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THE BACKGROUND DNA DAMAGE LEVEL IN PERIPHERAL BLOOD CELLS IN HALABJAS' SURVIVORS-KRG-IRAQ

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ABSTRACT

The inhabitants of communities in the Kurdistan area of Iraq, particularly Halabja, represent the greatest civilian populations ever exposed to chemical weapons. In several sections of the Kurdistan Region of Iraq between 1987 and 1991, the Iraqi government conducted a Genocide against Kurds. Various chemical warfare chemicals, such as sulfur mustard and the nerve agents Sarin, Tabun, and cyanide, were used in the act of war. There are scientific indications that chemical weapons alter DNA and causes genetic lesions and mutation which may eventually cause health problems. Survivors in this city are till now suffering from a variety of health complaints from mild to severe and fatal one as a result of the long-lasting effects of chemicals used in bombing the city. From the several health complains developed among survivors of Halabja bombardment were cancer, respiratory, ophthalmological, dermatological, reproductive and immunological complains. It was required for measuring the background DNA damage level in peripheral cells in some survivors of Halabja city. The Alkaline Comet Assay data reveals varying degrees of DNA damage level in Halabja survivors and in those born after the chemical attack in the same city. The damage level was significantly greater in Halabja survivors in comparison to those individual living in Duhok city (age/sex matched). However, the DNA damage measured in younger generation (those were born after the chemical attack in Halabja) is minimum, this could refer that only the exposed survivors at risk of genomic instability.

INTRODUCTION

Chemical warfare (CW) is the employment of the toxicity of chemical compounds as weapons. Chemical warfare has been deployed in numerous regions of the globe for millennia. (Fitzgerald, 2008). During World War I, chemical warfare entered its modern era. A chemical used in warfare is known as a chemical warfare agent (CWA), and over the 20th and 21st centuries, around 70 distinct compounds have been employed or stored as chemical warfare agents. These substances may exist in liquid, gas, or solid form. (Haber, 1986), (Burck & Flowerre, 1991). These agents have a direct and indirect toxic effect on plants, animals and humans (Geoghegan and Tong, 2006).

The inhabitants of communities in the Kurdistan region of Iraq, particularly Halabja, represent the greatest civilian populations ever subjected to chemical weapons. From 1987 to 1991, the Iraqi government conducted a "Genocide War." against Kurdish population in different parts of the Kurdistan Region of Iraq.

The act of war included use of various CWAs, including sulfur mustard (SM) and the nerve agents sarin, tabun, and VX, as well as cyanide, were used in the act of war, according to certain reports (Gosden, 1998). The strike killed approximately 5,000 people and injured between 7,000 and 10,000 others, the majority of whom were civilians; thousands more died of illnesses, infections, and birth deformities in the years that followed (Osman, 2002). The rising incidence of birth malformations in Halabja and elsewhere has persuaded specialists that the chemicals may have a lasting genetic influence on the victims of Halabja..

The increased incidence of malignancies is one of the chemical's consequences. Large

intestine cancer, colon cancer, lung cancer, breast cancer, and Leukemia There are scientific indications that chemical weapons impact DNA. and causes genetic lesions and mutation (Baban, 2000). Survivors in this city are till now suffering from a variety of health complaints from mild to severe and fatal one as a result of the long-lasting effects of chemicals used in bombing the city. From the several health complains developed among survivors of Halabja bombardment were cancer, respiratory, ophthalmological, dermatological, reproductive and immunological complains (Hama et al., 2009).

Sulphur mustard is genotoxic due to its interactions with DNA, which is an essential first step in the development of cancer (Mood et al., 2005). The exposure of humans to nitrogen mustard caused chromosomal damage (Fox and Scott, 1980). Individuals exposed to nitrogen mustard exhibited an increase in the incidence of secondary tumor (Rostam-Zadi et al., 1999), (Hassan et al., 2002). Typically, alterations in genes and chromosomes could remain for years. Nonetheless, the human gene pool can become subtly contaminated **(**Margery and Shaw, 1970). The toxicants that enter the human body disrupt the regular state and action of the chromosomes, leading to a rearrangement of hereditary material that results in chromosomal abnormalities and genetic mutation in somatic and germ cells (Higginson et al., 1992), (Al-Humadi, 2008).

