from various long-term complications due to their exposure to sulfur mustard (SM) during the Iran-Iraq war in 1983-88. The aim of the study was to investigate DNA damage of SM in veterans who were exposed to SM, 23-27 years prior to this study. **Materials and Methods:** Blood samples were obtained from the veterans and healthy volunteers as negative controls. Lymphocytes were isolated from blood samples and DNA breaks were measured using single-cell microgel electrophoresis technique under alkaline conditions (comet assay). Single cells were analyzed with "Tri Tek Comet Score version 1.5" software and DNA break was measured based on the percentage of tail DNA alone, or in the presence of H_2O_2 (25 μM) as a positive control. **Results:** A total of 25 SM exposed male veterans and 25 male healthy volunteers with similar ages (44.66 ± 6.2 and 42.12 ± 5.75 years, respectively) were studied. Percentage of the lymphocyte DNA damage was significantly ($P < 0.01$) higher in the SM-exposed individuals than in the controls (6.47 ± 0.52 and 1.31 ± 0.35, respectively). Percentages of DNA damage in the different age groups of 35-39, 40-44, 45-49, and 50-54 years in SM-exposed veterans (5.48 ± 0.17, 6.7 3 ± 1.58, 6.42 ± 0.22, and 7.27 ± 0.38, respectively) were all significantly ($P < 0.05$) higher than the controls (1.18 ± 0.25, 1.53 ± 0.22, 1.27 ± 0.20, and 1.42 ± 0.10, respectively). The lymphocytes incubated with H_2O_2 had much higher DNA damage as expected. The average of tail DNA is 42.12 ± 2.75% for control cells + H_2O_2 and 18.48 ± 2.14% for patients cells + H_2O_2; $P < 0.001$. **Conclusion:** SM exposure of the veterans revealed DNA damage as judged by the comet assay.

Key words: Comet assay, deoxyribonucleic acid damage, genotoxicity, human lymphocytes, sulphur mustard

INTRODUCTION

Sulfur mustard (SM), also known as mustard gas, has been used extensively during World War I and in the Iran-Iraq conflict (1983-88). There are more than 100,000 Iranian veterans and civilians who still suffer from various chronic health effects related to their exposure to SM.[1]

SM is an alkylating agent with cytotoxic, mutagenic, and vesicating properties.[2] In humans, it causes organic toxicity with delayed complications mainly on the respiratory, neuromuscular, dermal, and ocular and immune systems. It may also cause gastrointestinal, cardiovascular, hematological effects and even cancer.[3] Despite the fact that SM toxicity is not as lethal as nerve agents, disabilities produced by SM are a continuing problem that may last for the entire lifetime of the exposed veterans. Various cancers, as a consequence of genotoxicity induced by SM, were reported after World War I in SM-exposed veterans and in the Iranian victims many years after the initial exposure.[4-6] These findings suggest that there might be a continuing instability in their genetic systems that require more investigations. The basic theory of the toxicity induced by SM is alkylation reactions between SM and DNA, RNA, proteins, and lipid membranes.[7]

SM causes DNA cross links and mono- or bifunctional adducts and the majority of all alkylation is in the *N*-7 position of guanine (7-(2-hydroxyethylthioethyl) guanine).[8] In fact, one-third of the all cross links are interstrands and the cytotoxicity of SM is related to these cross links which prevent DNA replication.[9,10] DNA damage by SM exposure activates poly (ADP-ribose) polymerase-1 (PARP-1) and stimulates several DNA repair pathways, including base excision repair, nucleotide excision repair, and homologous recombination.[11] If this genotoxic stress cannot be repaired, the cell will start the apoptotic program.[12]

There are some studies that focused on DNA damage induced by SM *in vitro* or *in vivo* during the last decade. DNA fragmentation and apoptosis were reported in thymocytes, keratinocytes, and HaCaT cells exposed to SM.[13,14] Lakshmana also reported DNA damage in mice after inhalation exposure of SM[15] and it has been shown that SM caused DNA double strand breaks in the hairless mouse skin model.[16] Another study

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revealed a dose-dependent increase in DNA damage in a lymphoblastoid cell line exposed to an SM analogue.[11]

Evaluation of the DNA damage in humans 20 years after exposure to SM would increase our knowledge about human cellular response to SM and help to find an effective treatment for this cytotoxic agent. The comet assay is a sensitive method for measuring DNA strand breaks in eukaryotic cells. To the best of our knowledge, this is the first study that evaluates DNA damage in humans exposed to SM using comet assay.

