Bethlehem University

Deanship of Science

Biotechnology Master Program

Genotoxic Effects of Israeli Industrial Settlement Pollutants on Bruqeen Village (Salfit) residents.

By

Kholoud Mahmoud Khalil Hammad

In Partial Fulfillment of the Requirements for the Degree of

Master of Science in Biotechnology

September, 2011

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The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

" Genotoxic Effects of Israeli Industrial Settlement Pollutants on

Bruqeen Village (Salfit) residents."

By

Kholoud Mahmoud Khalil Hammad

In partial fulfillment of the requirements for the degree of Master of Science in Biotechnology

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Abstract:

Genotoxic Effects of Israeli Industrial Settlement Pollutants on Bruqeen Village (Salfit) residents.

Many industrial byproducts are genotoxic agents that induce cytogenetic changes and DNA damage. Occurrence of these abnormalities can lead to cancer in people living in areas affected with pollutants. Bruqeen village in Salfit is subjected to industrial waste products coming from Barqan Israeli industrial settlement. The objective of this study was to monitor and evaluate the genomic effects of these pollutants. We evaluated the extent of chromosomal breaks and DNA damage induced to human cells using whole blood samples from both test and control sites. Chromosomal breaks were assessed by routine cytogenetic methods and DNA damage assessed via the comet assay which is called also single cell gel electrophoresis (SCGE). Cytogenetic analysis for Bruqeen residents' sample (n=30) showed: 133 premature centromere separation (PCS), 43 chromosomal breaks (Csb), 40 chromatid breaks (Ctb) and 26 dicentric. In total, the average showed 4.08% of chromosomal aberrations (CA) and 3.81% of cells had CA. The results of controls (n=8) showed: 21 PCS, 2 Csb, 5 Ctb, 2 dicentric, and in total the average showed 1.97% of CA and 1.91% of cells had CA. Statistical analysis showed that there was no significant difference between exposed and control for PCS ,Ctb and Dicentric (P-value > 0.05). But there was a statistically significant difference for CA frequency, for cells that have CA and Csb (P-value < 0.05). Comet assay (data on 25 Bruqeen residents' sample) showed that there was significant difference from those of control sample (P-value < 0.05). Our study detailed below showed that there is significant effect on genetic material of residents of the polluted area, and that the control samples in Bethlehem showed higher values for the studied parameters than that found in literature for healthy subjects in other countries.

Key words: Genotoxicity, DNA damage, Cytogenetic analysis, Chromosomal aberrations, Comet Assay (SCGE).

"تقييم مدى التأثيرات السلبية للملوثات الصناعية لمستوطنة برقان الإسرائيلية على المادة الوراثية للمواطنين سكان قرية بروقين (سلفيت) "

خلود محمود خليل حماد

ملخص:

هناك العديد من المواد الكيميائية المصنعة واسعة الانتشار في البيئة التي تعتبر عوامل سامة للمادة الوراثية حيث أنها تحفز حدوث تغيرات كروموسومية غير طبيعية وتلف للمادة الوراثية. إن حدوث هذه التغيرات غير الطبيعية يؤدي إلى تطور السرطان عند الأشخاص القاطنين في المناطق المتأثرة بالملوثات. يتم تنفيذ العديد من الدراسات منذ العام 1978 لتقييم التأثيرات الوراثية السامة التي تسببها العديد من الملوثات. قرية بروقين التابعة لمحافظة سلفيت تعاني من تلوث تسببه مستوطنة برقان الإسرائيلية الصناعية. إن هدف هذا البحث هو تقييم ودراسة مدى التأثيرات السلبية للمخلفات الصناعية لمستوطنة برقان الإسرائيلية الملقاة في القرية على المادة الوراثية للمواطنين سكان قرية بروقين. لقد تم في البحث تقييم مدى التلف على المادة الوراثية (DNA) من خلال جمع عينات دم كاملة من أفراد عينة الدراسة, وتم تطبيق تحليلين: التحليل الكروموسومي والتحليل بالفصل الكهربائي للخلية الواحدة (التحليل المذنب). نتائج التحليل الكروموسومي للعينة العشوائية من سكان القرية (عدد أفرادها30) ما يلي: 133 انفصال سابق لأوانه للسنترومير, 43 تكسر في الكروموسومات, 40 تكسر في الكروماتيدات, 26 كروموسومات ذات سنترومير, 4,08% درجة تكرارالاختلالات الكروموسومية, 3,81% نسبة الخلايا التي تضمنت اختلالات كروموسومية. أما نتائج العينة الضابطة (عدد أفرادها 8) فهي كالتالي (على الترتيب): 21, 2, 5, 2, 1,97%, 1,91%. التحليل الإحصائي أظهر أنه لا توجد فروق ذات دلالة إحصائية بين العينة المعرضة للتلوث والعينة الضابطة بالنسبة لما يلي: الانفصال السابق لأوانه للسنترومير, التكسر في الكروماتيدات, الكروموسومات ذات سنترومير (P-value > 0.05), لكن توجد فروق ذات دلالة إحصائية بالنسبة لما يلي: التكسر في الكروموسومات, نسبة الاختلالات الكروموسومية, نسبة الخلايا التي تضمنت اختلالات كروموسومية (P-value < 0.05). نتائج تحليل المادة الوراثية بالفصل الكهربائي للخلية الواحدة (التحليل المذنب) أظهرت أنه يوجد فروق ذات دلالة إحصائية بين العينة العشوائية لسكان القرية (عدد أفرادها 25) والعينة الضابطة (عدد أفرادها 5) حيث أن (P-value < 0.05). يتميز التحليل الوراثي المذنب بأنه حساس, سريع وقليل التكلفة نسبيا, وأداة جيدة للحكم على وجود التكسر في المادة الوراثية على مستوى الخلية الواحدة. أظهرت دراستنا المفصلة أدناه أن المادة الوراثية لسكان المنطقة المتأثرة بالتلوث تعاني من تأثر ملموس, وأن المادة الوراثية لأفراد العينة الضابطة تعاني من تأثر أكثر مما هو منشور علميا عن مدى التأثر الطبيعي للمادة الوراثية للأشخاص الأصحاء.

DECLARATION

I declare that the Master Thesis entitled " Evaluation of the Genotoxic Effects of Barqan Settlement Industrial Pollutants on residents existing in Broqeen Village (Salfit)" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgement is made in the text.

Name and signature: Kholoud Mahmoud Khalil Hammad_

Date: _____

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Dedication

To

My Dear Parents, Sisters, Brothers, Husband and My Friend Aysha Hamdan

For their patience, support and

encouragement, with love and respect .

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List of Abbreviations:

ROS	Reactive oxygen species
CA	Chromosomal aberrations
PCS	Premature Centromere Separation
Csb	Chromosomal break
Ctb	Chromtid break
Dic	Dicentric chromosome
SCGE	Single Cell Gel Electrophoresis

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Chapter 1: Introduction:

1.1. Background:

Genotoxicity:

Genotoxic stress can be defined as the cellular response to the effects and results of extra and intracellular damage of DNA (Bajak, 2005). The genotoxic chemical carcinogens which can interact with their cellular targets were classified into nonalkylating agents and alkylating agents (Maronpot, 1991). While according to Bajak (2005) they were classified into ones that cause oxidative stress and others cause stress induced due to the effect of alkylating agents. The exposure to genotoxins like ionising irradiation, and to chemicals like alkylating agents can induce chromosomal aberrations (CA) (Evans & Scott, 1968 cited in Major, 2000).

Oxidative stress or the damage caused by non alkylating agents: Nitric oxide and reactive oxygen species (ROS) play vital roles in different physiological processes and cause modifications for several biological molecules, particularly, DNA. When intracellular imbalance between pro-oxidants and antioxidants occurs, oxidative stress results. Since the living organisms are normally and always exposed to free radical species that might be dangerous, keeping in mind that such free radical species might originate endogenously or exogenously. A wide range of base- and sugar- derived DNA lesions in addition to DNA-protein cross links were identified as results of DNA oxidation (Bajak, 2005). Maronpot (1991) pointed that the nonalkylating agents substitute for the nucleosides exocyclic amino groups directly, which might takes place either by oxidative mechanisms or by direct electrophilic attack. Such substitution results in alteration in base-pairing due to deamination or change in the isomeric structure. Nitrous oxide is considered an example for oxidative deamination. Formaldehyde is considered an example that causes cross links within DNA (it also causes hydroxyl methyl adducts so it is considered alkylating agent too).

Stress induced by alkylating agents: Maronpot (1991) stated that alkylating agents react mainly and firstly with guanine and adding their alkyl group to the purine ring at nitrogen atom number (7). This is because they have electron–deficient (i.e. positively charged which are electrophilic) groups which are capable of making covalent linkages (adducts) with the negatively charged parts of biological molecules like DNA through nonenzymatic reactions. Such adducts might be mutagenic or carcinogenic. The formed adducts might be small or large (bulky). Regardless of the adduct size, it causes conformational or configurational alterations in the DNA, that result in DNA replication infidelity. Such interactions of electron–deficient groups may cause lethal adducts or mutations. If they were not lethal, then they are more associated with cancer since initiation of it occurs if such adduct occurred at critical genomic site.

Biomonitoring studies:

Industrial human activities cause contamination of the environment by toxic pollutants. Various researches have been studying their genotoxic effects on humans and other living organisms. Genetic damage can lead to health problems which is an area of concern since it is often associated with carcinogenesis.

Populations are occasionally exposed to mutagenic/carcinogeneic chemicals environmentally and/or occupationally which can impact health (Natarajan, 2005). There is a threat in all exposed species of alterations in the normal DNA structure due to such exposure with direct and indirect effects on functioning of cells and organisms (Sviezena, et al., 2004). Studies attempted to develop tools to investigate exposure to genotoxic factors and health risk assessment. According to Natarajan (2005), the studies that aim to monitor the effects of genotoxins and carcinogins in human populations are increasingly taking place to assess risk.

There are now a wide variety of techniques for biomonitoring genetic damage induced in humans by environmental factors (Anwar, et al., 1992). Some researchers studied the effect of pollutants in field-collected organisms while others examined the effect of chemicals/substances on the cells or organisms in a laboratory setting (Cotelle and Ferard, 1999; Lee and Steinert, 2003 cited in Wirzinger, et al., 2007, p.28).

Cytogenetic and molecular tests have been utilized on wild animals living in polluted areas. Cytogenetic methods has been utilized on wild rodents since 1978 to investigate the genetic damage induced by environmental pollutants. Significant correlations between pesticides, radioactivity, heavy metal contamination and chromosome aberration frequencies have been detected in wild rodents living in contaminated areas (MacGregor, et al., 2006).

Wirzinger, et al. (2007) studied the effect of sewage – treatment effluent on three locations that differ in the amount that they receive in Germany using three-spined sticklebacks (*Gasterosteus aculeatus* L.). They did not look for detailed information about the chemical characterization of the polluted locations since their objective was to find a relation between the occurrence of genotoxic damage and the exposure to sewage. I followed their point of view about not referring to the chemical analysis of polluted water. Their study showed that comet assay of blood cells is a good choice in environmental pollutants' evaluation. They recommended two types of tests in monitoring study: one that measures short-term genotoxic damage and other that measures more persistent genotoxic agents' effects. They depended in their conclusion on the fact that the Single Cell Gel Electrophoresis (SCGE) test detects recent pollution effects that appear early and might remain for short time since they might be repaired through DNA-repair system. Through the study they considered micronuclei more persistent in the cell and remain for longer time than single-strand breaks and alkali-labile sites. In this study combining between Comet assay and chromosomal aberration assay as an alternative for micronucleus test was applied. The two tests are not specific biomarkers, but assess any genotoxic effect.

Similarly, Bueno, et al. (2000) examined the capability to use cytogenetic end points including the frequency of cells with chromosome aberrations as biomarkers in the early detection of genotoxic factors for populations that exist under higher risks. They suggested that it is not enough to depend only on one cytogenetic end point to have a real picture on the environmental factors' effects on the genetic material. The use of chromosomal aberrations in addition to cytogeneic end points can be useful in a biomointoring study.