Chromosomal deviation represents apparent DNA damage in stained cells. Typically, lymphocytes from exposed populations are taken and analyzed for varying sorts of chromosomal damage. In addition to significant animal research, this methodology has been applied to several occupational and environmental exposures to chemicals and radiation. Furthermore, cytogenetic endpoints in peripheral blood lymphocytes have been utilized as biomarkers to facilitate an acceptable epidemiological evaluation of cancer prognosis (Hagmar et al., 1998). There are promutagenic DNA adducts in the p53 tumor suppressor gene caused by mustard gas. DNA point mutations were identified in lung malignancies of Japanese mustard gas manufacturing employees (Vähäkangas, 2003).

The chemicals utilized during the attack may have an effect on the body that is not comparable to that of ionizing radiation. As result, the DNA of the Kurds has been transformed, as have their genes (Gosden, 1998). Numerous various genes can be altered in the body, increasing the chance of cancer or sickness, and in eggs or sperm, they can cause congenital defects or fetal death (Baban, 2000).

It is believed that Halabja is still polluted, as no cleanup has occurred; contamination of soil and water may be responsible for some of the bad health impacts experienced by Halabja people even today (Gosden, 1998). Christine Gosden, a professor of medical genetics, traveled to Halabja in 1998. he was concerned about the severe health impacts of this chemical combination on mothers and children in particular (Battrick, 2001). Even in minute doses, the chemical proved fatal, according to research. Therefore, the gassing of Halabja and Anfal worked as a mutagen and altered the DNA of the victims. (Alan, 2009) and destroying their genetic integrity (Eklow et al., 1984).

As a part of Iraq, Kurdistan Region has been subjected to numerous environmental and epidemiological changes that raise the likelihood of cancer in this region. It's therefore, important to further study, the risk at genomic level in exposed individuals (survivors) to chemical weapons through investigating the DNA damage level in their lymphocytes.

Aim of the study: To measure and compare the background DNA damage among survivors living in Halabja city comparing in those who living in Duhok city.

MATERIALS AND METHODOLOGY:

Methodology:

This study contains two groups of individuals living in Halabja city. The first group of individuals those were born before 1988 and the second group of individuals were born after 1988 (after chemical attack); Group 1 (35 individuals) Halabja Residence (Survivors) their age ranging between 33-83 years (14 females, 22 males) . Group 2: (34 individuals from Duhok city) their age ranging between 36-74 years (10 females, 24 males). Group 3: (23 individuals were born after chemical attack) Halabja residence their age ranging between 27-32 years (8 females, 15 males). Group 4: (17 individuals from Duhok city) their age ranging between 21-31 years (7 females, 10 males). The entire cohort appeared to be healthy and had no history of taking cytotoxic drugs. All are non-smokers and abstainers. (A customized questionnaire (Appendix-1) was administered in order to record the direct responses of individuals

throughout multiple visits to Halabja. Each patient was given a questionnaire containing fields for the following information: (Name, Address, Telephone Number, Gender, Age, Exposed to chemicals or not, Route of exposure, Marital Status, Number of children and their health). In addition, the questionnaire forms were filled up through direct interviews with patients and their relatives, as well as through the use of hospital medical data, with the assistance of doctors and other Halabja Chemical Victims Society personnel.

Variables	Group 1 Halabja Survivors (35)	Group 2 Duhok Participants (34)	P value
Age (years)	53.55 ± 12.84	49.32 ± 11.92	0.7126
BMI	26.65 ± 1.188	24.12 ± 1.074	0.6515

Variable	Group 3 Halabja Participants (23)	Group 4 Duhok Participants (17)	P value
Age (years)	25.15 ± 3.89	26.55 ± 3.115	0.3002
BMI	21.94 ± 0.74	24.71 ± 1.32	0.0923

Materials:

After completing the consent document, blood samples were collected from each participant in this study. Blood samples were frozen in EDTA tubes and transported on ice to the Genotoxicity Research Unit at the DMRC-College of Medicine-University of Duhok-Duhok-KRG-Iraq. All other demographic features were recorded in a special questionnaire during sampling.

