Telomere shortening associated with increased levels of oxidative stress in sulfur mustard-exposed Iranian veterans

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Running title: Telomere shortening in SM-exposed individuals

Abstract

Sulfur Mustard (SM) is the most widely used chemical weapon. It was used in World War 1 and in the more recent Iran-Iraq conflict. Genetic toxicity and DNA alkylation effects of SM in molecular and animal experiments are well documented. In this study, lymphocytic telomere lengths and serum levels of isoprostane F2α were measured using q-PCR and enzyme immunoassay-based methods in 40 Iranian veterans who had been exposed to SM between 1983-88 and 40 non-exposed healthy volunteers. The relative telomere length in SM-exposed individuals was found to be significantly shorter than the non-exposed individuals. In addition, the level of 8-isoprostane F2α was significantly higher in the SM-exposed group compared to controls. Oxidative stress can be caused by defective antioxidant responses following gene mutations or altered activities of antioxidant enzymes. Chronic respiratory diseases and infections may also increaseoxidative stress. The novel finding of this study was a the identification of 'premature ageing phenotype'. More specifically, telomere shortening which occurs naturally with aging is accelerated in SM-exposed individuals. Oxidative stress, mutations in DNA repair genes and epimutaions may be among the major mechanisms of telomere attrition. These findings may help for a novel therapeutic strategy by telomere elongation or for validation of an exposure biomarker for SM toxicity.

Keywords: Telomere shortening, Sulfur Mustard, Oxidative stress

1. Introduction

Sulfur mustard (SM) is a highly toxic chemical warfare agent which was widely used in World War One and during the Iran-Iraq conflict from 1983-88 [1]. There are different reports of long-term health effects, organ damage and cancer progression after a single exposure to SM [1-4]. Genotoxic and mutagenic properties of SM are well-documented in molecular and animal experiments [5] but only few studies focused on the potential delayed genotoxicity of SM in human beings. The critical issue is how a single exposure to SM can lead to chronic inflammatory and degenerative diseases many years after exposure. A variety of cancers were reported in chemically injured individuals with a history of exposure to SM in the First World War and in the Iran-Iraq conflict, which indicates that genetic change takes place in SM-exposed individuals [3, 4, 6]. These findings may lead to continuing/ongoing genetic instability in exposed individuals. The toxicity of SM was postulatedto be mediated by alkylation of DNA and proteins, although the precise mechanisms are not clear yet. DNA damage induced by SM has been previously shown in vitro after acute exposure [7-9] and in peripheral lymphocytes of Iranian veterans many years after exposure, with the latter being consistent with ongoing genetic instability [10].

Telomeres are unique DNA-protein structures containing noncoding hexanucleotide repeats (TTAGGG) which serve as protective caps at the ends of chromosomes. These specialized structures are essential to maintain genomic integrity [11]. They play an important role in preserving chromosome stability and telomere shortening is considered to be associated with cellular senescence, aging and mortality [12]. Progressive shortening of telomeres can lead to apoptosis or oncogenic transformation of somatic cells, affecting the health and lifespan of an

individual. Shorter telomeres than normal, have been associated with cellular stress (including reactive oxygen species) and increased incidence of disease and poor survival [13].

It wasproposed that the cytotoxicity of SM is predominantly due to DNA/protein alkylation, as well as an increase in the level ofreactive oxygen species (ROS). SM forms a highly reactive carbonium ion which reacts with DNA, proteins and other biological molecules such as glutathione. Glutathione depletion increases the level of ROS production [14]. Furthermore, conversionof ROS to highly toxic oxidants can cause lipid peroxidation in membrane phospholipids, leading to loss of membrane functions [15]. Stimulation of poly(ADP-ribose) polymerase-1 (PARP-1) following SM-induced DNA damage leads to consumption of cell energy and generation of reactive oxygen species [16].

To increase our understanding of the toxic mechanism of SM and how this might contribute to the long-term health issues after exposure, this study investigated telomere length as a potential novel target of SM toxicity. The findings demonstrate accelerated telomere shortening and increased levels of oxidative stress in a SM-exposed population compared to non-exposed controls.