Silva, et al. (2000) used comet assay to detect genotoxicity in a region close to a strip coal mine by examining wild rodents *(Ctenomys torquatus)* in their natural habitats. They assured that comet assay can detect DNA damage caused by coal effects, and that it is useful in such monitoring studies.

Alink, et al. (2007) used comet assay to study the mutagenecity of Rhine water using mudminnow *(Umbra pygmaea L.)* as fish model.

The disposal of Uranium refining residue in Texas attracted Au, et al. (1995) to study effects on people residing near them. They found that the mean frequency of cells with chromosome aberrations and the mean frequency of cells with deletion frequency were higher for people residing adjacent the Uranium refining and mining activities than those residing far away although the difference was not statistically significant.

Laqqan, et al. (2011) conducted their research to determine the frequency of chromosomal aberrations in Gaza Strip workers assuming that they are exposed to various chemical pollutants. The study group included farmers, plumbers, taxi drivers, paint factory workers and gas station workers, while the control group consisted of healthy individuals not directly exposed to pollutants in their jobs. They found that the occurrence of chromosomal aberrations in the study group was significantly higher than the control group.

Anwar (1994) applied a study on peripheral blood lymphocytes to monitor the cytogenetic effects in individuals exposed to automobile exhaust represented by traffic policemen in Cairo, Egypt. She chose policemen with exposure time more than ten years as a study group. The study showed that the percentage of chromosomal aberrations of the study group was significantly higher than that of control group.

Similar work was done by Kamboj & Sambyal (2006). They aimed to evaluate the chromosomal aberrations formed in traffic policemen exposed to automobile exhaust through their daily work in Amritsar city, Punjab (India). They found that the mean percentage frequency of metaphases with structural aberrations that they tested in the study were significantly higher in traffic policemen than that of control sample.

Leite-Silva, et al. (2007) used chromosomal aberration and Comet assay to study genotoxic and antigenotoxic effects of bladder wrack *(Fucus vesiculosus*) extract on cultured human lymphocytes. For the validation of comet assay results they used chromosome aberration assay (Hartmann, et al., 2003 cited in Leite-Silva, et al., 2007).

Chromosomal aberration and the alkaline comet assay were used in the work of Zeljezic & Garaj-Vurhovac (2001) to assess the degree of DNA damage in persons in

fields of pesticide production using peripheral blood lymphocytes. They found that the exposed group showed significant increase in: aberrant cells, chromatid breaks, chromosome breaks and dicentric chromosomes than the control group. Similarly, the degrees of DNA damage in terms of tail length were significantly higher than those of control one. They also reported that when the exposed group individuals were transferred to spend eight months out of the pesticide zone, the extent of DNA damage in forms of chromosomal aberrations and tail length were reduced but still higher than those of the control group.

Albertini, et al. (2000) attempted to provide guidance for monitoring individuals exposed to genotoxins by both chromosomal aberrations (using the classical chromosomal aberration analysis (CA)), and DNA damage (using comet assay).

Some researches studied DNA damage in healthy persons to generate reference data in environment monitoring studies. Gundy and Varga (1983) studied the frequency of chromosomal aberrations in peripheral blood lymphocytes of healthy persons of ages between 18 and 45 years old. The study showed that 113 subjects appeared with no aberrations, but 62 persons had a total of 154 aberrations. The chromosomal aberrations were 80 chromatid breaks, 62 chromosome fragments and 12 dicentric.

Kopjar, et al. (2006) used chromosome aberration test and the alkaline comet assay to evaluate DNA damage in peripheral blood lymphocytes of healthy persons to be used in the determination of the level of variability in the baseline damage of control samples. They assured the positive relation between the found amount of chromosomal aberrations from one side and the found amounts of long tails and DNA migration in comet assay on the other side. Their results are mentioned later in the discussion part too.

According to Bechmann (2002/010), genotoxicity tests are divided into two main categories: the first one includes those which measure effects at the chromosomal level, while the other one includes those that measure effects at the molecular level. Chromosome aberration tests which are cytogenetic tests are examples of the first category, while comet assay (SCGE) which measures DNA strand breaks is an example of the second one. Parallel studies between DNA and cytogenetic damages caused by different genotoxic agents revealed a very strong association between the two types of damage (Rozgaj, et al., 2005).

Piperakis (2009) mentioned that comet assay is conducted by many researchers in many fields nowadays. He mentioned part of such applications. One application is through human studies (e.g. biomonitoring, nutrition studies, aiding diagnosis (e.g. xeroderma pigmentosum, Nijmegen breakage syndrome), in addition to evaluate the background levels of DNA damage in individuals. Another application is ecological monitoring (using organisms as biosensors for measuring contamination of the environment with genotoxins). Genotoxicity testing is another field in which the comet assay used as a standard test to evaluate new chemicals' or pharmaceuticals' safety. Another application is the estimation of DNA repair by measuring repair capacity at the cellular level.

There is significance for the techniques that enable the sensitive detection of DNA damage and chromosomal breaks in environmental research because of the long latent period between exposure to environmental effectors and the appearance of the genetic alterations effects (Błasiak &Trzeciak, 1998).

Taking all the above into consideration, it was obvious that using a combination of cytogenetics and comet assay to examine the area of Burqeen would add significantly to our understanding of the impact of the industrial waste on the human population directly.

1.2. Chromosome aberration assay (CA):

History & Background:

Cytogenetics is the field of research that deals with chromosomes. In 1956 the researchers were able to count the human chromosomes, and so to declare that the number of human chromosomes is 46. Several techniques were conducted before that date, but the main two steps that led to fast advances in human cytogenetics in that time (i.e. in the 1950s) were two main ideas. Firstly, the use of phytohemagglutinin (PHA), a plant originated material, that stimulates white blood cells' division in order to increase the number of cells in metaphase. Secondly, the use of hypotonic solution that forces cells to absorb water and swell, so that they spread and can be visualized. In the early 1960s the developments led to finding cytogenetic changes associated with both constitutional genetic syndromes (e.g. Down) and cancer (e.g. CML) (Tseng, 1995).
Chromosome studies revealed tumor−specific cytogenetic rearrangements in the vast majority of human neoplasms. Studies led to the identification of pathogenetically important translocations, deletions, and other rearrangements that are intimately associated with the tumorigenic process (Massey University, 2008).

Human chromosomes are numbered from 1-22 in addition to the sex chromosomes. Characteristics of chromosome morphology are shown in figure (1). Chromosomes can be solidly stained or treated to produce a banding pattern. Classification and identification are done according to the International System of Cytogenetic Nomenclature (ISCN, 2005).

Chromosomes are classified according to size and centromere position into seven distinguishable groups (A-G):

Group A (1-3)	Large metacentric chromosomes
Group B (4-5)	Large submetacentric chromosomes
Group C (6-12,X)	Medium-sized metacentric or submetacentric chromosomes, The X chromosome looks like the long longer chromosomes in this group.
Group D (13-15)	Medium –sized acrocentric chromosomes with satellites
Group E (16-18)	Relatively short metacentric or submetacentric chromosomes
Group F (19-20)	Short metacentric chromosomes
Group G (21-22,Y)	Short acrocentric chromosomes with satellites. The Y chromosome bears no satellites.

Some structural chromosomal abnormalities can be distinguished, such as:, chromatid break, chromatid gap, chromosome break, chromosome gap, dicentric and premature centromere separation (ISCN, 2005; Major, 2000).

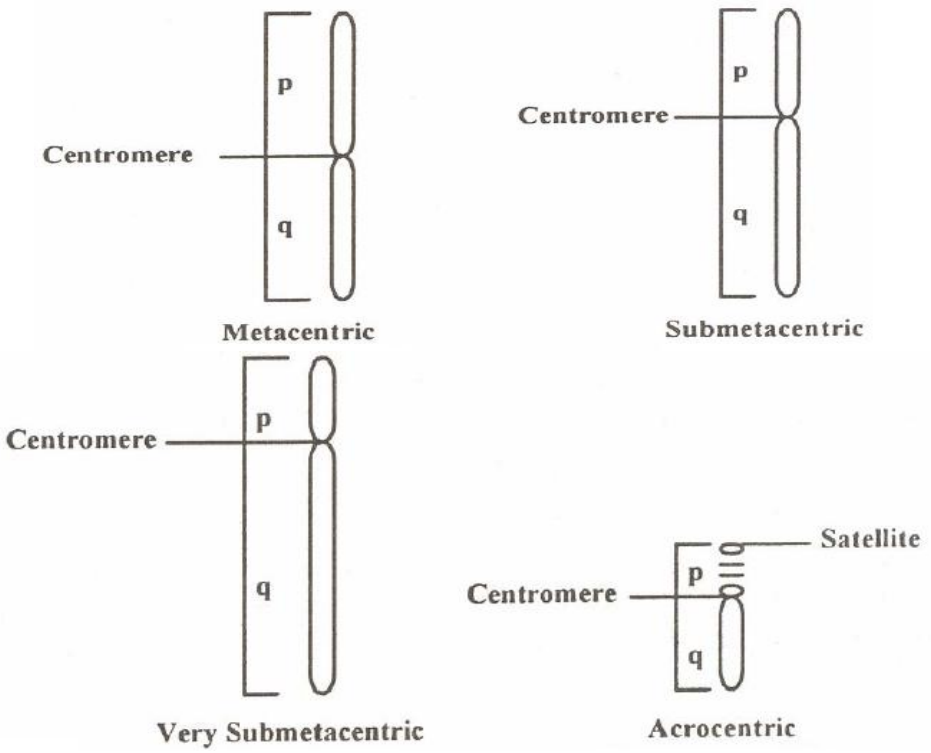

Figure 1: Chromosome morphology (Tseng, 1995)

Literature Review:

CAs are changes in normal chromosome structure or number that can occur spontaneously or as a result of chemical or radiation **(**MacGregor, et al., 2006).
Anwar (1995, pp.132) stated that the conventional cytogenetic technique for peripheral lymphocytes using geimsa staining is one of the most sensitive and suitable techniques for carcinogens identification. It gives the ability for fast and direct study of tested cells since it evaluates the whole genome and so suitable for monitoring of populations**.**

The chromosome aberration assay is considered to be a strong classical cytogenetic method for testing genotoxicity and can be used as a validation test for the results of comet assay (Hartmann, et al., 2003 cited in Leite-Silva, et al., 2007, p.2).

The most classically applied methods for risk evaluation are conducting cytogenetic methods (Tucker & Preston, 1996 cited in Major, 2000). The studies that investigate the effects of occupational exposures to genotoxic agents and result in that the CAs increase in exposed samples in comparison with unexposed controls are increasing yearly, and nowadays there are databases that include what such researches

revealed about agents and/or occupations with increased cancer risk according to the cytogenetic data (Major, 2000).

According to Natarajan (2005), although there are now many biomarkers that can be used to detect the degree of exposure to the genotoxins, the most powerful indicator of the potential for the future development of cancer is the degree of chromosomal aberrations.

Bueno and her collegues (2000) used analysis of the cytogenetic end points of two species of wild rodents residing in industrial, agricultural and a control area as a method of environmental genotoxicity evaluation. In our study we used human populations directly.

In the work of Testa, et al. (2002) chromosome aberrations (including dicentric and breaks), and chromatid aberrations (including breaks) were used in addition to other end points to detect the degree of risk of chronic occupational exposure to low levels of chemicals of workers in clinical analysis laboratories. Their research showed that the degree of DNA damage of exposed group was significantly higher than that of control one. Besides, the frequencies of chromatid breaks and chromosome breaks of exposed group were higher than those of control group.

Chromatid breaks and chromosome gaps were used to examine the genotoxic and antigenotoxic effects of *Fucus vesiculosus* extract on cultured human lymphocytes in the work of Leite-Silva, et al. (2007).