Alkaline Comet Assay *ACA

Alkaline Comet Assay Technique (ACA) was used to determine the level of DNA damage in lymphocytes of blood samples from residents of Halabja city and Duhok city, KRG, Iraq. The ACA method, also known as single cell gel electrophoresis, has been regarded as a sensitive and versatile approach for measuring DNA damage in single cells. The ACA was modified based on earlier studies (Kumaravel and Jha, 2006), (Olive et al., 1990) to measure genetic damage in all subjects involved in the current study. Beginning with slide preparation, precoating, and gel formation, the experiment proceeded to electrophoresis and scoring of the data. Briefly, 10 L of whole blood was placed to an Eppendorf tube and mixed with 180 L of low melting point agaros (LMP) solution that had been pre-wormed. Then, 80 l of the mixture was poured onto each half of a microscopic slide that had been coated with 1% agarose with a normal melting point. The two gels were directly covered with cover-slips, allowed to freeze on ice for 15 minutes, and shielded from light. After removing the coverslips, the slides were placed carefully into a pre-cold lysis buffer (100 mM disodium EDTA, 2.5 M NaCl, 10 mMTris HCl, pH 10) containing 1% Triton-X-100 (v/v) in coupling jars and stored overnight at 4c. After the lysis process, the slides were rinsed with ice-cold double distilled water (dH2O) once, followed by two more rinsings with ice-cold dH2O, each left for 20 minutes in the dark. After washing processes, the slides were placed on an electrophoresis tank (Cleaver scientifica) and the ice-cold alkaline buffer solution (300 mM NaOH, 1 mM disodium EDTA, pH > 13) was added and allowed for 20 minutes to allow the supercoiled DNA to unwind. Following the alkaline buffer incubation, the electrophoresis was conducted for 20 minutes with a 300 MA and 30VA current. After electrophoresis, the slides were transferred to trays and washed twice with ice-cold dH2O and neutralizing buffer. Before scoring and measuring DNA damage, slides were allowed to dry overnight and stained with syber green dye (Thermo Fisher Scientific). Under a fluorescence microscope, the head and tail of each DNA strand is visible. The intact DNA with a circle-shaped head indicates no damage Fig. 1A. The length of the tail indicates the extent of DNA damage (%Tail DNA) Fig. 1B. Each gel was scored by selecting and capturing fifty comets for assessment using a fluorescent microscope (Leica fluorescence microscope) at 20X magnification.