MATERIALS AND METHODS

Patients and sample

After coordination with Khorasan Razavi Veterans Affairs Foundation, we studied all files of 34 veterans with disability >25% due to SM poisoning during the Iraq-Iran war in 1983-88. Medical Committee of the Foundation had already verified the disability percentages of the veterans due to late complications of SM poisoning and recorded in their files.[17] The study was approved by the Research Ethics Committee of Mashhad University of Medical Sciences (E.C.151/88787) and conducted in accordance with the Declaration of Helsinki and guidelines on Good Clinical Practice. Written and signed informed consents were obtained from all veterans who volunteered and participated in the study.

The unexposed control group ($N = 25$) were male participants from healthy volunteers or the first degree relatives of the veterans at the same age levels who lived in the same region (city of Mashhad) as the study group. There are a range of confounding factors, such as age and smoking, which may have effect on the level of DNA damage of peripheral blood lymphocytes. The case and control groups were matched in these factors. The age range in this study was 35-54 (42.12 ± 5.75) years and we categorized them into four different age groups: 35-39, 40-44, 45-49, and 50-54 years old. There might be variation in the amount of SM exposure by each veteran, but was not possible to estimate it. Based on their current health status and the severity of SM-toxicity-induced disability, they have been categorized in three different groups based on the disability percentages as: Mild (25-40), Moderate (40-70), and Severe (>70).[17,18]

The classification method is mainly based on the chronic health effect of exposure to SM in three organs which are the most common targets of this toxic agent: respiratory system, eyes, and skin. Since the pattern of the late toxic effect of SM is almost similar to chronic pulmonary obstructive disease (COPD), the severity of pulmonary damage was assessed by the GOLD (Global Initiative for Chronic Obstructive Lung Disease), which is a global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease.[17]

The evaluation of the ocular injuries was based on the slit lamp findings with focus on corneal damage and the late keratopathy as well as the disorders in conjunctive vessels.[19] The assessment of the skin lesions was based on the clinical objective findings such as color changes and pigmentation disorders as well as scars and dry skin.[20,21]

Chemicals and reagents

Agarose (Mol. Biol. grade) was purchased from Invitrogen (USA). Low melting point agarose was purchased from Fermentas (Lithuania). Hydrogen peroxide, ascorbic acid, ethidium bromide, Triton X-100, Tris, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lymphoprep (1.077 ± 0.001 g/ml) was obtained from Progen Biotechnik, Germany. All basic reagents were of analytical grade.

Isolation of human lymphocytes

Whole blood was obtained from the SM-exposed individuals and the negative controls. Briefly, 5 ml of whole venous brachial vein blood from each subject was drawn into heparinized tubes and was diluted with PBS (Phosphate buffered Saline) (1:1). Lymphocyte separation medium (Lympho Prep.) (5 ml) was added to each clean tube and blood samples were carefully layered over them. Samples were centrifuged for 20 min at 2800 rpm, 4°C. The gradient-separated lymphocytes were harvested gently from the interphase and centrifuged at 1500 rpm for 10 min. The lymphocytes were then counted in a Neobauer chamber using Trypan Blue solution 0.4%. The numbers of viable cells were measured to be more than 85%. Duplicate lymphocyte samples were prepared for each study subject.