Studying chromosomes requires examining them at their clearest structure, which is at mitosis or meiosis, mainly when the cells are in the late prophase or metaphase. Bone marrow and peripheral blood lymphocytes are usual sources for chromosomal analysis, but peripheral blood lymphocytes are mostly preferred since their sampling is less invasive. To make sure of having adequate amount of actively dividing cells, the peripheral blood sample is cultured with B- or T- cell stimulants (i.e. mitogens) for about 48 to 69 hours (Qumsiyeh, et al., 2001).

Colcemid is added before harvest to block cells at the metaphase stage of the cell cycle (Au, et al., 1995).

Centrifugation step is followed to harvest cells, and the pellet is kept for the following step. A hypotonic solution is added to swell the cells, thereupon getting elongated metaphase chromosomes for clearer observation. After further centrifugation step and keeping the pellet again, a fixative solution is added three times, these steps of fixation are needed to preserve the cells so that the chromosomes remain in their mitotic state at a fixed based material instead of water based material. The cells are now ready for slide preparation, where the cells are placed on slides to be observed. The slide have to be put on a hot water bath for short time, so that the evaporating fix solution pops out the cell membrane that enables the cell contents to be flattened on the slide. Humidity is important factor, i.e. it is preferred to be above 40-50% in the lab. For chromosome hardening on the slides, the slides after that are kept on hot plate at 90°C for one hour or at 37°C over night, then stained to be visualized using light microscope.

According to Bayani & Squire (2004) the well spread metaphase with few overlapping chromosomes that are of good length is a critical parameter for the good application of the technique.

Types of CAs aimed to be detected in chromosomal aberration test in this study:

According to Major (2000), the most frequent aberrations noticed in the genotoxicology monitoring studies are the following: achromatic lesion (gap), terminal deletion (break) which are considered chromatid type aberrations, and terminal deletion (acentric fragment), dicentric chromosome (with one acentric fragment) which are considered chromosome type aberrations. Premature centromere separation is considered a possible genotoxicology end-point for biomonitoring studies.

Therefore, the following CA types are used as end points in this biomonitoring study: chromosome breaks, chromatid breaks and gaps, dicentric and premature centromere separation.

1.3. Comet assay or Single Cell Gel Electrophoresis (SCGE):

History:

According to Piperakis (2009) new approaches interested in monitoring DNA damage have been developed in the last two decades. Cook et al.(1976 cited in Piperakis, 2009) examined the nuclear structure based on cells' lysis with nonionic detergent and high-molarity sodium chloride to remove membranes, cytoplasm and nucleoplasm, and to disrupt nucleosomes, usually all histones become soluble by the high salt. By this, the nucleoid (composed of nuclear matrix or scaffold composed of RNA and proteins), is left with the negative supercoiled DNA (as a result of the turns made by the double helix around the histones of the nucleosome) and that such supercoils prevent DNA from free rotation, therefore, DNA is not a linear model but it is composed of successive loops. But, when an intercalating agent is added (e.g. ethidium bromide) or radiation affects the molecule, the supercoiling becomes unwounded, relaxed and the loops extend out the nucleoid.

Later on in 1978, Rydberg and Johanson (cited in Piperakis, 2009) tried to examine the strand breaks directly by embedding cells in agarose on slides and lysing under mild alkaline conditions.

After that, Ostling and Johanson (1984 cited in Piperakis, 2009) developed the comet assay depending on the same principle but the lysis and electrophoresis were performed under neutral conditions, and by using acridine orange for staining. Comet assay took its name since the resulted image looked like a “comet” with a distinct head, which represents intact DNA, and a tail, which includes damaged or broken fragments of DNA. In that procedure, only double-strand breaks could be analyzed. Actually, the microgel electrophoresis technique was first developed by Ostling and Johanson for DNA damage detection at the level of single cell (Industrial Toxicology Research Center, n.d.).

Important modifications were done by Singh, et al. (1988) and Olive, et al. (1990) (cited in Piperakis, 2009). Singh et al. (1988) applied the electrophoresis under highly alkaline conditions (pH>13) to enable relaxation and unwinding of the DNA supercoils and so made it possible to detect the alkali labile sites and single strand breaks in DNA during electrophoresis. By this modification the method can measure low levels of strand breaks with high sensitivity. Olive, et al. (1990) applied electrophoresis under neutral or mild alkaline conditions to detect single strand breaks, thereupon, this method was optimized to detect a subpopulation of cells with varying sensitivity to drugs or radiation (Piperakis, 2009).

Literature Review:

Nowadays, various studies applied comet assay using human peripheral blood lymphocytes whether in vivo or in vitro (Collins, et al., 1997 cited in Silva., et al. 2000),

Wirzinger, et al. (2007); Silva,et al. (2000); Leite-Silva, et al. (2007) and others used comet assay or Single Cell Gel Electrophoresis (SCGE) for detecting DNA damage in organisms. Many researches applied this technique on mammalian cells (Wirzinger, et al., 2007; Leite-Silva, et al., 2007; Silva,et al., 2000; Bilgici et al., 2005).

Piperakis (2009) mentioned that the alkaline comet assay has been widely spread since the 1990s, and probably it is one of the mostly used assays for DNA damage repair assessment nowadays. The popularity and acceptance of it can be shown by the entries total number in the PubMed database when using “comet assay” as search term (3575 entries by March 2008).

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Rozgaj, et al. (2004) considered comet assay a sensitive and a strong technique so used to evaluate the effects of mercury chloride in rats` blood samples. Comet assay showed to be a highly sensitive technique in the evaluation of DNA damage.

Recently, SCGE is considered a very valuable alternative for the cytogenetic tests (Sviezena, et al., 2004; Wirzinger, et al., 2007). Additionally, it is suitable for environmental monitoring (Silva, et al., 2000).

Comet assay is a method used to detect single and double strand-breaks in addition to alkali–labile sites and excision-repair events (Wirzinger, et al., 2007; Silva, et al., 2000).

Leite-Silva, et al. (2007) used comet assay in their study since it detects DNA damage like single and double strand-breaks and alkali–labile lesions in single cells after exposure to environmental genotoxins whether for short or long period of exposure time (Tice et al., 2000 cited in Leite-Silva, et al., 2007).

Jha, A., (2008) presented the single-cell gel electrophoresis or comet assay as the technique that has revolutionized the field of genetic ecotoxicology or eco-genotoxicology. He mentioned that it is a rapid, sensitive method providing the opportunity to study DNA damage (including oxidative damage), repair and cell death (apoptosis) in different cell types without prior knowledge of karyotype and cell turnover rate.

SCGE has many advantages: it is rapid since it gives the results within a few hours, economic and simple that it can be applied to various cell types and gives the ability to examine single cells (Wirzinger, et al., 2007).

Piperakis (2009) referred the rapid popularity and the large benefits of this technique nowadays among researchers to its various characteristics including: the appropriate cell lines, its applicability with good results in several types of eukaryotic cells in any organ, including plants and many prokaryotic cells, its capability to detect damage at the level of single cells. Additionally, it is noninvasive technique, rapid, simple and inexpensive so the results can be obtained on the same day. Other advantage, it is highly sensitive i.e. can detect 50–15,000 breaks/cell, and that the damage can be detected in cycling and non cycling cells. About the sample, a sample of a very small size (from 10,000 to 50,000 cells) can be used successfully, in addition to that, whether the sample is fresh or frozen it is considered appropriate.

Tice et al. (2000) as cited in Leite-Silva, et al. (2007), stated that the alkaline comet assay is very useful for examination of genotoxicity in the cells that exposed to different chemical and physical effects whether in vivo or in vitro.

Silva, et al.(2000) stated that in addition to being applied using human blood lymphocytes, other cell types and organisms were used in the use of comet assay

It is characterized by being a straightforward visual method for quantitative detecting of DNA damage in single cells, and is considered as a valuable tool in fundamental DNA damage and repair studies (Visvardis et al., 2000 cited in Leite-Silva, et al., 2007).

The alkaline version of comet assay was commonly used in many genotoxic studies and was preferred for its sensitivity (Bilgici et al., 2005).

The comet assay can be used to detect several classes of DNA damage by varying its conditions which cause nicks at the specific DNA lesions sites (Piperakis, 2009).

On a microscope slide precoated with agarose layer (Industrial Toxicology Research Center, n.d.), the nucleated cells are embedded in low melting point agarose.

Then, these slides are covered by a solution of high salt concentration to remove the membrane and histones, so what remains from each embedded cell is a nucleoid lying within a cavity in the gel (Singh et al., 1988; Tice and Strauss, 1995;
Tice et al., 2000 cited in Wong et al., 2005).

In the case of using blood samples, it is recommended to use Dimethyl sulfoxide (DMSO) in the lysing solution so that radicals resulted by the release of hemoglobin iron can be scavenged (Tice & Vasquez, 1999). .

The remained DNA (nucleoid) is exposed to unwinding usually under alkaline conditions to force the DNA supercoils for relaxation and expression of DNA strand breaks and alkali-labile sites. Besides, the use of highly alkaline conditions induces denaturation and unwinding of the duplex DNA, also the expression of alkali labile sites as single strand breaks (Industrial Toxicology Research Center, n.d.).

Electrophoresis might occur under neutral, mildly alkaline or strongly alkaline conditions (Tice and Strauss, 1995; Singh, 1996; Collins et al., 1997; Angelis et al.,

1999 cited at Wong, et al., 2005). By this step, the relaxed loops of damaged DNA which include breaks are pulled towards the anode during electrophoresis due to the effect of electric field (keeping in mind that the phosphate groups of DNA molecule are negatively charged at alkaline pH). Accordingly, a comet tail will be formed due to the migration of broken DNA, and a comet head will be formed due to the remaining coiled DNA in the nucleoid (Singh, 1996; Clingen et al., 2000; Tice et al., 2000 cited in Wong, et al., 2005). Figure (2) shows the comet components.

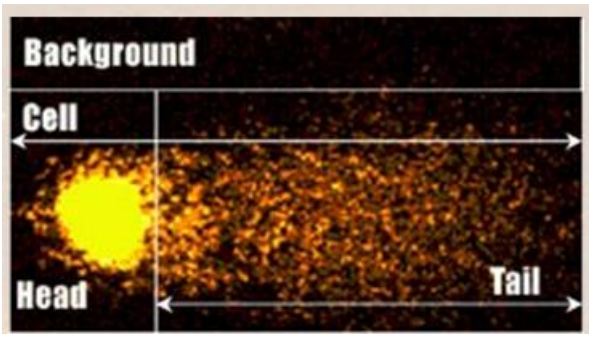


Figure 2: Illustration of comet components (Industrial Toxicology Research Center, n.d.).

According to (Industrial Toxicology Research Center, n.d.) getting the comets depends on two principles, firstly, the size and the amount of broken ends of the DNA determines DNA migration. Secondly, at the beginning of migration tail length increases with damage, but then reaches a maximum length depending on the conditions of electrophoresis, not the size of fragments.

Piperakis (2009) stated that breaks increase DNA migration, but DNA binding and crosslinks can retard DNA migrations. Accordingly, in the SCGE, the increased migration can indicate strand breaks, alkali labile sites and incomplete excision repair sites, but decreased DNA migration can indicate crosslinks DNA–DNA or DNA–protein interactions.

Generally, the amount of strand breaks is proportional to the amount of DNA in the tail relative to the DNA in head (Wong, et al., 2005).

A DNA staining fluorescent dye can be used such as ethiduim bromide to visualize the DNA (Tice and Strauss, 1995; Tice, et al., 2000 cited in Wong, et al., 2005). Other dyes used include propidium iodide (Olive, 2002 cited in Wong, et al., 2005), 4,6-diamidino-2- phenylindole (DAPI) (Panayiotidis and Collins, 1997 cited in Wong, et al., 2005) and YOYO-1 (Singh, 1996 cited in Wong, et al., 2005). Silver stain can be used too (Clingen, et al., 2000 cited in Wong, et al., 2005). Scoring of DNA damage can be done either by visual or computerized image analysis (Piperakis, 2009).