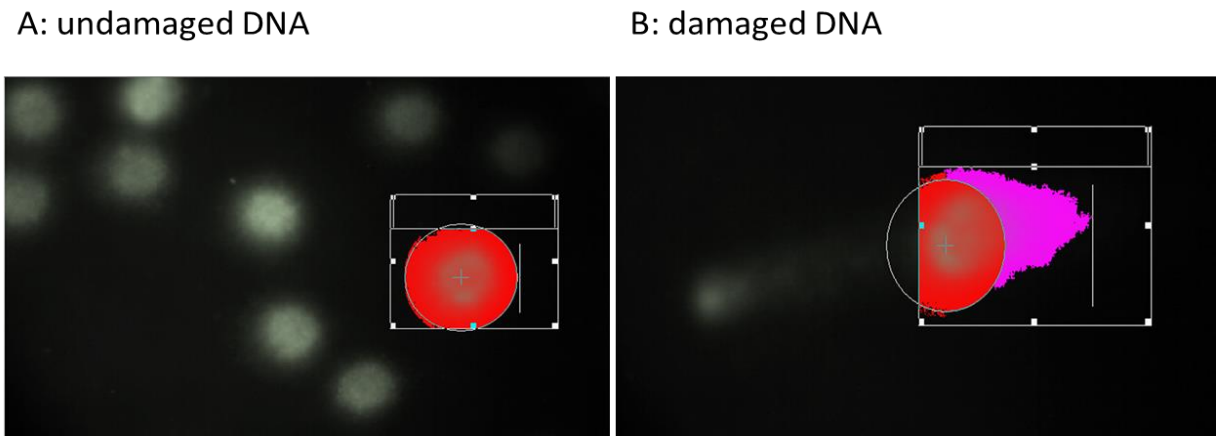


Fig. (1): (A) Showing undamaged DNA (Intact DNA) and (B) shows DNA with migrated tail (damaged DNA). The two captured DNA were analyzed by CASPLab software.

Statistical Analysis:

Prism-GraphPad-5 was used to compare the Mean and frequencies of the parameters among the group. Two groups' parameter means were compared using the T-independent test. P 0.05 indicated statistical significance.

RESULTS & DISCUSSION

Results:

In order to investigate the impact of a chemical on background DNA damage in the Halabja community, exposed people were recruited for this study**.** The first group included (36 individuals) which represent Halabja Residence (Survivors) their age ranging between 33-83 years, while the second group included (34 individuals) from Duhok city and their age ranging between 36-74 years. The third group included (23 individuals) which represent Halabja residences their age ranging between 27-32 years, while the fourth groups included (17 individuals from Duhok city) their age ranging between 21-31 years.

Figure (2) shows DNA damage level (%) in those born before 1988 (group-1) (before the chemical attack), the results show out of (35 individuals) who their age range between 33-83 years old there were (9 individuals) minimum amount of DNA damage, and (10 individuals shows mild levels of DNA damage, the rest with higher amount of DNA damage level represent up to 20% of the DNA was damaged.

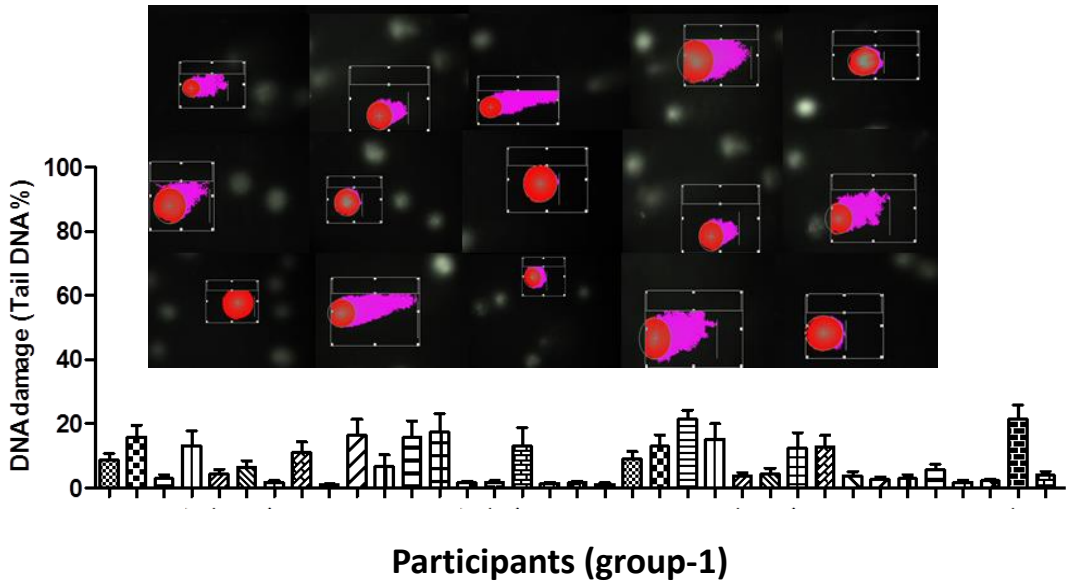


Fig. (2): shows DNA damage level in individuals (group-1) living in Halabja city, those born before the chemical attack in Halabja. The results show out of 35 individuals there were 14 individuals with high to moderate degrees of DNA damage.