2. Materials and Methods

2.1 Study Population

The study was a historical cohort investigation with Iranian veterans who were exposed to SM between 1983 and 1988. It was not possible to estimate individual SM exposure levels, therefore they were categorized in three different groups as: Mild (25-40%), Moderate (40-70%), and Severe (>70%), based on veterans' current health status and the severity of SM toxicity-induced disability [10]. Briefly, the classification method was mainly based on the chronic health effect of exposure to SM in three organs which are the most common targets of SM exposure:

respiratory system, eyes and skin. [10, 17]. The evaluation of the respiratory system, eyes and skin injuries was based on guidelines that was reported in the previous studies [18, 19]. The study group was 40 male veterans (age 35–74 y) with disability of >25% due to complications of SM poisoning. Controls were veterans' male relatives as non- SM-exposed healthy volunteers. For those who didn't have male relatives, healthy participants with no exposure to SM, were selected to achieve 40 control cases. Since smoking is associated with accelerated telomere shortening [13], the cases and controls were matched 1:1 on aging (±1) and smoking (up to 25% pack/year). 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 participants of the study. All the following laboratory experiments were repeated three times in duplicates.

2.2 Telomere Length Measurement using relative comparative real time PCR

Three milliliters of whole blood sample were collected in EDTA contained venoject tubes from each individual and genomic DNA was extracted from buffy coat using the QIAamp DNA Mini kit (Qiagen, Chatsworth, CA). The amounts of leukocyte DNA were quantified using a Nanodrop2000 spectrophotometer (Thermo Scientific, USA). Total DNA was then kept at -80℃ until further use. Leukocyte telomere length was measured in blood genomic DNA using the quantitative real-time PCR [20, 21]. To standardize the experiments, which run at different times and to eliminate the differences between experiments, a pooled DNA was prepared. Five microliters of each control samples were mixed and the amount of DNA was quantified and aliquoted. In each experiment, a dilution series (1:4) of pooled DNA ranging from 8 ng/µl to 0.5 ng/µl were run and fresh standard curve was created. Single-copy gene human β-Globin (HBG)

was used to normalize the sample-to-sample variation in template DNA. All PCRs were performed on a Step One Thermal Cycler (Applied Biosystems, USA).

The PCR primer sequences used in PCR reactions are presented in Table 1 [22].

Each10 µl of single copy gene reaction contained 300 nM and 700 nM of the forward and reverse human β-Globin primers (HBG) (Metabion International AG, Germany), respectively, and 5 µl of 2 × Power SYBR® Green PCR Master Mix (Applied Biosystems, USA). The following temperature profile was applied: initial denaturation step of 95°C for 10 min, followed by 40 cycles of 95°C for 15s, 58°C for 20s, and 72°C for 28 s. Each 10 µl of telomere reaction contained 100 nM and 900 nM in the forward and reverse Tel primers (TEL) (Metabion International AG, Germany), respectively, 5 µl of 2 × Power SYBR® Green PCR Master Mix (Applied Biosystems) and 0.3 µl of DMSO PCR Reagent (Merck, Germany). The PCR profile for telomere (TEL) amplification was: initial denaturation step of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 54°C for 2 min. Each plate was composed equally of interdigitated cases and controls, and included negative control wells (containing no DNA). At the end of each reaction, a melting curve was obtained for both TEL and HBG PCRs.

2.3 Oxidative Stress Measurement

The measurement of the concentrations of F2-isoprostane (sum of esterified and non-esterified isomers) in serum was carried out as described previously [23] with some modifications. Serum was collected by centrifugation of coagulated blood at 2500 X g for 10 min and collecting the supernatant. To hydrolyze esterifies lipids, the serum samples were treated with KOH at the ratio of 4:1 at 45 °C for 2 hours. Thereafter, the samples were neutralized by addition of HCl (0.1 mol/L), and the pH was adjusted to 3.5 with NaOH (1 mol/L). The samples were centrifuged at 500 × g at 5°C for 15 min, and the supernatants were removed for the measurement of

Isoprostane F2α using direct 8-iso-Prostaglandin F2α kit (Assay designs, USA). The concentration of total Isoprostan F2α in each sample was recorded as pg/ml. Each sample was tested in duplicate.