This principle is summarized by figure (3) which represents alkaline comet assay:

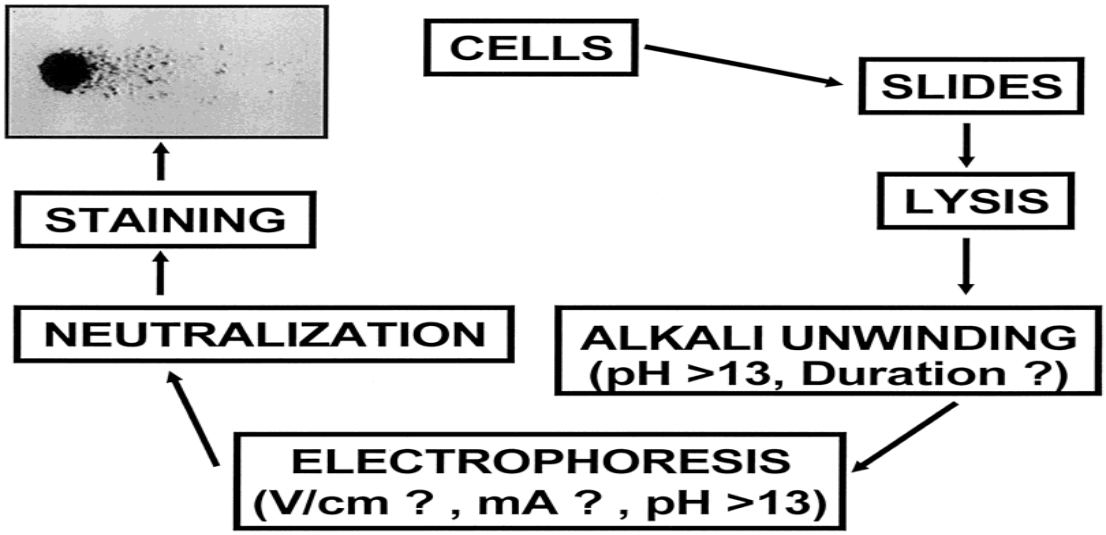


Figure 3: Major steps in alkaline comet assay method (Tice et al., 2000).

Alkaline comet assay:

The alkaline comet assay version is the one by which the DNA is enabled to unwind at pH >13 (Singh, et al., 1988 cited at Wong, et al., 2005). The use of high and strong alkaline conditions provides images of better clarity, and a response of steeper gradient (Tice, et al., 2000).

According to Dhawan, et al. (n.d.) and Wong, et al. (2005) the alkaline version of comet assay enables the detection of Single Strand Breaks (SSBs) (i.e. frank strand breaks and incomplete excision repair sites), the alkali-labile sites and crosslinking. Olive, et al., (1990, cited in Wong, et al., 2005) mentioned that at pH> 13 SSBs are formed from alkali-labile sites, therefore, the use of milder pH (i.e. pH= 12.3) does not enable the detection of ALS and because they will not be transformed into SSBs.

Tice, et al. (2000) paper argued that the alkaline version (pH.13) is considered the best to detect genotoxic agents since it is able to express SSBs, ALS, DNA-DNA/DNA protein cross-linking and SSB associated with incomplete excision repair sites. Additionally, the paper mentioned that there are several versions for the alkaline comet assay, but all have to meet the major critical steps in the version.

Wong, et al. (2005) mentioned that the comet assay is a relatively simple, sensitive and well validated tool for measuring strand breaks in DNA in single cells. Tice, et al. (2000) pointed that it is sensitive to detect low levels of DNA damage, the Industrial Toxicology Research Center (n.d.) mentioned that the assay is sensitive to detect 1 break in 10^{10} daltons.

In addition to that, it is fast, relatively not expensive and a small number of cells (about less than 10,000 cells) are required (Wong, et al., 2005), i.e. about only few microlitres (5-10μl) of blood or any other suitable tissue (e.g. nasal & buccal mucosal cells, epithelial cells, male germ cells, fine needle biopsy) are enough to perform the required strong statistical analyses (Industrial Toxicology Research Center, n.d.).

Since genotoxic and genoprotective agents might be cell type specific or tissue specific (Singh, et al., 1988; Burdon, 1999 cited in Wong, et al., 2005) it is a benefit of comet assay that a wide range of eukaryotic cells, whether proliferating or non-proliferating can be used successfully to apply the assay (Tice, et al., 2000; Collins,

2004 cited in Wong, et al., 2005) which is not a characteristic of cytogenetic biomonitoring studies that use mainly lymphocytes (Valverde, et al., 1999).

Besides, an image analysis software can be used, by which counting of about 50 to 100 cells/individual or treatment group is capable for a computerized picture of the stained cells (Industrial Toxicology Research Center, n.d.).

Flexibility is another feature of comet assay, since there are nowadays several versions available, each of which enables particular application of the assay. The principle of many of them is incubating the cells *in vitro* with the required agent before conducting the comet assay, then measuring the DNA damage. The required agent might be a genotoxic, antigenotoxic agent or a putative genotoxic or protective agent in order to be studied and determined. By this the cells can be collected before and after the addition of the agent to compare its effects (Wong, et al., 2005).

A table showing overview of comet versions is included in appendix d.

Piperakis (2009) mentioned that comet assay is conducted by many researchers in many fields nowadays. He mentioned part of such applications. One application is through human studies (e.g. biomonitoring, nutrition studies, aiding diagnosis (e.g. xeroderma pigmentosum, Nijmegen breakage syndrome), in addition to evaluate the background levels of DNA damage in individuals. Another application is ecological monitoring (using organisms as biosensors for measuring contamination of the environment with genotoxins). Genotoxicity testing is another field in which the comet assay used as a standard test to evaluate new chemicals' or pharmaceuticals' safety. Another application is the estimation of DNA repair by measuring repair capacity at the cellular level.

Types of DNA damage detectable by comet assay:

Double-strand breaks (DSBs) which appear as a result of DNA fragmentation. They are detectable by introducing them to neutral pH electrophoresis. Single-strand breaks (SSBs) which do not result in DNA fragments if the two strands of the DNA are not separated/ denatured. This can be achieved by DNA unwinding pH 12.1. Alkali labile sites (ALS) are expressed when DNA is treated with alkali at pH above 13. Also, by treating the DNA with lesion specific glycosylases/endonucleases, breaks can be introduced at the sites of DNA base modifications, then the fragments produced can be detected (Piperakis, 2009).

It is worth to be mentioned that the alkali labile lesions are able to be transformed into SSB's when using under alkaline conditions (Industrial Toxicology Research Center, n.d.).

Chapter 2: Study Area and Objectives:

Bruqeen village is located to the southwest of Salfit City at a distance of 7 kms, and at the foothills of the Barqan Israeli Industrial Park. The industrial park is one of many colonial settlement activities established in the occupied Palestinian territories since 1967. Bruqeen is one of the Palestinian villages that are affected by the industrial wastewater stream that is discharged from the industrial Park (ARIJ, 2001).

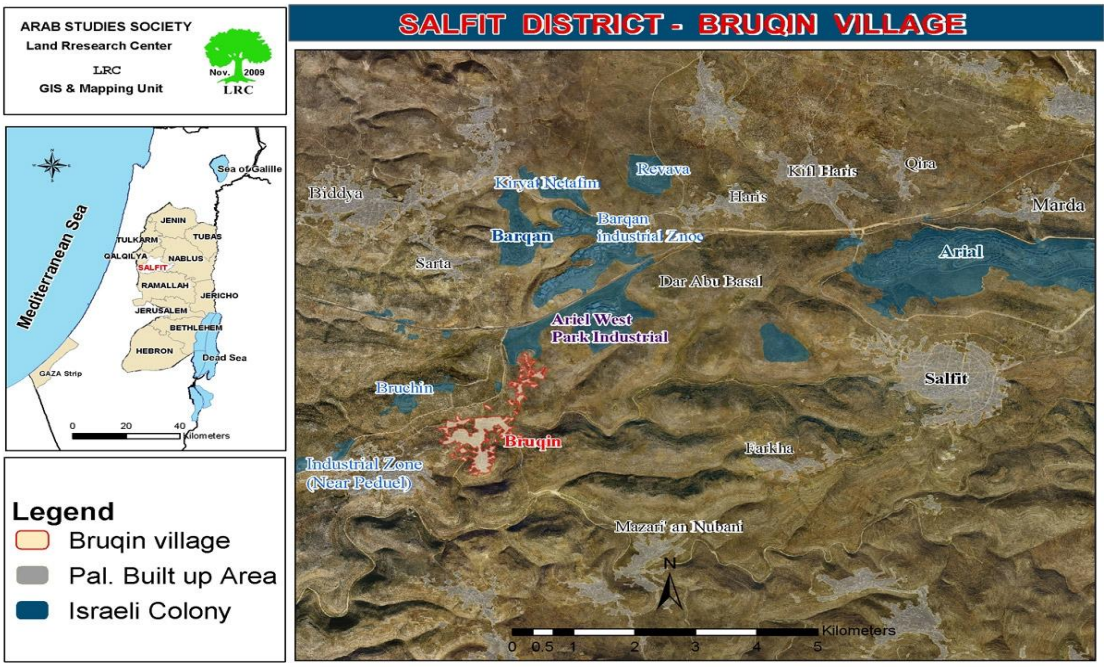


Figure 4: Map for Salfit district showing Bruqeen village location (ARIJ, 2004)

The Barqan residential settlement was established in 1981 northwest of Salfit City on about 649 dunums (1 dunum=1000 meters) of land from Haris Palestinian village. There were around 1262 Israeli settlers residing in this colony (ARIJ database, 2007 cited in Isaaq, 2007). The Barqan Industrial Park was established within the same area in 1982, it occupies an area of 1417 dunums and is considered to be one of the largest Israeli industrial Parks in the West Bank (ARIJ, 2007).

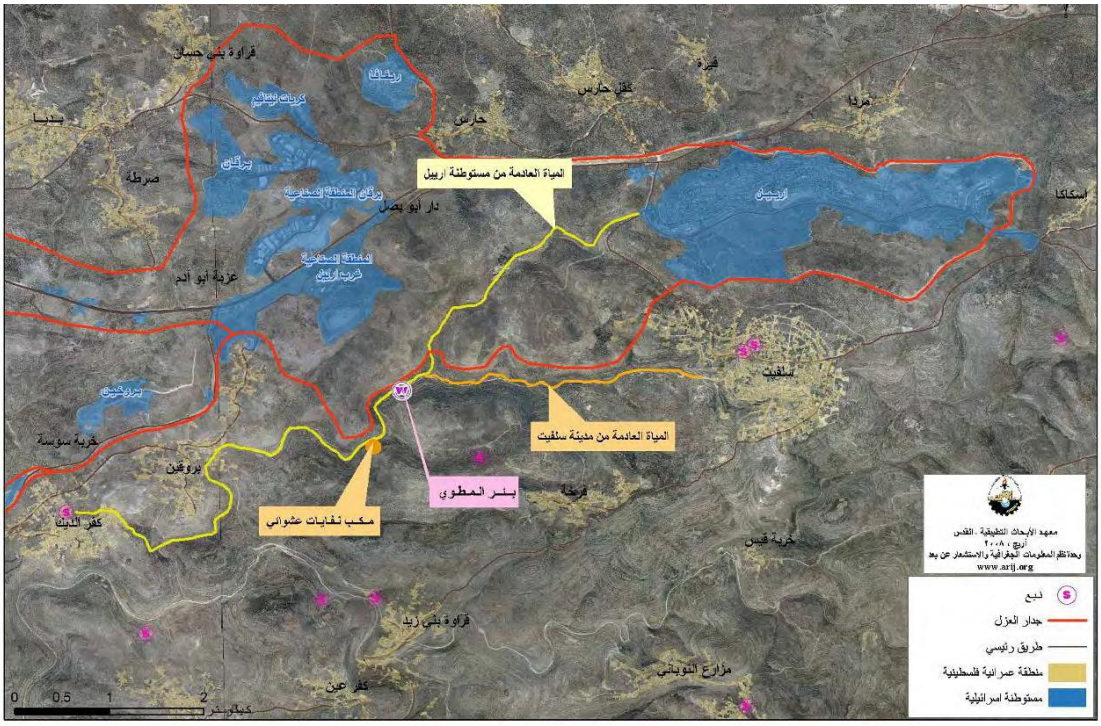


Figure 5: Wastewater flowing from Salfit city and Israeli settlements in the valley folded down to Bruqeen and Kafr Ad Dek villages (ARIJ, 2008).