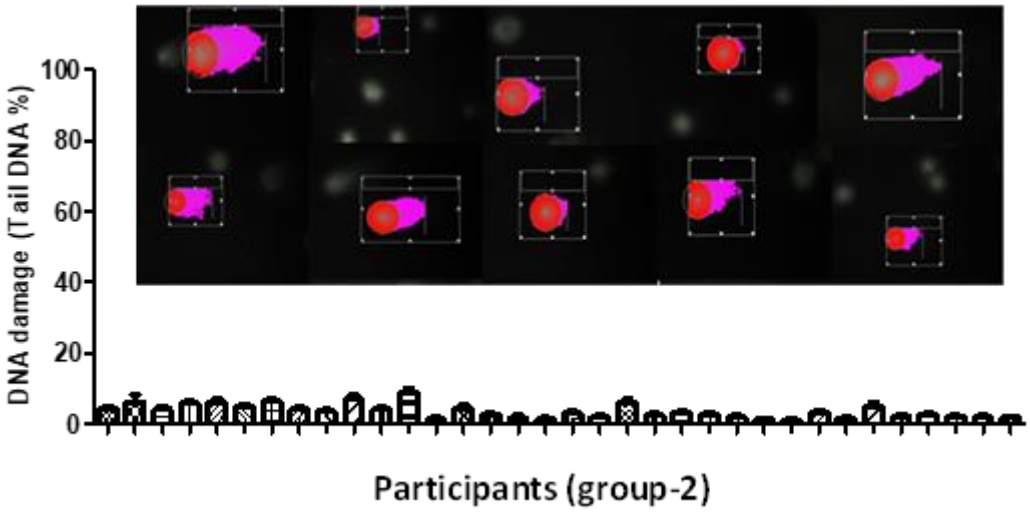


Fig. (3): Shows DNA damage level in individuals (group-2) living in Duhok city. The results show out of 34 individuals there were only few individuals with mild degrees of DNA damage, the others with a very minimum amount of DNA damage.

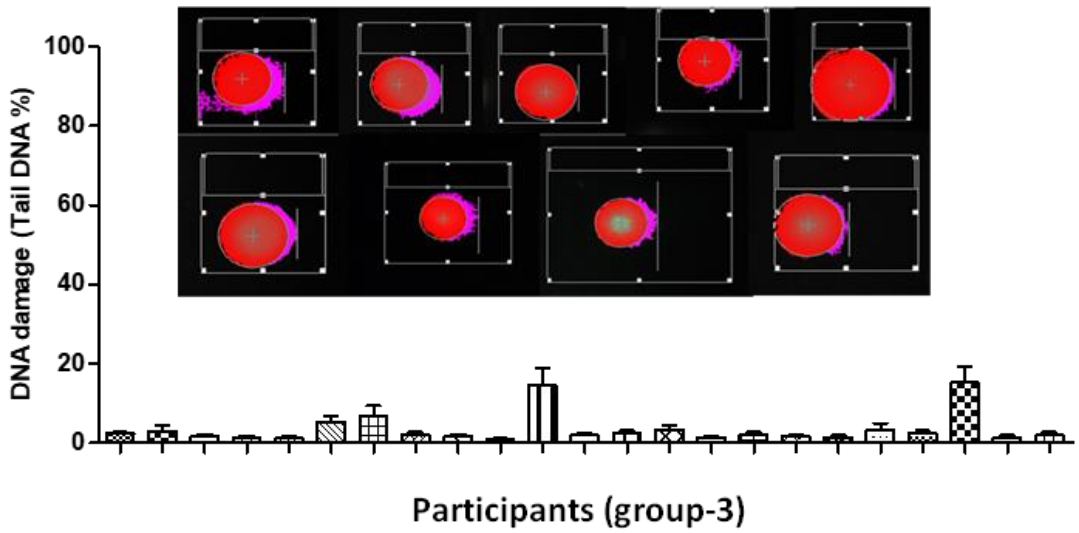


Fig. (4): Shows DNA damage level in individuals (group-3) living in Halabja city, those were born after the chemical attack in Halabja. The results show out of 17 individuals there were only 4 individuals with mild degrees of DNA damage, the others only with very minimum amount of genomic damage.