2.4 Statistical Analysis

Statistical analyses were performed using SPSS version 15 (Chicago, USA). Data were analyzed for normality and demographic distribution of the groups by means of student's t test and expressed as mean ± SEM. SEM of relative telomere length for each exposure category were calculated with generalized linear regression models, adjusted for age (± 1 year) and cigarette smoking (pack/day/year). Association between telomere length and age was determined by Pearson correlation test. The Association between relative telomere length and level of Isoprostane with severity of chemical injury was measured using ANOVA with post-test Tukey. A P-value <0.05 was considered significant.

3. Results

3.1 Demographic information

General information of the patients were summarized as shown in Table 2. In this study, controls and SM exposed individuals were matched by age (± 1 year) and cigarette smoking (pack-years) and were similar for these characteristics and therefore, controls and patients did not differ significantly in age mean ± SEM (44.37± 9.70 and 46.85 ± 6.31 respectively).

3.2 Relative Telomere lengths

Telomere length was determined in DNA isolated from peripheral blood leukocytes as the T/S ratio (Telomere to Single copy gene), using a relative comparative qPCR method [24] with a 78mer TTAGGG repeat as a standard curve (Absolute).

The results showed that the relative telomere length in SM-exposed veterans was significantly lower than non-exposed controls; (Mean ± SD: 0.80 ± 0.06 vs. 1.02 ± 0.01, P< 0.05, Figure 1) (Mean Age ± SEM 46.85 ± 6.31, N = 40) (Mean Age ± SEM 44.37 ± 9.7, N = 40). P value is based on the student t-test and *$P < 0.05$ is considered significant. We divided the veterans to three different categories as mild (25-40 %) (n=18), moderate (40-70 %) (n=10) and severe (>70 %) (n=12) disability. Mean relative telomere length in lymphocyte DNA in SM exposed individuals with the history of severe toxicity and the highest disability (>70%) had shorter telomeres compared to mild and moderate injury groups and non-exposed controls (*P< 0.05, Figure 1). Data are expressed as Mean± SEM and P value is based on ANOVAs with post-hoc Tukey-Kramer and *$P < 0.05$ is considered significant.

There was an inverse correlation between T/S and age. The association of telomere length with age in SM exposed individuals were significant (r= -0.39, P<0.05), while this association for healthy controls was near to be significant (r=-0.22, P= 0.08). Data represents the relationship between mean telomere lengths (T/S ratio from qPCR) of DNA samples prepared from lymphocytes of SM exposed individuals with age (Figure 2).

3.3 Oxidative stress

Direct 8-iso-Prostaglandin F2α Enzyme Immunoassay kit (Assay designs, USA) was used to determine levels of isoprostanes which is correlated to the levels of oxidative stress in both SM-exposed and control groups. The results shows that levels of 8-isoprostane F2α in serum of SM exposed group are significantly higher compared to the controls (429 pg/ml and 198 pg/ml, respectively) (*** P<0.001) (Figure 3). Also, we observed a significant relationship between severity of SM toxicity and serum level of isoprostane, asshown in Fig.3.

4. Discussion

SM is the most used chemical weapon and its genotoxic effects (predominantly via monofunctional and bifunctional DNA adduction) have been confirmed in several in vitro, in vivo, and clinical studies. [25].

In order to find suitable diagnostic tools and possible therapeutic options for SM toxicity, it is necessary to have a detailed understanding of the molecular mechanisms mediating SM genotoxicity and the associated cellular response. In vitro studies have shown that telomeres are highly susceptible to oxidative stress. Oxidative stress-mediated DNA damage is an important determinant of telomere shortening [26]. Kawanishi showed an accelerated telomere shortening in human cell cultures induced by oxidative stress and formation of 8-oxodG at the GGG triplet in the telomere sequence has been revealed [27].