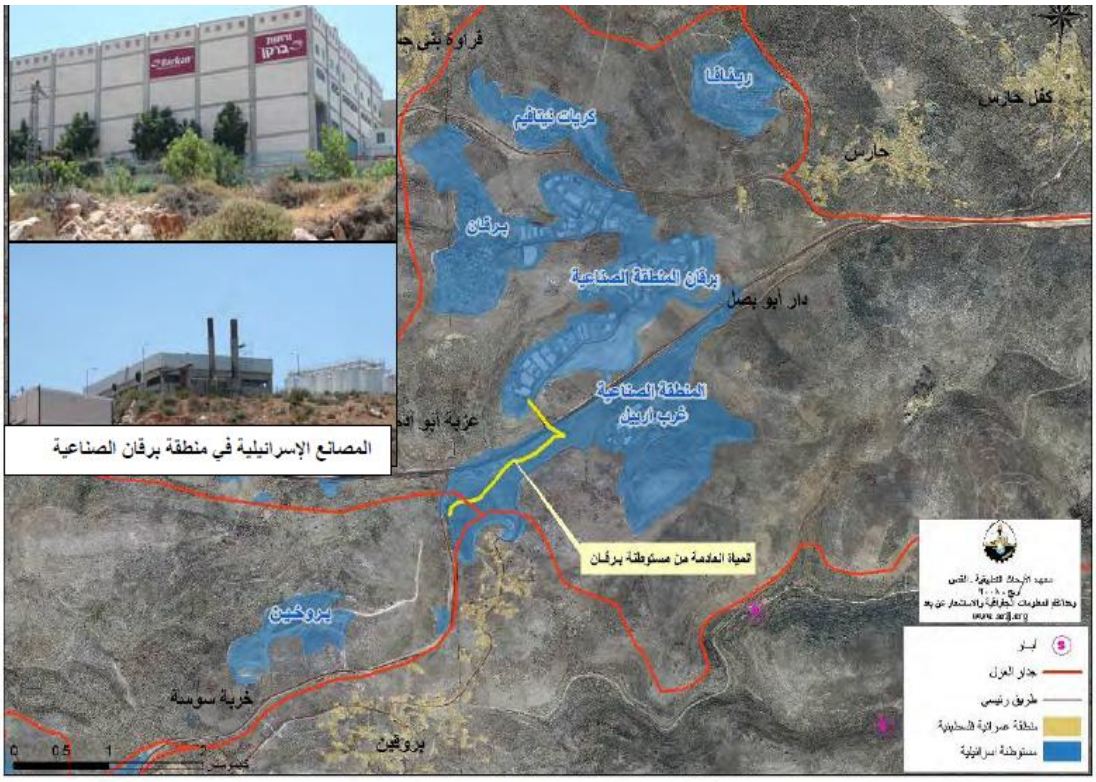


Figure 6: Wastewater flowing from the Barqan industrial zone (ARIJ, 2008).

The Barqan Industrial Park releases untreated waste water that includes hazardous waste and could be a source of pollution for air, soil and for the water Aquifer (Isaaq, 2007).

Figure 7: Agricultural land affected by the flow of industrial wastewater from the Barqan industrial Zone (ARIJ, 2008).

Figure 8: Picture for sheep and cows feeding on the plants growing around the dump site (ARIJ, 2008).

Figure 9: Barqan sewage pumped in Palestinian Bruqeen (ARIJ, 2004).

Figure 10: Bruqeen drowning in surrounding colonies sewage (ARIJ,2004).

Figure 11: Waste water flowing next to residential houses in Bruqeen village (ARIJ, 2008).

A report by ARIJ (2001) stated that there are about 73 factories in Barqan Industrial Park which vary in activities including: plastic manufacturing, painting manufacturing, electroplating, metal fabrication, wood furniture manufacturing, aluminum manufacturing, electronics manufacturing, surface coating industries and others. These industries could be dangerous to humans and to the environment. Such industries produce harmful substances such as heavy metals (including zinc, lead, cadmium, chromium, nickel, copper and others). Other pollutants include volatile organic compounds, acids, cyanides, arsenic, mercury, nitrogen oxides and others.

Pollutants produced by such industrial activities and unregulated waste disposal may pose costs and threats on society and individuals. Adverse impacts of these activities may be irreversible and diverse.

The existence of this industrial area may explain the preliminary findings and residents' reports of high occurrence of diseases in the affected villages, including:

- Skin diseases,
- Cancer,
- Mental and physical disabilities,
- Infertility,
- Respiratory diseases.

Worries about the impact of the Barqan Industrial Park practices on the health are expressed by the Technical Report of the Preliminary Study for the Barqan Israeli Industrial Park and its impacts. It included the complains of the Palestinians against the environmental, economical, health and other problems that resulted from these Israeli activities (ARIJ, 2001).

Palestinians interviewed in those areas expressed their concern to us about several areas:

1. Houses are located near a stream of discharged untreated industrial wastewater,

2. Spread of mosquitoes even in winter,

3. Bad odors,

4. Some of the trees appear to be burnt by the industrial wastewater,

5. The disappearance of some plants that used to grow in the area where the industrial wastewater is now discharged and the replacement by other kinds of plants,

6. The death of some animals in the area near the discharged industrial wastewater,

7. The high occurrence of certain diseases (appendix a),

8. The safety of animal products (e.g. milk) grazed in the area near the discharged industrial wastewater.

A visit was made to the Oncology Section at the Nablus National Hospital, and the social worker was met, who enabled me to collect the data shown in table (1) by manual searching from patients' records. The social worker mentioned that this information might not be informative as it is expected, because some patients registered as Salfit residents even they are from its villages, besides, many patients go to Al-Muttala'a Hospital in Jerusalem and others go to other hospitals rather than Nablus Hospital (e.g. Jordan), so these numbers might not reflect accurately the cancer incidence of these locations.

	Bruqeen	Kafr Ad Dek	Haris	Farkha	Snereiih	Sarta	Salfit	Kifl Haris	Deir Isteia	Iskaka	Bedia	Azzawia
2001		**2**	**1**	**1**	**1**		**3**	**1**	**2**			**1**
2002		**2**		**1**	**1**						**1**	
2003	**2**	**3**	**1**				**1**		**3**	**1**	**1**	**1**
2004		**5**	**1**	**2**			**3**	**1**			**2**	**2**
2005	**3**			**1**		**1**	**9**		**3**	**1**	**2**	**2**
2006	**2**		**2**			**5**	**9**	**3**	**2**	**1**		**2**
2007	**2**	**1**	**1**	**1**		**1**	**1**		**5**			**1**
2008	**2**		**1**	**1**	**1**		**4**	**1**	**1**		**1**	**2**
2009 Until 23/6/2009			**1**		**2**	**2**	**1**	**1**				**2**
Population # (Palestine General Federation of Trade Union,2009**)**	**2800**	**4000**	**2800**	**1200**	**2400**	**2500**	**7500**	**3500**	**3200**	**1000**	**6500**	**4500**
Frequency of cancer occurrence relative to population #	**.004**	**.003**	**.003**	**.006**	**.002**	**.0036**	**.004**	**.002**	**.005**	**.003**	**.001**	**.003**

Table 1: Numbers of registered cancer cases in chosen villages in Salfit governate (Nablus National Hospital, 2009).

But the literature reports that cancer rates may increase in areas subjected to pollution many years and even decades after exposure. So, current incidences may not reflect the impact of the industrial settlements on human health. Because of that, we thought it is appropriate to conduct a direct assay on chromosomal abnormalities and DNA damage in the exposed individuals.

The distinct advantage in applying genetic tests is that it opens the door to the possibility of detecting future health problems before they arise as major issues for protecting and warning the people from the danger of current pollutants (i.e. Early detection). Mainly, the results point out to the future carcinogenic dangers of the industrial pollutants components.

It is necessary to monitor the hazardous effects of the Israeli pollutants on the health of Palestinians residing around the Barqan Israeli Industrial Park in order to alarm the residents of the future hazards of these pollutants.

Chapter 3: Materials and Methods:

We collected blood samples in sodium heparin tubes (9 ml Sodium Heparin, greiner bio-one) subjects from the test area that had an average age of (15- 40 years old). They were chosen to meet the following criteria: residing in Bruqeen, their occupations do not require dealing with genotoxic materials and not being smokers. They included persons of various occupations (students, housewives, employees, workers, taxi drivers, unemployed and others). Signed informed consent forms were obtained from all participating individuals prior to the blood donation. Blood donors were also informed about the aim of the study. Each donor completed a standardized questionnaire in order to obtain relevant details of current health status, health history and lifestyle. The questionnaire included inquiring about: health, occupation and being smoker or not (Consent and questionnaire forms are included in appendixes b and c).

Field sampling was conducted at two main sites: Bruqeen village for the exposed samples, and Bethlehem city for control samples. The first sampling trip from Bruqeen village was in 5th of April 2009 (14 samples), while the second one was in 24th of February 2010 (29 samples). Chromosome aberration (CA) test was possible on 12 samples from the 1^{rst} sampling and 18 from the 2nd sampling (20 males and 10 females in general). On the other hand, Comet assay was conducted using 6 samples from the 1rst sampling and 19 from the 2nd sampling (15 males and 10 females in general). Control group for CA test was composed 8 samples (4 males and 4 females) while the control sample for comet assay composed of 5 samples (2 males and 3 females). Figure (12) show sampling process in Bruqeen:

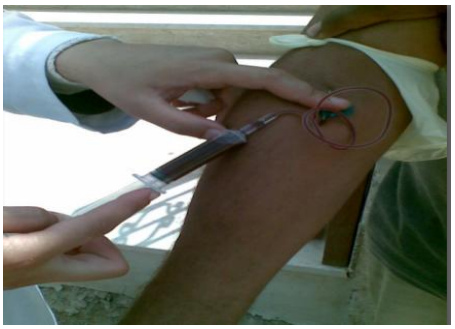


Figure 12: Sampling process implemented by the assistance of Mrs. Tahani Abdullah the Director of the Laboratory of medical tests at the Directorate of Salfit Health.

Peripheral blood samples (Volume= 5 ml) were collected by venipuncture into heparinised tubes (9 ml Sodium Heparin, greiner bio-one) between 10 a.m. and 2.00 p.m. After collection, all blood samples were coded, transported in a dark cool container (room temperature to Bethlehem University laboratory and processed as quickly as possible.

3.1. Cytogenetic analysis (Chromosome aberration (CA) test):

0.5 ml whole blood was incubated for at 37 ± 1^{o}C for 72 h with 5ml blood culture medium with a PHA (Peripheral Blood Karyotyping Medium with Phytohemagglutinin (500 ml), Biological Industries, Cat.# 01-201-1A). To arrest dividing lymphocytes in metaphase, 40µl Colceimed solution (Biological Industries, 10µg/ml in DPBS, Cat.# 12-004-1D) was added 1 h prior to the harvest, then centrifuged (1000 r.p.m.,10min). The supernatant was carefully removed with a pipette and the tubes were vortexed briefly to loosen the pellet. The cells were resuspended in a 10 ml hypotonic solution (0.075 M KCl) at 37^{o}C for 18-20 min incubation.