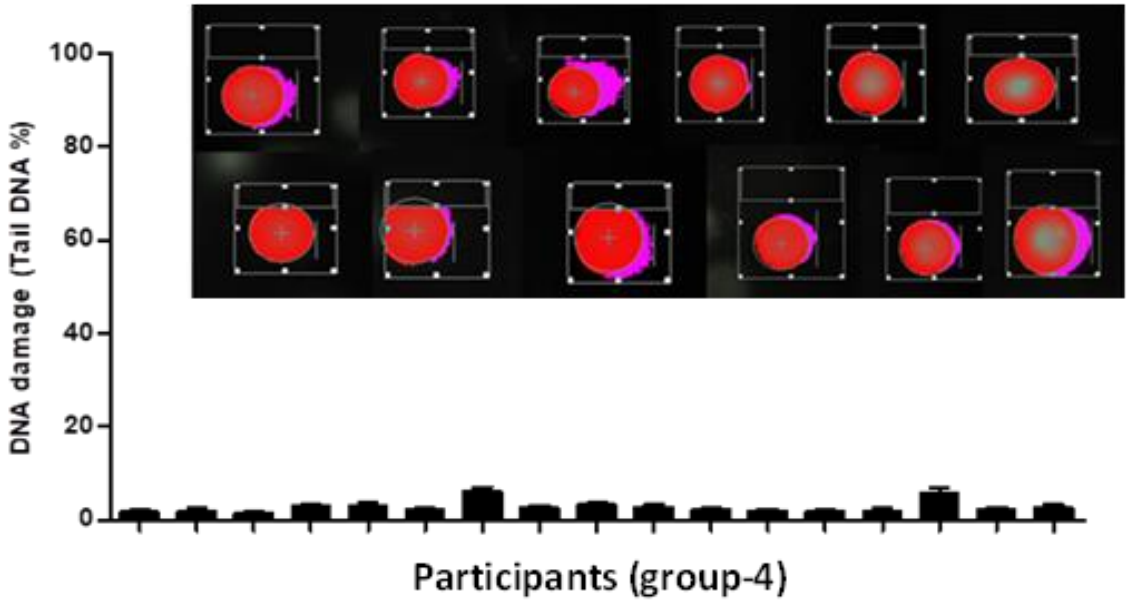


Fig. (5): Shows DNA damage level in individuals (group-4) living in Duhok city. The results show only very minimum amount of DNA damage.

Additional analysis of the results reveals that the mean level of background DNA damage in Group-1 individuals: (36 individuals) Halabja Residence (Survivors) their age ranging between 33-83 years was significantly greater than that in the Group-2: (34 individuals from Duhok city) their age ranging between 36-74 years. (**Figure-6**). The Mean ± SE of the tail DNA damage in lymphocytes were 7.943 ± 2.767 and 5.177 ± 1.271, respectively, *p=0.0331*.