Determination of DNA damage (comet assay)

For detecting DNA damage, we used the alkaline comet assay which is the most sensitive, valuable, and standard method for assessing DNA damage.[22] The alkaline comet assay was done based on the method described by Singh with some modifications.[23] Alkaline conditions cause unwinding of the two DNA strands and reveal single- and double-strand breaks as well as alkali labile sites.[24] This is the most appropriate technique for measuring a range of DNA damage forms in a single-gel electrophoresis.[25]

In order to establish the optimal concentration for H_2O_2, 50 µl of cell suspension of 10 healthy volunteers were incubated with H_2O_2 (0, 25, 50, 100, and 200 µM) at 4°C for 15 min. The lowest concentration of H_2O_2 (25 µM), which induced significant DNA damage (% tail DNA = 42.12 ± 2.75) ($P < 0.001$) with cell viability >85%, was chosen as the positive control.

Nine hundred fifty microliters of PBS or H_2O_2 (25µM) were mixed with 50 µl of cell suspension and incubated

at 4°C for 15 min. A mixture of 50 µl cell suspension and 50 µl low melting point agarose (LMA) 1.5% at 37°C was added to microscopic slides and a third layer of 100 µl LMA 0.75% (w/v) was applied and allowed to set for 10 min on ice. Slides were soaked in cold final lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% Triton X-100, 1% dimethyl sulfoxide, and pH 10.0). DNA was allowed to unwind for 30 min in fresh electrophoresis buffer (1mM Na_2EDTA, 0.3 N NaOH, and pH 13.0) and then washed three times with cold neutralizing buffer (Tris-HCl 0.4 M and pH 7.5) and stained with ethidium bromide (20 µg/ml). To minimize extra DNA damage, all steps were carried out under low light conditions. Cells were analyzed by Tri Tek Comet Score Version 1.5 software. The DNA damage was expressed as % DNA tail. % tail DNA = [tail DNA/(head DNA + tail DNA)] ×100. A higher number indicates a higher level of DNA damage.

Statistical analysis

Data were checked for normality and they were normally distributed. Demographic distribution of the groups was evaluated by means of Student *t*-test (for two groups) and Kruskal Wallis (for more than two groups) and expressed as mean ± SD. Differences between the patients and controls in four experimental groups and also between four different age groups in the percentage of DNA damage were measured by analysis of variances with the *post Hoc* test of Kruskal Wallis using SPSS version 13 (Chicago, USA). Association between percent of DNA damage and severity of disabilities was tested by Spearman's correlation coefficient. Values were expressed as mean ± standard error (SE). Statistical significance was accepted at $P < 0.05$.

RESULTS

Demographics and the study population

After taking history and physical examination, eight cases of the 34 patients' files were excluded from this study due to high age, which might have effects on the amount of DNA damage or the interference of possible unknown elderly diseases. One case with a history of consumption of immunosuppressive drugs was also removed from the study. Therefore, 25 patients were studied. Controls (not exposed to SM) were all healthy male individuals; seven of them were first degree relatives of the exposed cases. As some veterans did not have male first-degree relatives at the same age, or it was not possible for them to participate in this study, we chose other 18 healthy volunteers, all of a similar age range (±2.5 years) (mean = 42.12 ± 5.75) as the veterans. The demographic characteristics of the 25 cases and 25 controls are summarized in Table 1.

The cases and the controls were all male with a similar age range and did not differ significantly in terms of cigarette smoking or exposure to environmental risk factors (such as hazardous fumes or particles in workplace). For the measurement of DNA damage, cases and controls were matched according to smoking for each age group. In complete blood count, the numbers of lymphocytes in the veterans were lower than in the controls but it was not significant (2300 ± 100 and 2370 ± 100, respectively).