Cells became fragile during this incubation, therefore, rough handling and strong repipetting were avoided. About 1-2 ml fresh Carnoy's fixative (1: 3 Glacial Acetic Acid: absolute methanol) were added to the top of each tube. The tubes were centrifuged for 10 min at 1000 r.p.m. The supernatant was aspirated off and the pellet was resuspended (by tapping with finger tip or gently with pipette) in the remaining few drops. 10 ml of fix was added to the tube (slowly at first) then the tube was inverted gently to be mixed. Again, spinning for 10 min at 1000 r.p.m. was applied. The supernatant was aspirated off, 8 ml of fresh fix was added. Fixation and centrifugation were repeated several times (mostly 3 times) or until the pellet was clear white. At this step, the pellet is suspended in a small volume of fixative (about 1 ml, i.e. to appear cloudy). The cell suspension was dropped onto microscopic slides (Approx. 76x26 mm./3x1 inch, SB, ground edges, frosted ends, Germany) handled with 45^{o}-angle. The slides were put on a hot water bath for about 30 seconds with blowing directly onto the slide, then kept on warm plate (about 45°C until completely dry. Each slide was kept on hot plate at 90°C for one hour, or at 37°C over night for chromosome hardening. Slides were stained using Giemsa solution (HiMedia, Cat. # S011). Each slide was covered with Geimsa solution in pH 6.8 Gurr's Buffer for 3 minutes. All slides were coded and scored. Two hundred metaphases per subject were analysed for chromosomal aberrations using light microscope (Leica ATC 2000) with 100x magnification. Only metaphases containing 45–47 centromeres were analysed. Total numbers and types of aberrations for each sample were evaluated.

3.2. Comet assay (SCGE):

The Comet assay was performed according to Tice & Vasquez (1999) with modifications depending on trial and error in Bethlehem University lab.

A. Preparation of base slides: A day before conducting the assay, clean dry microscopic slides (Approx. 76x26 mm./3x1 inch, SB, ground edges, frosted ends, Germany) were dipped up to one-third the frosted area in hot 1.0% prepared normal melting point agarose (NMPA) (500 mg NMPA (Gibco, Cat. # 15510-019) per 50ml in Milli Q water) then were gently removed. The underside of each slide was wipped to remove agarose then slides were laid on a flat surface and air dried. They were

stored at room temperature until usage with avoiding high humidity conditions. Also, before the day of conducting the assay, a lysing solution and stock alkaline buffer were prepared.

B. Preparation of the lysing solution: To about 35 ml dH_2O the following ingredients were added: 7.305 g NaCl (2.5M) (Bio lab, Cat #: 19030291), 1.86 g EDTA (100mM) (Ethylene diamine tertra acetic acid, Sigma, Cat. # ED), 0.06 g Trizma base (10 mM) (Sigma, Cat. # T4661), then stirring the mixture was began, through that 0.4 g NaOH (Bio lab, Cat. # 19080391) was added, then the mixture was left to dissolve (about 20 min.). After that, the pH was adjusted to 10.0 using conc. HCl (Sigma, Cat. # 25814) or NaOH (MERCK, Cat #: 64271), (it was prepared a day before conducting the assay).

On the day of usage, final lysing solution became ready by adding fresh 1% Triton X-100 (i.e. 0.5 ml) (Triton X-100, Sigma, Cat. # T8532) and 10% DMSO (i.e. 5 ml) (Dimethyl sulfoxide, Sigma, Cat. # D84118) and then was refrigerated for at least 30 minutes prior to slide addition.

C. Preparation of alkaline buffer: Stock solutions: 10 N NaOH solution (10 g/25 ml dH_2O), and 200 mM EDTA solution (0.7445 g/10 ml dH_2O, pH 10) were prepared and stored at room temperature (Both stock solutions could be prepared every 2 weeks). 1X Buffer was made fresh before each electrophoresis run as follows: per 250ml: 7.5 ml NaOH and 1.25 ml EDTA, q.s. to 250 ml and mixed well. Before usage pH of the buffer was measured to ensure >13.

D. ***Preparation of Electrophoresis solution:*** Stock 10X TBE was prepared as follows: 5.4 g Trisma base, 2.75g Boric acid (Bio Chemika, Cat #: 15665) and 0.465 g EDTA were dissolved in 45 ml dH_2O. The volume was adjusted to 50 ml and then stored at room temperature. The working solution was prepared by diluting 10X to 1X in dH_2O (The preparation occurred 1-2 days before the following steps).

E. Cells isolation and slide preparation and treatment: To the coated slide, 100 µl of 0.8% (37ºC) Low Melting Point Agarose (LMPA) (prepared by dissolving 0.2 g LMPA (Sigma, Cat. # A9414-5G) in 25ml PBS (Dulbecco's Phosphate Buffered Saline, 10X, Sigma, Cat.# D1408), mixed with 15 µl of whole blood sample (after keeping the tube to settle to increase lymphocytes' collection potential). A coverslip was placed on the slide then the slide was put resting on ice until the agarose layer hardened (~10 minutes). The coverslip was gently removed, then a third LMPA agarose layer (about 75 µl or less, i.e. just to cover the slide) was put on the slide. Again, a coverslip was put on the LMPA layer, then the slide was returned on ice until the agarose layer hardened (~10 minutes). Slide preparation was applied under dim yellow light. The coverslip was removed, and the slide was gently and slowly lowered into cold, freshly made Lysing Solution, and kept in refrigerator (~4ºC) away of light (to avoid additional DNA damage) over night.

F. Expression of DNA damage: The slides were gently removed from the lysing solution, then placed side by side as close together as possible in horizontal container filled with freshly made pH>13 alkaline buffer (slides were completely covered with the buffer). The slides were kept for 20 minutes in the alkaline buffer (to enable DNA unwinding and the expression of alkali-labile damage).

G. Electrophoresis of Microgel Slides: The slides were removed gently from the alkaline solution and washed by being immersed in 1X TBE buffer. A horizontal gel box was filled with 1X TBE buffer, then the slides were then placed side by side as

close together as possible in the box. The power supply was turned on to 15 volts (~0.74 V/cm), and the slides were electrophoresed for 30 minutes. The power was turned off, then the slides were gently removed from the buffer and placed on a drain tray.

Slides were stained with 80µL 1X Ethidium Bromide (Sigma, Cat. # E7637), left for 5 min and then dipped in chilled distilled water (to get rid of excess stain), then a coverslip was placed over each slide, and was scored immediately using fluorescent microscope (Olympus BX41).
Time did not enable us to examine all slides immediately, so before staining, some slides were dried as follows: Slides were drained and kept for 20 min in cold 100% methanol (Bio Lab, Cat. # 13680521) for dehydration. The slides were air dried and placed at 50^0C for 30 min, then stored in a dry area. When we wanted to examine them, they were rehydrated with chilled distilled water for 30 min and stained with EtBr as previously mentioned, then covered with a fresh coverslip. Before viewing them, excess liquid on the back and edges was removed.

H. Evaluation of DNA Damage:
Observations were made of stained DNA using a 10X and 20X objectives on the fluorescent microscope. Infinity Microscopy Camera was used for taking pictures for cell samples, then Infinity software (Canada) was used to measure tail length and nucleus length. 100 randomly selected cells were analyzed per sample. All statistical analyses were done using SPSS 13.0 for Windows (Copy right(c) SPSS Inc. 1984-2004).

Chapter 4: Results:

4.1. Chromosomal analysis:

The data is divided into two groups: control group (n=8), and exposed group (n=30). For each individual in each group, the following parameters were examined: Premature centromere separation (PCS), Chromosome break (Csb), Cromatid break (Ctb), Dicentric, Frequency of CA and percentage of cells that have CA (% of cells with CA).

The objective of the study was to examine whether there are significant differences between the control and exposed groups by comparing these parameters.

For each, the following measurements were used through comparisons:

PCS: detected # of PCS out of 200 scored metaphases for each subject,

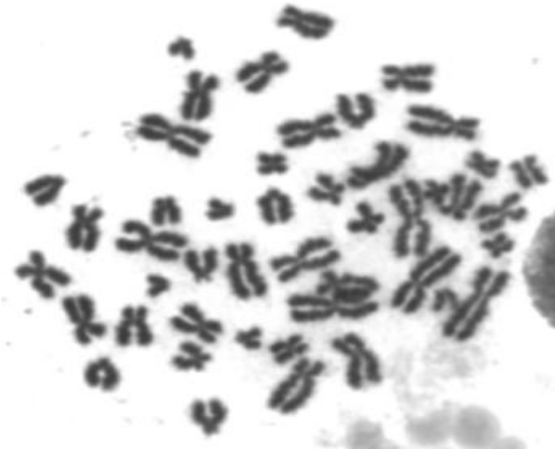
Csb: detected # of Csb out of 200 scored metaphases for each subject,

Ctb: detected # of Ctb out of 200 scored metaphases for each subject,

Dicnetric : detected # of Dicentric / 200 scored metaphases for each subject,

Frequency of CA : Total # of CA for each subject was scored in 200 metaphases) and the frequency is this number divided by 2 (i.e. Frequency per 100 cells)

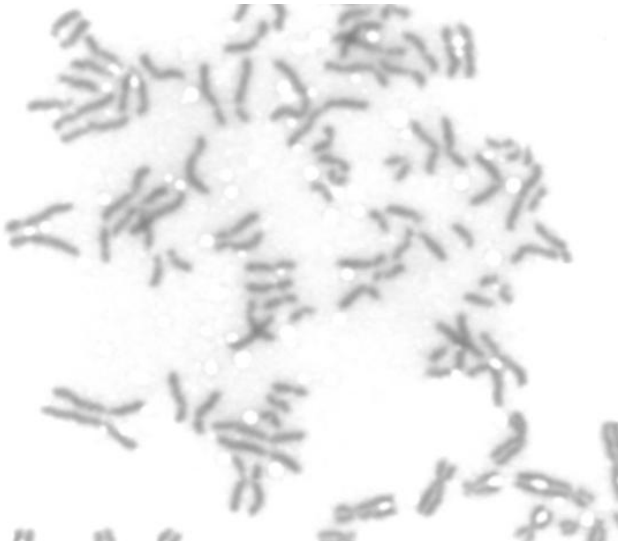
% of cells that have CA: Total # of cells with CA in 200 scored metaphases adjusted as frequencey of cells with any abnormality in 100 cells.



a

b

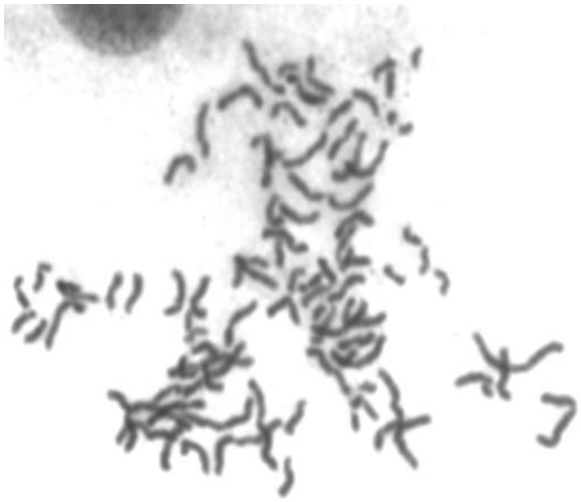
Figure 13: Representative pictures (a,b) for normal cases that do not include any cytogenetic abnormalities.



a

b

c



d

Figure 14: Representative pictures (a,b,c,d) for the occurrence of PCS.

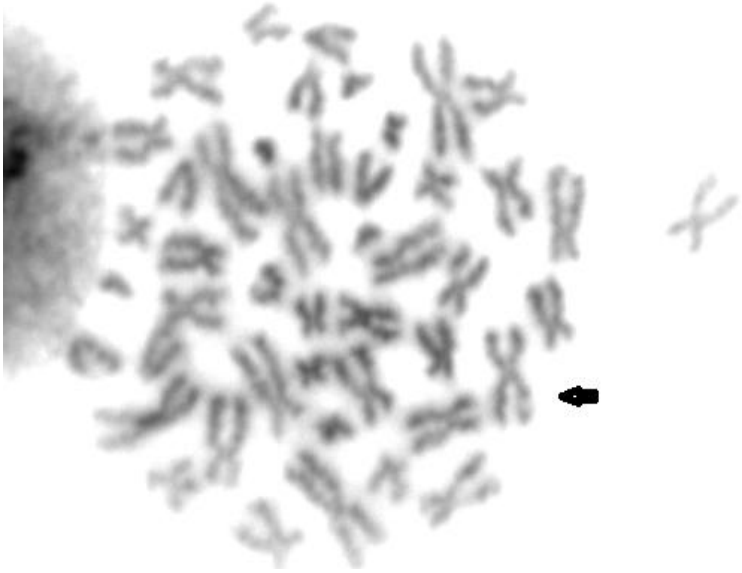


Figure 15: Representative picture for the occurrence of Ctb.