Our findings indicate that exposure to SM results in telomere shortening and increased oxidative stress in human beings. In this study, mean relative telomere length in the SM exposed individuals was lower compared to the healthy controls. To the best of our knowledge, this is the first report elucidating an association between shortening of telomere length and SM toxicity in human subjects. Our investigation shows that the inverse relationship between telomere length and age is even more significant after SM exposure, indicating accelerated aging phenotype after SM exposure. However, it is likely that theresponses vary between individuals due to the genetic background and environmental factors. The accelerated telomere shortening/cell aging could be a novel causative factor for the delayed health effects and organ degeneration observed in SM-exposed individuals.

A possible explanation for the mechanisms of delayed toxicity would be epigenetic perturbations caused by SM exposure. The epigenotype can be transmitted to daughter cells maintaining a specific epigenotype within the cell lineages. Mutations of genes involved in DNA methyl

transferase (DNMT) synthesis and histone deacetylases (HDAC) due to SM exposure, may lead to gene malfunction in SM-exposed cells. Although this is a speculative hypothesis at this stage, SM may change the activity of important enzymes involved in epigenetic regulation such as HDAC and suppress DNMT, leading to epigenetic perturbations. DNMT and HDAC are responsible for p53 regulation, as well as many antioxidants and anti-inflammatory products; and mutation in these enzymes could be responsible for p53 activation and increasing oxidative stress [16]. This may explain delayed SM complications, genotoxicity and even cancer.

Previous studies showed a decrease of the activities of certainantioxidant enzymes and an increased oxidative stress in peripheral lymphocytes of Iranian veterans who were exposed to SM. [28, 29]. In this study, the level of Isoprostane F2α was measured in the serum of the patientsas a marker of oxidative stress. Isoprostanes are biologically active and long-lasting chemicals which are produced in high quantities during oxidative stress [30] and currently considered as a useful measure of oxidative injury in vivo [31].

There could be a possible correlation between telomere length and oxidative stress, which shouldbe investigated in future studies. It is notable that the level of 8- Isoprostane F2α, a marker of oxidative stress, was significantly higher in SM group compared to the controls. Using a chemical analogue of SM in mice can induce general oxidative stress and activation of transcription factors such as NF-Kappa B through the MAPK pathway [32]. In a study with 250 SM-exposed veterans, significant glutathione depletion was observed. Furthermore, a correlation was detected between glutathione-related enzyme activity and pulmonary disorders due to SM exposure [2, 29]. It was found that chronic exposure to SM in rats, decreases glutathione reductase and glutathione peroxidase activity and increases lipid peroxidation [33]. As an alternative mechanism, chronic respiratory diseases, infections and common health problems in

SM-exposed veterans, may lead to greater levels of oxidative stress. Increased levels of IgM, disruption in cellular and humeral immunity and increased interleukin 1, 2 (twenty years after single exposure to SM) are other signs of progressive and chronic effects of SM toxicity, which can cause oxidative stress [1]. Oxidative stress can induce different types of DNA damage including base oxidation, as well as DNA single- and double-strand breaks. (SSBs and DSBs, respectively) [34]. An increased level of DNA damage has been shown previously in SM exposed individuals using comet assay [10]. Oxidative stress is the most possible cause for such DNA damage and our current findings support our previous results.

Telomeres typically shorten with aging by a few dozen to hundreds of base pairs per cell division [35]. Compared with aging, oxidative stress is considered to be a more important cause of telomere shortening [27]. Telomere contains Guanine rich regions and GGG repeats which are highly sensitive to oxygen species. Therefore, telomeric DNA is a preferential target for oxidative damage [36]. Accelerated telomere shortening in SM-exposed individuals may lead to premature cell senescence and can therefor cause degenerative disorders to be developed sooner than in normal cells/non-exposed individuals. If the telomere shortening induced by SM, persists occurrs at the time of exposure, would be permanent and would mean that the normal telomere shortening which occurs with aging would take less time to reach a critical point (Fig. 2). This would manifest itself as a 'premature ageing phenotype' and this hypothesis is supported by thefindings of the present study; showing an increased rate of age-related telomere shortening in SM-exposed individuals compared to non-exposed controls. Interestingly, there was a significant association between telomere shortening and severity of SM disability, which confirms the accelerated telomere shortening after single exposure to SM and a dose-dependent correlation. Adose-dependent relationship was also observed between severity of disability and levels of