COMET ASSAY RESULTS

For evaluation of the amount of DNA damage, we measured the percentage of DNA in tail of comets (intensity of tail), which is the most commonly used method to analyze Comets in clinical and research studies.[25]

The comet assay results indicated that the amount of DNA damage observed in SM-exposed individuals was significantly higher than in the controls (6.47 ± 0.52 and 1.31 ± 0.35, respectively, $P \leq 0.05$). The percentage of DNA damage in different age groups of the controls (Tail DNA: 1.18 ± 0.25%, 1.53 ± 0.22%, 1.27 ± 0.20%, and 1.42 ± 0.10%) were lower than the SM-exposed veterans (Tail DNA: 5.48 ± 0.17%, 6.73 ± 1.58%, 6.42 ± 0.22%, and 7.27 ± 0.38%). The differences between all age groups were significant ($P < 0.05$, $P < 0.01$, $P < 0.01$, and $P < 0.05$, respectively), as shown in Figures 1 and 2. DNA damage was increased by higher ages, from 5.48 ± 0.17% to 7.27 ± 0.38%, but this increase was not significant ($P = 0.49$), as shown in Figure 2.

As predicted, peripheral blood lymphocytes of the controls and the veterans, when incubated with H_2O_2 (25 µM), showed significantly higher DNA damage than those without H_2O_2 treatment in all four age groups of the patients. (The average of tail DNA of 42.12 ± 2.75% and 18.48 ± 2.14%, respectively; $P < 0.001$.) According to the severity of chemical injury, the Spearman correlation coefficient between percentage of damage and severity of disabilities was not significant ($P = 0.75$, $r_s = 0.555$). Also, there were not any significant differences in the amount of DNA damage in the mild, moderate, or severe disability groups of patients (Tail DNA = 6.13% ±0.53, 6.75% ±1.14,

Table 1: Demographic data of patients with delayed complications of sulphur mustard poisoning and the control group

Parameter	SM-exposed individuals (*n*=25) (%)	Controls (*n*=25) (%)	*P* value
Age in year (Mean±SD)	42.12±5.75	44.66±6.2	0.16[a]
Age groups: *N* (%)			
35-39	5 (20)	4 (16)	
40-44	7 (28)	7 (28)	0.9[b]
45-49	6 (24)	8 (32)	
50-54	7 (28)	6 (24)	
Cigarettes (pack*/year) (mean±SD)	4.25±3.75	6.41±3.65	0.152[a]

[a]Student *t*-test, [b]Kruskal-Wallis, *Pack of 20 cigarettes

and 7.19% ±0.98 SEM, respectively; $P > 0.05$, Figure 3). Figure 4 illustrates photographic images of lymphocytes in a healthy control (A), two SM-exposed veterans (B and C), and H_2O_2-treated lymphocytes (D).

DISCUSSION

Our results indicated that the percentage of DNA damage in the SM-exposed veterans were significantly higher than their same age control group [Figures 1 and 2]. The first studies on DNA damage induced by SM were performed on bacteria such as *E. coli*[26] and revealed that SM-induced interstrand DNA crosslinks and inhibited DNA replication, which is necessary for cell survival. DNA alkylation including mono adducts and cross links blocked DNA replication; therefore, it causes cell cycle arrest and DNA single- and double-strand breaks.[27,28] *In-vitro* studies have shown an increase in DNA damage after treatment of human lymphocytes and keratinocytes with SM using the comet assay.[29,30]

White blood cells have a limited life span and direct effects of DNA damaging agents are normally repaired quickly.[22] Mutations in DNA repair genes (for example, in hematopoietic cells) at the time of the initial exposure are a possible explanation for the observations in this study. To address the exact mechanisms, western blotting studies are designed to investigate the activation of DNA damage response pathways as well as the expression level of DNA damage response proteins and data showed the higher expression of some proteins involved in DNA damage and repair pathways (data are not presented here).