The following tables show descriptive data of exposed and control samples:

Table 2 : Descriptive data of Bruqeen sample (exposed sample), including data obtained about:PCS, Csb, Ctb, Dicentric (/ 200 metaphase / person), frequency of CA (taken as total aberration in 200 metaphases divided by 2) and % of cells that have CA (number of cells with any aberrations in 200 metaphases divided by 2)).

Participant	Gender	Age	PCS	Csb	Ctb	Dicentric	Total CA #	CA frequency	% of cells that have CA
1	M	35	4	0	1	0	5	2.5	2.5
2	M	20	3	0	0	1	4	2	2
3	M	38	3	0	0	0	3	1.5	1.5
4	M	36	0	0	0	2	2	1.26	1.26
5	M	15	2	1	0	0	3	2.6	2.6
6	M	30	7	2	0	1	10	5	4.5
7	M	22	3	2	0	0	5	2.5	2.5
8	F	33	3	3	0	1	7	3.5	3.5
9	M	22	3	0	2	2	7	3.5	3.5
10	F	20	4	1	1	0	6	3	3
11	F	19	6	4	1	2	13	6.5	5
12	F	39	12	3	0	0	15	7.5	7.5
13	F	18	9	7	8	0	24	12	10
14	F	32	3	0	0	1	4	2	2
15	F	40	7	4	2	1	14	7	6.5
16	F	30	1	0	0	0	1	0.5	0.5
17	F	26	4	1	3	3	11	5.5	5.5
18	M	20	2	0	0	0	2	1	1
19	F	22	4	4	0	2	10	5	4.5
20	M	33	4	1	5	2	12	6	6
21	M	37	5	1	2	0	8	4	4
22	M	31	20	1	6	1	28	14	12
23	M	30	3	2	0	0	5	2.5	2
24	M	31	2	0	2	0	4	2	2
25	M	25	2	0	0	2	4	2	2
26	M	21	3	3	1	2	9	4.5	4.5
27	M	27	7	1	4	0	12	6	5.5

28	M	39	3	2	1	2	8	4	4
29	M	25	3	0	1	0	4	2	2
30	M	20	1	0	0	1	2	1	1
Total	30	-	133	43	40	26	242	Average =4.08 %	Average= 3.81%

Table 3 : Descriptive data of control sample, including data obtained about:PCS, Csb, Ctb, Dicentric (/ 200 metaphase / person), frequency of CA (taken as total aberration in 200 metaphases divided by 2) and % of cells that have CA (number of cells with any aberrations in 200 metaphases divided by 2)).

Participant	Gender	Age	PCS	Csb	Ctb	Dicentric	Total CA #	CA frequency	% of cells that have CA	Residence
1	M	22	0	0	1	0	1	0.5	0.5	Bethlehem
2	M	37	3	0	1	0	4	2	2	Bethlehem
3	M	44	5	0	0	0	5	2.5	2.5	Hebron
4	M	26	2	0	0	0	2	1.5	1.5	Hebron
5	F	35	4	0	1	0	5	2.5	2.5	Bethlehem
6	F	19	2	0	0	0	2	1.26	1.26	Bethlehem
7	F	38	4	0	2	2	8	4	4	Bethlehem
8	F	26	1	2	0	0	3	1.5	1	Hebron
Total	8	-	21	2	5	2	30	Average= 1.97%	Average= 1.91%	-

The following table (4) shows descriptive information about the parameters of the two groups' data.

Table 4 : Descriptive information (Mean, Median, Standard Deviation) about the parameters of the exposed and control data: PCS, Csb, Ctb, Dicentric (/ 200 metaphase / person), frequency of CA (taken as total aberration in 200 metaphases divided by 2) and % of cells that have CA (number of cells with any aberrations in 200 metaphases divided by 2).

Parameters	Groups	Mean	Standard Deviation	Median
PCS	Exposed	4.4333	3.28677	3
	Control	2.6250	1.68502	2.5
Csb	Exposed	1.4333	1.71572	1
	Control	0.250	0.70711	0
Ctb	Exposed	1.333	2.02286	0.5
	Control	0.625	0.74402	0.5
Dicentric	Exposed	0.8667	0.9371	1

	Control	0.25	0.70711	0
CA Frequency	Exposed	4.201	3.13289	3.5
	Control	1.97	1.05495	1.75
% of cells with CA	Exposed	3.8673	2.6656	3.5
	Control	1.9075	1.1	1.75

Accordingly, the means, medians and standard deviations for all parameters of exposed group are higher than that of control group (except that the medians for Ctb of exposed and control are equal). To evaluate wether these differences are significant in chromosomal aberrations between the two groups or not, data should be examined. Normality was tested with considering the null hypothesis: Data follow normal distribution.

Table(5) shows the results.

Table 5 : Results of applying normality tests on exposed and control CA data: PCS, Csb, Ctb, Dicentric (/ 200 metaphase / person), frequency of CA (taken as total aberration in 200 metaphases divided by 2) and % of cells that have CA (number of cells with any aberrations in 200 metaphases divided by 2).

	1:Control group, 2: Exposed group	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Premature Centrome Separation	Control group	.168	8	.200*	.966	8	.862
	Exposed group	.278	30	.000	.723	30	.000
Chromosomal Breaks	Control group	.513	8	.000	.418	8	.000
	Exposed group	.233	30	.000	.806	30	.000
Chromatid Breaks	Control group	.300	8	.033	.798	8	.027
	Exposed group	.265	30	.000	.709	30	.000
Dicentric	Control group	.513	8	.000	.418	8	.000
	Exposed group	.289	30	.000	.797	30	.000
Frequency of Chromosomal Aberration	Control group	.183	8	.200*	.945	8	.661
	Exposed group	.140	30	.140	.862	30	.001
Percentage of Cells that have Chromosomal	Control group	.170	8	.200*	.950	8	.707
	Exposed group	.149	30	.086	.882	30	.003

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

From the table (5) it is clear that P-value for Csb, Ctb and dicentrics are <0.05, so normality is rejected, (even that the P-value for PCS, CA frequency and percentage of cells that have CA are > 0.05).

The following descriptive table (6) shows that mean ranks for all parameters of exposed samples are higher than that for control ones as are medians and means..

Tables 6 : Descriptive information (Mean ranks) about the parameters of the exposed and control data: PCS, Csb, Ctb, Dicentric (/ 200 metaphase / person), frequency of CA (taken as total aberration in 200 metaphases divided by 2) and % of cells that have CA (number of cells with any aberrations in 200 metaphases divided by 2).

	1:Control group, 2:	N	Mean Rank	Sum of Ranks
Premature Centrome Separation	Control group	8	15.25	122.00
	Exposed group	30	20.63	619.00
	Total	38		
Chromosomal Breaks	Control group	8	12.38	99.00
	Exposed group	30	21.40	642.00
	Total	38		
Chromatid Breaks	Control group	8	17.88	143.00
	Exposed group	30	19.93	598.00
	Total	38		
Dicentric	Control group	8	13.75	110.00
	Exposed group	30	21.03	631.00
	Total	38		
Frequency of Chromosomal Aberration	Control group	8	12.25	98.00
	Exposed group	30	21.43	643.00
	Total	38		
Percentage of Cells that have Chromosomal Aberrations	Control group	8	11.94	95.50
	Exposed group	30	21.52	645.50
	Total	38		

Accordingly, 2-independent samples test (Mann-Whitney Test) was used for comparisons between the exposed and control data for all variables, the Null hypotheses (Mean ranks for exposed and control are equal) was put under investigation.

The results of Mann-Whitney Test are shown in table (7) as following:

Table 7: Results of applying 2-independent samples test (Mann-Whitney Test) on exposed and control CA data: PCS, Csb, Ctb, Dicentric (/ 200 metaphase / person), frequency of CA (taken as total aberration in 200 metaphases divided by 2) and % of cells that have CA (number of cells with any aberrations in 200 metaphases divided by 2).

	Premature Centrome Separation	Chromosomal Breaks	Chromatid Breaks	Dicentric	Frequency of Chromosomal Aberration	Percentage of Cells that have Chromosomal Aberrations
Mann-Whitney U	86.000	63.000	107.000	74.000	62.000	59.500
Wilcoxon W	122.000	99.000	143.000	110.000	98.000	95.500
Z	-1.239	-2.193	-.502	-1.828	-2.083	-2.176
Asymp. Sig. (2-tailed)	.215	.028	.616	.068	.037	.030
Exact Sig. [2*(1-tailed Sig.)]	.235[a]	.041[a]	.661[a]	.104[a]	.038[a]	.028[a]

a. Not corrected for ties.

b. Grouping Variable: 1:Control group, 2: Exposed group

It seems from the table above that P-value for PCS, Ctb, Dicentric > 0.05, so there is no significant difference between exposed and control for those parameters. P-value for CA frequency, for % of cells that have CA and Csb < 0.05, so there is significant difference between exposed and control for these variables.

In order to examine the effect of gender on the degree of DNA damage, the same test was applied on the whole sample (exposed and control), The following descriptive table(8) shows that mean ranks for all parameters of females > of that for males. So the medians and means of females > of that of males. So, it seems that there is apperant difference for all parameters.

Tables 8 : Descriptive information (Mean ranks) about the parameters of males and females data: PCS, Csb, Ctb, Dicentric (/ 200 metaphase / person), frequency of CA (taken as total aberration in 200 metaphases divided by 2) and % of cells that have CA (number of cells with any aberrations in 200 metaphases divided by 2).

	Gender	N	Mean Rank	Sum of Ranks
Premature Centrome Separation	Male	24	17.56	421.50
	Female	14	22.82	319.50
	Total	38		
Chromosomal Breaks	Male	24	16.83	404.00
	Female	14	24.07	337.00
	Total	38		
Chromatid Breaks	Male	24	19.38	465.00
	Female	14	19.71	276.00
	Total	38		
Dicentric	Male	24	18.83	452.00
	Female	14	20.64	289.00
	Total	38		
CA Frequency	Male	24	17.04	409.00
	Female	14	23.71	332.00
	Total	38		
Percentage of Cells that have Chromosomal Aberrations	Male	24	17.69	424.50
	Female	14	22.61	316.50
	Total	38		

To test wether these differences are significant, the following Null hypothesis was put under investigation: Mean ranks for males and females are equal,

The results of Mann-Whitney Test are shown in table (9):

Table 9: Results of applying 2-independent samples test (Mann-Whitney Test) on males and females CA data: PCS, Csb, Ctb, Dicentric (/ 200 metaphase / person), frequency of CA (taken as total aberration in 200 metaphases divided by 2) and % of cells that have CA (number of cells with any aberrations in 200 metaphases divided by 2).

	Premature Centrome Separation	Chromosomal Breaks	Chromatid Breaks	Dicentric	CA Frequency	Percentage of Cells that have Chromosomal Aberrations
Mann-Whitney U	121.500	104.000	165.000	152.000	109.000	124.500
Wilcoxon W	421.500	404.000	465.000	452.000	409.000	424.500
Z	-1.433	-2.081	-.098	-.537	-1.791	-1.322
Asymp. Sig. (2-tailed)	.152	.037	.922	.591	.073	.186
Exact Sig. [2*(1-tailed Sig.)]	.161[a]	.054[a]	.940[a]	.643[a]	.076[a]	.191[a]

a. Not corrected for ties.

b. Grouping Variable: Gender

It seems from the table above that P-value for all parameters (except for Csb) > 0.05, so there is no significant difference between males and females for those parameters.