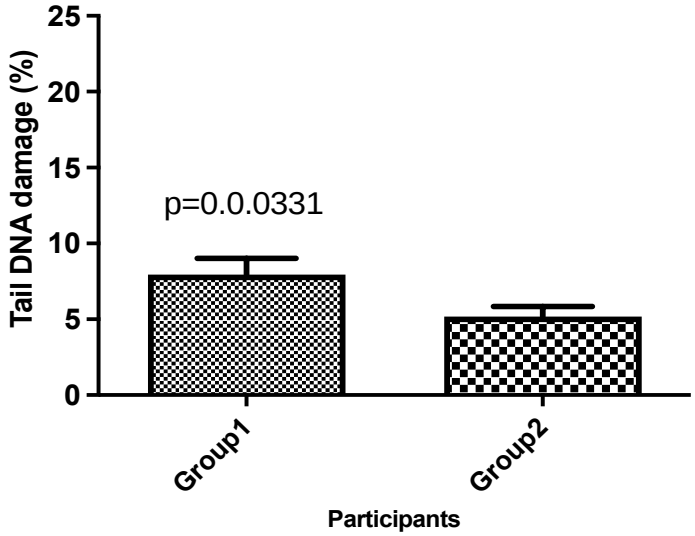
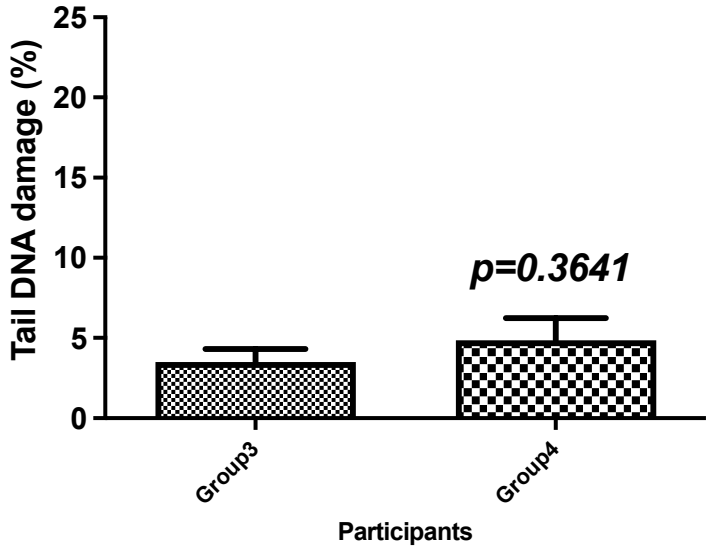


Fig. (6): The difference between the means of DNA damage in individuals exposed directly to chemical attack in Halabja (Group-1) and in the same age group living in Duhok city (Group-2).

Moreover, when the mean of comet assay scores of group-3 and group-4 (those were born after the chemical attack in Halabja and Duhok) it shows a nonsignificant difference with Mean ± SE 3.501 ± 1.373 and 4.874 ± 1.498 respectively, p=0.3641(**Figure-7**). DNA damage occurred more in those individuals who were born before chemical attack (those directly exposed to chemicals, while third group represent those individuals in Halabja who were not exposed to chemical attack and they have a minimum amount of background DNA damage.



Fig/ (7): The difference between the means of DNA damage in individuals living in Halabja city (those born after chemical attack) (group-3) and in those with the same age born in Duhok city (group-4).

DISCUSSION

It has long been known that sulfur mustard causes DNA inter-strand cross-links (Roberts et al., 1971). (Shahin et al., 2001). which were identified first in E. coli (Lawley & Brookes, 1965). When sulfur mustard combines with DNA, one of the resulting compounds consists of two guanines connected by a mustard molecule. This crosslink can develop from a pair of guanines in opposite DNA strands, and it suppresses cell proliferation. However, considerable cross-linking can also occur between two adjacent guanines on the same strand) (Walker, 1971). (Remington, 2010).

Lin et al. (1996) reached the conclusion that sulfur mustard generated dose-dependent interstrand cross-links in the DNA of rat epidermal keratinocytes in primary monolayer culture, thereby altering the cell cycle and DNA synthesis. HeLa cells (Ball and Roberts, 1972) and rat cutaneous keratinocytes demonstrated comparable outcomes (Ribeiro et al., 1991). DNA mismatch-repair in African green monkey kidney cells has also been demonstrated to be affected by sulfur mustard (Fan & Bernstein, 1991). It has been demonstrated that sulfur mustard forms DNA adducts in vitro (Van der Schans et al., 1994). Upon incubation of double-stranded calf-thymus DNA or human blood with [35S]-labeled sulfur mustard, the following adducts were discovered: N7-[2-[(2-ydroxyethyl) thio] ethyl]-guanine, bis[2-(guanin-7-yl) ethyl] sulfide, and N3-[2-[(2-hydroxyethyl) thio -adenine, and O6-[2- [(2-hydroxyethyl)thio]ethyl] - guanine and its 2′-deoxyguanosine derivative (Fidder et al., 1994). The N7 position of deoxyguanosine is the major location at which sulfur mustard alkylates DNA. The base adduct N7-(2-hydroxyethylthioethyl)-guanine (N7-HETE-Gua) is liberated upon depurination of the resultant N7-(2-hydroxyethyl)-2′-deoxyguanosine. DNA adducts such as N7-hydroxyethylthioethylguanine, 3-hydroxyethylthioethyl adenine, and the cross-link di-(2-guanin-7-yl-ethyl) sulphide have been linked to the harmful effects of sulfur mustard (Saladi et al., 2006). DNA isolated from human leukocytes and treated in vitro to [14C]-labeled sulfur mustard contained the adduct N7-(2-hydroxyethylthioethyl)-guanine (Ludlum et al., 1994).