Cellular DNA is subjected to numerous attacks constantly,

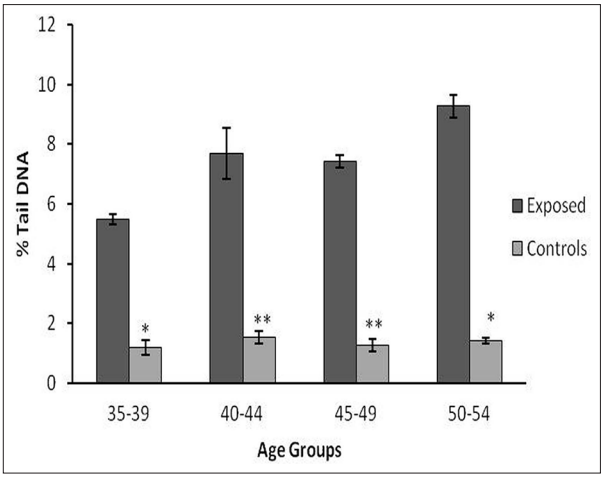

Figure 1: Human lymphocyte DNA damage in 25 SM-exposed individuals and 25 healthy controls in different age groups. Results are expressed as the mean ± SEM ($n = 2$ slides × 50 lymphocytes for each individuals). $^{**}P < 0.01$ and $^{*}P < 0.05$

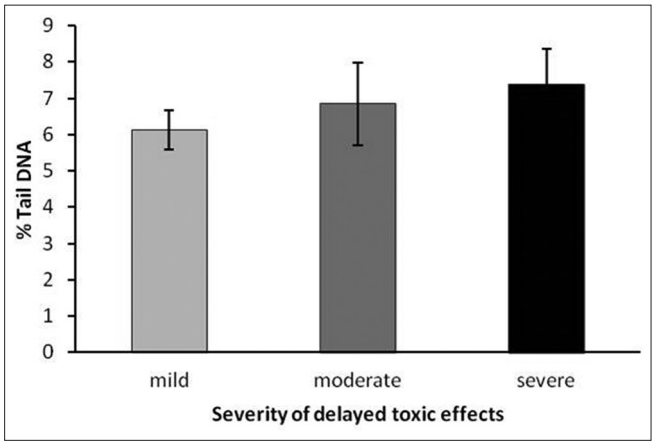

Figure 3: Human lymphocyte DNA damage in three different categories of SM-exposed individuals based on the severity of chemical exposure. The disability percentages are Mild (25-40), Moderate (40-70), and Severe (>70). Results are expressed as the mean ± SEM ($n = 2$ slides × 50 lymphocytes for each individuals)

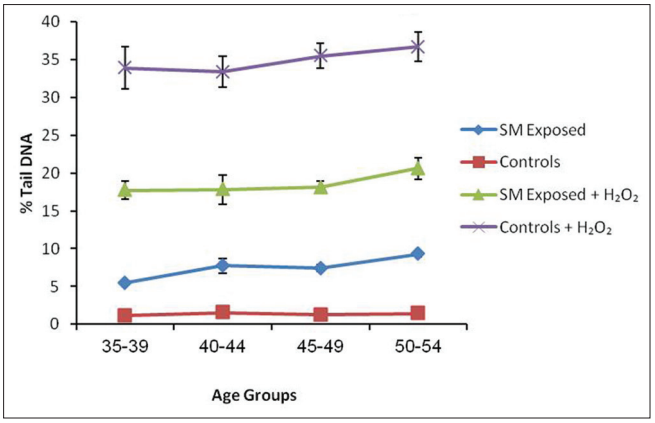

Figure 2: Human lymphocyte DNA damage in 25 SM-exposed veterans and 25 healthy subjects and H_2O_2-treated cells in different age groups (years). Results are expressed as the mean ± SEM ($n = 2$ slides × 50 lymphocytes for each individuals). $P < 0.001$ for each point