oxidative stress marker, although it was not statistically significant. In a study with the same group of patients, increased DNA damage was detected in lymphocytes from SM-exposed individuals using the comet assay [2]. Mutations in DNA repair genes following exposure to SM has been proposed as a possible reason for this DNA damage [10]. Such mutations may also occur in the epigenetic system. Apart from genetic factors, telomeres are under epigenetic control [37, 38]. Mammalian telomeres contain nucleosomes and can be subjected to many types of histone modifications such as acetylation, methylation and phosphorylation [39]. Changes in either histone modification at the telomeres or DNA methylation at subtelomeres are associated with telomere length deregulation [40].

It is known that disturbs the epigenetic environment of transcription factors such as NF-κB, AP-1 and pro-inflammatory genes such as TNF-α and ILs. It has been shown that epigenetic modifiers have an influence on gene expression in the pathogenesis of SM-induced lung toxicity [16]. Furthermore, inflammatory reactions induce oxidative stress. TNF-α significantly decreases telomerase activity and reduces telomere length in leukemic cells [41]. Therefore, oxidative stress and chronic inflammation may be among the major mechanisms of telomere attrition.

According to our findings, SM can cause DNA damage, oxidative stress, gene silencing and expression of some proteins. It will be interesting to investigate the mechanisms mediating the increased levels of oxidative stress in SM-exposed individuals.

5. Conclusion

In conclusion, we found shorter telomere length and increased levels of a biomarker of oxidative stress in veterans many years after their exposure to SM. These findings confirm delayed genotoxicity of SM in patients over 25 years after single exposure to SM. Accelerated aging after SM was another novel finding of this study, which may contribute to chronic and progressive

organ damage/dysfunction and different cancers many years after SM exposure. There has long been an interest in manipulating telomere length in cancer and age-related disease. Although a long way down the line, it can be considered that these findings may help for a novel therapeutic strategy for SM toxicity utilizing telomere elongation methods.

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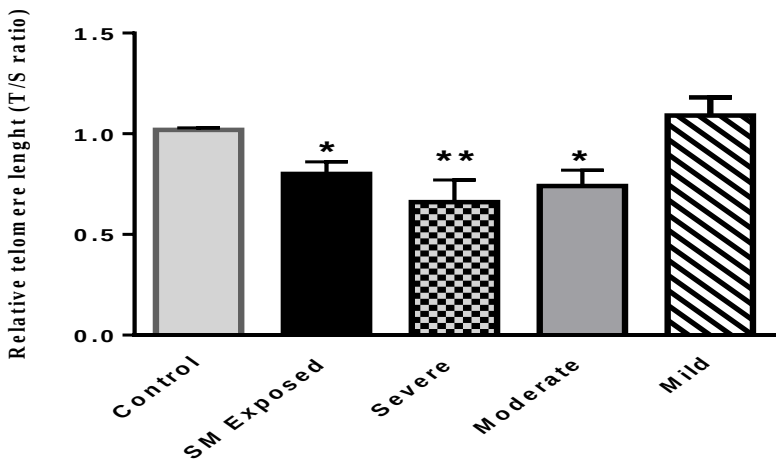

Fig. 1-Relative telomere length in SM exposed individuals and non-exposed controls and based on disability percentages due to complications of SM poisoning. The Mean relative telomere length in lymphocyte DNA in SM exposed individuals were significantly shorter (mean age ± SEM 46.85 ± 6.31, N = 40) than non-exposed controls (mean age ± SEM 44.37 ± 9.7, N = 40). P value is based on the student t-test and $*P < 0.05$ is considered significant. The mean relative telomere length in lymphocyte DNA in SM exposed individuals in three different categories as mild (25-40 %)(n=18), moderate (40-70 %)(n=10) and severe (>70 %)(n=12) disability and controls. SM exposed individuals in the group of severe injury have significantly shorter telomeres compared to mild and moderate injury groups and non-exposed controls. Data are expressed as Mean± SEM and P value is based on ANOVAs with post-hoc Tukey-Kramer and $*P < 0.05$ is considered significant.