Somani and Babu (1989) found that alkylation by sulfur mustard influences transcriptional processes and may result in shortened transcripts by inhibiting RNA polymerase via an alkylated promoter. The analysis of shortened transcripts revealed that sulfur mustard alkylates the DNA-template strand preferentially at the 5′-AA and 5′-GG regions. Low concentrations of sulfur mustard can limit cell division by cross-linking complementary DNA strands or induce mutagenesis by causing replication or repair mistakes. Nerve agents are sturdy and straightforward to disseminate., extremely poisonous, and have quick effects when absorbed through the skin or inhaled. The chemical structures of the five nerve agents tabun (GA), sarin (GB), soman (GD), cyclohexylsarin (GF), and VX are comparable to the organophosphate pesticide Malathion. These compounds initially excite and then paralyze certain nerve transmissions throughout the body, in addition to causing additional hazardous consequences such as convulsions (Gunderson et al., 1992).

Chronic vocational exposure to OPs has been associated with an increased risk of developing cancers such as non-Hodgkin lymphoma and several kinds of leukemia (Waddell et al., 2001) concluded from study the cytogenetic effects of insecticides (Lorsban, Endosulfan and Carbaryl) on male albino mice , that all pesticides are capable of causing chromatid and chromosomal abnormalities in mice and raising sperm abnormalities in male laboratory mice. Many carcinogenic are genotoxic and cause cancer development by producing DNA damage and mutation. According to Abdullah (2009), the use of various weaponry was altering the background activity in Halabja. Since these radioactive compounds are prevalent in the environment, they may be a major contributor to the high incidence of cancer in the Halabja region. Oliveira et al., (2007) Exposure to chemical substances may have a range of effects, from instantaneous death to a slow process of chemical carcinogenesis.

Our data in the present work show different levels in DNA damage in the peripheral blood cells (Lymphocytes). The mean of damaged DNA level in survivors of Halabja chemical attack is significantly greater than that observed in their opponent living in Duhok city. The background DNA damage levels in most of those born before chemical attack in Halabja city are relatively high. It shows that out of 36 survivors only nine of them with minimum amount of DNA damage, the others with mild to greater damage level in their peripheral cells.

When the results were further analyzed statistically, we found that the mean level of DNA damage is significantly higher, ($p=0.0331$), than in those with the same ages but living in Duhok city. Interestingly, the younger generation living in Halabja city (Group-3) with only a very minimum amount of DNA damage in their peripheral blood cells. Data show the background DNA damage in those individuals living in Halabja, those born after the chemical attack, is less than 5% compared to the damage level in DNA (group-4), in those born. This may indicate that the generation born after the chemical attack in Halabja are less likely to expose to the damaging effect of chemicals.

CONCLUSION & RECOMMENDATION

The endogenous DNA damage level in peripheral blood cells in Halabja survivors is relatively high compared to the levels of genomic damage in their opponents living in Duhok city. Surprisingly, the genomic instability in younger generations in Halabja city are less likely to have more genomic instability.

Further studies are needed to illustrate the damaging effect of chemical exposure in survivors in Halabja city by including a larger sample size and using different techniques for detection of genomic damage and DNA mutations.

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