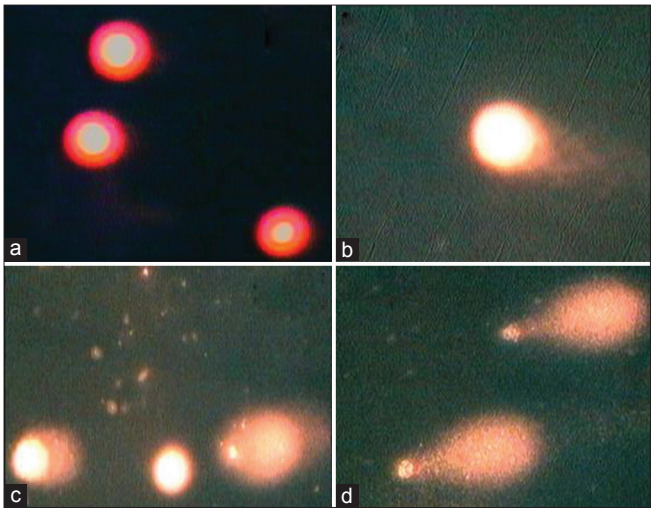

Figure 4: DNA comet images of lymphocytes: (a) Lymphocytes of a healthy volunteer with no induced damage, (b) and (c) Lymphocytes of two SM-exposed veterans, (d) Control lymphocytes treated with H_2O_2 (25 μM). Picture C shows a slide of lymphocytes of an SM-exposed veteran with different levels of DNA damage. (Slides were stained with ethidium bromide and exposed to UV radiation for imaging)

both by endogenous or environmental agents; however, the mutagenic effects are reduced by DNA repair genes. Mutations in DNA repair genes following exposure to SM may increase levels of endogenous or exogenous DNA damage or increase the speed of mutation process. The DNA damage may also be due to a general inflammatory/oxidative stress mechanism. Another explanation for the delayed SM damage in leukocytes is the findings by Meier *et al*. (1987) that showed a time dependant and consistent drop in lymphocytes NAD + levels after exposure to SM, which inhibit DNA repair pathway.[31] Future studies will be required to identify the nature of the DNA damage detected in this study. For example, the measurement of 8-hydroxy deoxyguanosine (8-OHdG) as a marker of oxidative damage. In addition, neutral or enzyme-linked comet assays would allow the measurement of specific forms of DNA damage. Small number of subjects is also a limitation of this study and future studies with greater population of SM veterans, would help to reach a strong conclusion.

SM veterans usually show leucopenia[32] and abnormal lymphocytes in blood smear.[33] In our experiment also, DNA damage was not observed in all veterans' lymphocytes. Mutation in some, but not all cells could be a possible explanation for this diversity. The association between increasing age and DNA damage has been reported previously. Goukassian *et al*. have shown a decrease in proteins involved in DNA repair pathway as a function of increased age.[34] Ageing may also alter the functional capacity of human hematopoietic stem cells to repair DSBs, leading to higher DNA damage accumulation. Decreased repair processes contribute to genomic instability and the accumulation of mutations.[35] We observed a slight increase in the pattern of DNA damage with increasing age. However, this was not statistically significant, perhaps due to the low number of cases. Another important finding of this study was that the level of DNA damage in patients at three categories of disability (mild, moderate, and severe) did not reveal significant differences. There were patients among the exposed group with history of subclinical exposure to SM who had developed long-term health effects and are currently receiving medical care and categorized as mild patients in this study. Even in these cases, we observed that the level of DNA damage was significantly higher than the control group. According to Figure 3, it seems that veterans' cells did not show the same genotoxicity of H_2O_2 as healthy individuals. We propose one possible hypothesis for this finding. It seems that veterans' cells respond more quickly to the hydrogen peroxide, possibly because of the activated DNA damage responses in SM-exposed individuals. It has been reported that following DNA damage and in response to molecular changes, which induce damage signaling, a series of repair enzymes are activated including poly (ADP-ribose) polymerase-1 (PARP-1). This result in the cellular depletion of ATP and induction of pro-inflammatory necrotic cell death.[36] DNA-PK might also be involved in DNA repair following DNA strand breaks.[37] The exact repair pathway after SM exposure is not clearly known yet; however, we think that the amount of damage right after the exposure had been much higher. Mutation in a number of genes involved in DNA repair changes the pattern of natural repair pathway and induces DNA fragmentation. This is also in relevance with different late toxic effects of SM veterans like cancers long time after exposure.[4,38]

This study confirms that SM toxicity in human results in DNA damage two decades after the initial exposure, which might have a key role in life lasting chronic health effects among the exposed individuals. The exact mechanisms mediating this increase in DNA damage remain to be investigated in future studies.

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