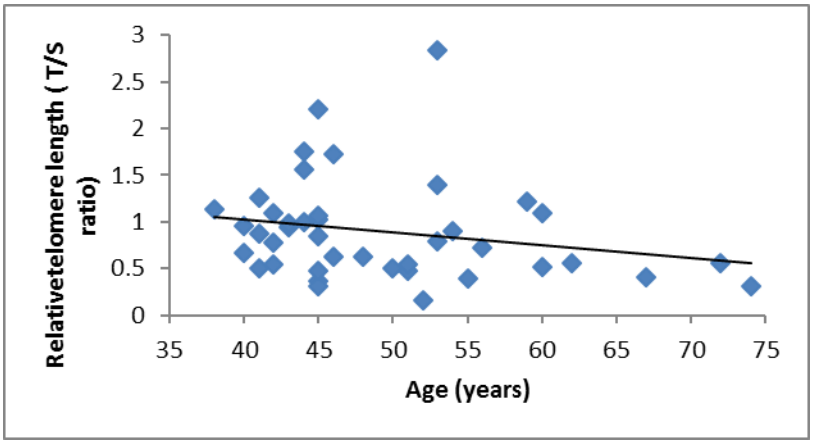

Fig. 2- Correlation between mean telomere length and age in SM exposed individuals. Each point represents an individual (Pearson correlation test) (r= -0.39, P < 0.05).

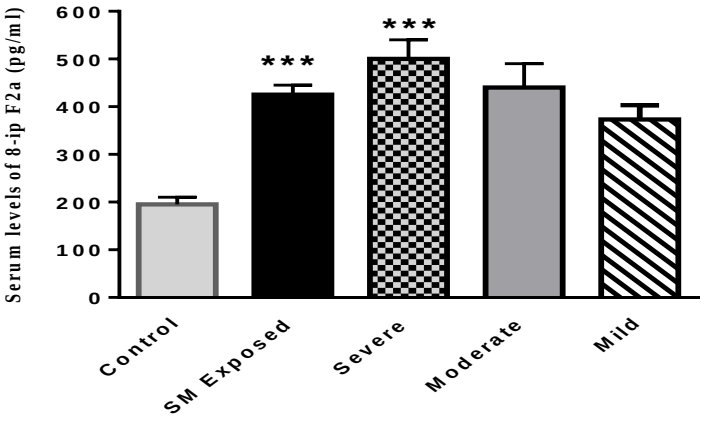

Fig. 3- Concentration of 8-isoprostane $F_2\alpha$ in serum samples of SM exposed individuals and non-exposed controls and based on disability percentages due to complications of SM poisoning.

Table1: Primer sequences used for Real time-PCR assay.

Gene	Primer	Sequence
Telomere	Forward	(5'-CGGTTTGTTTGGGTTTGGGTTTGG GTTTGGGTTTGGGTT-3')
	Reverse	(5'-GGCTTGCCTTACCCTTACCCTTAC CCTTACCCTTACCCT-3')
HBG	Forward	(5'-CTTCTGACACAACTGTGTTCACTAGC-3')
	Reverse	(5'-CACCAACTTCATCCACGTTCACC-3')

Table 2: Demographic characteristics of patients with delayed complications of sulfur mustard poisoning with different severity of disability (mild, moderate and severe) and the control group.

Parameter	Control	SM Exposed Individuals	P value
Number	40	40	
Mild	-	18	
Moderate	-	10	
Severe	-	12	
Sex	Male	Male	
Age	44.37 ± 9.7*	46.85 ± 6.31	0.185
Pack-years of smoking	6.65 ± 4.15	8.25 ± 5.50	0.12

*Mean ± SD

Student t test for difference in medians