The effect of age was noticed through the comparisons between average values of the studied parameters data. Tables (10) and (11) show that.

Table 10: Mean values for CA data: PCS, Csb, Ctb, Dicentric (/ 200 metaphase / person), frequency of CA (taken as total aberration in 200 metaphases divided by 2) and % of cells that have CA (number of cells with any aberrations in 200 metaphases divided by 2) of exposed group.

Parameter	**Age (Mean values for mentioned parameters)**			
	< 20 (3 participants)	**20-29** (12 participants)	**30-39** (14 participants)	**>39** (1 participant)
PCS	5.6667	3.2500	5.0000	7
Csb	4.0000	1.0000	1.0714	4
Ctb	3.0000	1.0000	1.2143	2
Dicentrics	0.6667	1.0833	0.7143	1
Frequency of CA	6.6667	3.3058	4.2400	7
% of cells that have CA	5.8667	3.2217	3.8043	6.5

Table 11: Mean values for CA data: PCS, Csb, Ctb, Dicentric (/ 200 metaphase / person), frequency of CA (taken as total aberration in 200 metaphases divided by 2) and % of cells that have CA (number of cells with any aberrations in 200 metaphases divided by 2) of control group.

Parameter	**Age (Mean values for mentioned parameters)**			
	< 20 (1 participant)	**20-29** (3 participants)	**30-39** (3 participants)	**>39** (1 participant)
PCS	2.0	1.0000	3.6667	5
Csb	0	0.6667	0.0000	0
Ctb	0	0.3333	1.3333	0
Dicentrics	0	0.0000	0.6667	0
Frequency of CA	1.26	1.1667	2.8333	2.5
% of cells that have CA	1.26	1.0000	2.8333	2.5

It appears from the two tables above that is no age associated effect noticed in our samples.

4.2. Comet analysis:

The data is divided into two groups: control group (n=5), and exposed group (n=25).

For each, two measurements were determined:

The ratio of (tail length: nucleus length) represented as (TL: NL),

The ratio of (tail length: total length) represented as (TL/ (TL+NL)).

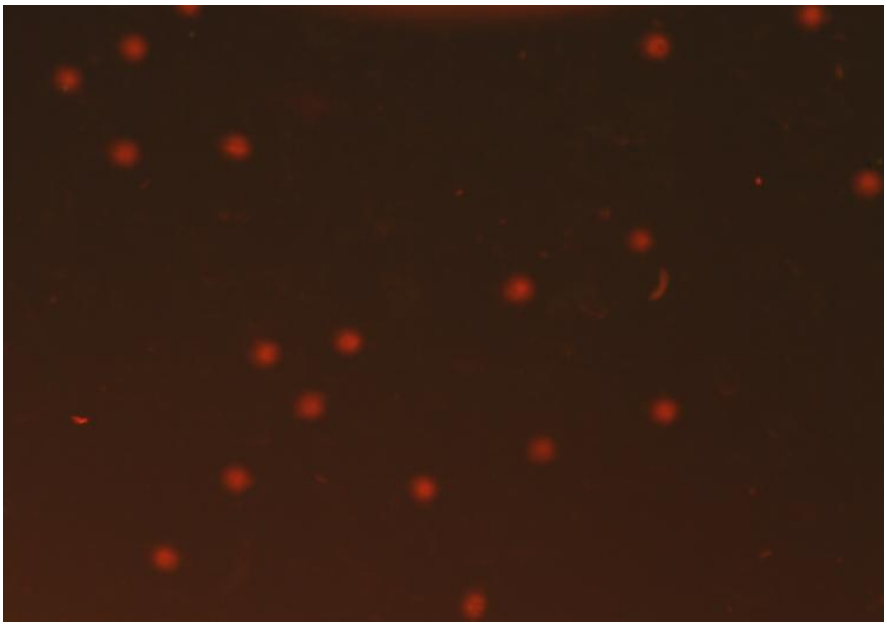
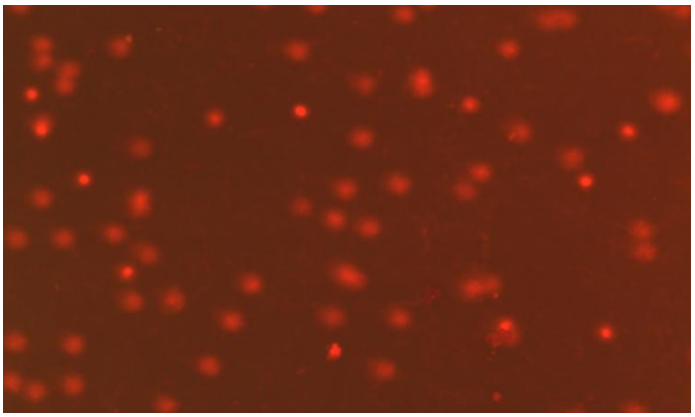


Figure 16: Representative picture for the control samples' comets.



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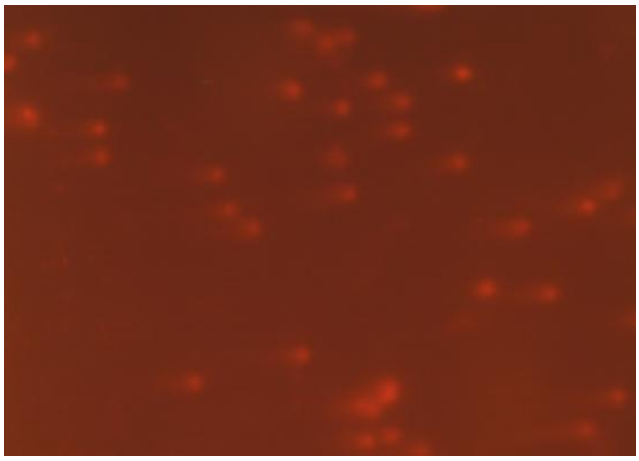

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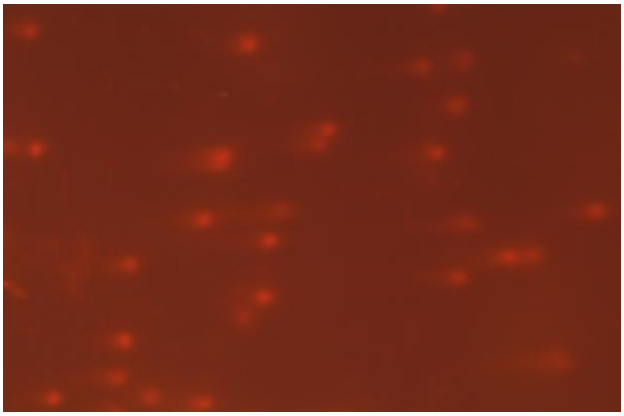

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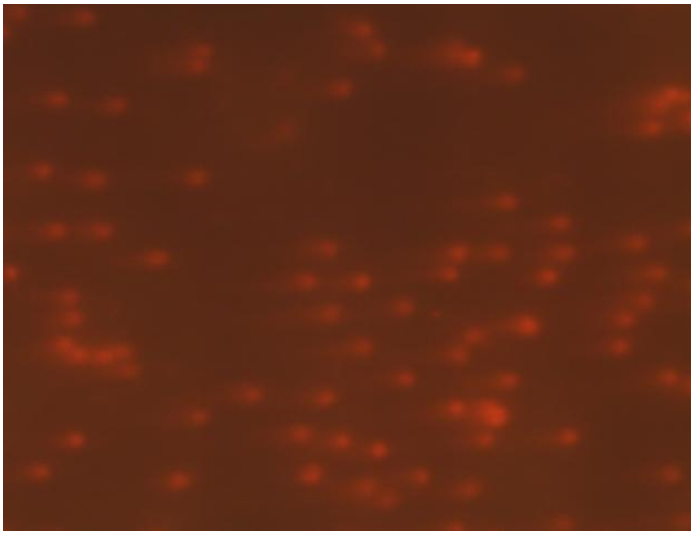

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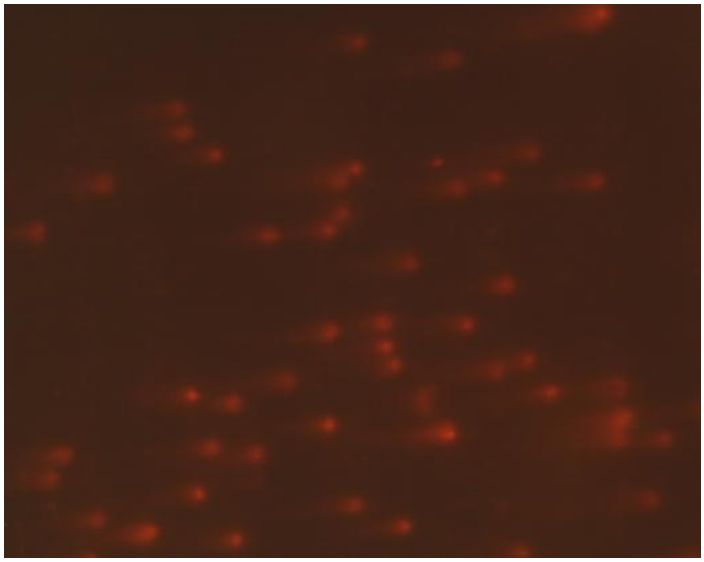

e

Figure (17): Representative pictures for the samples' comets, including different degrees of DNA damage.

Table (12) shows descriptive data about the: (TL: NL), (TL/ (TL+NL)) of the exposed and control groups' data.

Table 12: Descriptive data about the: (TL: NL), (TL/(TL+NL)) of the exposed and control groups' data.

Parameter	Group	Mean	Standard Deviation	Median
TL/NL	Exposed	1.2256	1.26258	0.9226
	Control	0.7356	1.29012	0.3292
TL/(TL+NL)	Exposed	0.4523	0.21931	0.4799
	Control	0.2801	0.24519	0.2477

Both mean and median data show increased DNA damage in the test versus control group (Table 12). To evaluate wether these differences in the two parameters between the two groups are significant or not, the data was tested for normality (Null hypothesis) and normality was rejected (Table (13)).

Table 13: Results of applying normality tests on exposed and control comet data ((TL: NL), (TL/(TL+NL))).

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	1:exposed, 2:control	Statistic	df	Sig.	Statistic	df	Sig.
TL : NL	Exposed group	.166	2500	.000	.732	2500	.000
	Control group	.284	500	.000	.561	500	.000
TL:(TL+NL)	Exposed group	.054	2500	.000	.970	2500	.000
	Control group	.135	500	.000	.916	500	.000

a. Lilliefors Significance Correction

The following descriptive table(14) shows that mean ranks for all parameters of exposed samples is higher than of that for control ones as are medians and means.

Table 14: Descriptive information(Mean ranks) about the parameters of the exposed and control data ((TL: NL), (TL/(TL+NL))).

	1:exposed, 2:control	N	Mean Rank	Sum of Ranks
TL : NL	Exposed group	2500	1602.99	4007475.50
	Control group	500	988.05	494024.50
	Total	3000		
TL:(TL+NL)	Exposed group	2500	1602.99	4007475.50
	Control group	500	988.05	494024.50
	Total	3000		

2-independent samples test (Mann-Whitney Test) was used for comparisons between the exposed and control data for the two parameters to test whether the apparent difference is significant and indeed it is (Table 15).

Table 15 : Results of applying 2-independent samples test (Mann-Whitney Test) on exposed and control comet assay data (((TL:NL), (TL/(TL+NL))).

Test Statistics[a]

	TL : NL	TL:(TL+NL)
Mann-Whitney U	368774.5	368774.500
Wilcoxon W	494024.5	494024.500
Z	-14.500	-14.500
Asymp. Sig. (2-tailed)	.000	.000

a. Grouping Variable: 1:exposed, 2:control

It seems from table (15) that P-value for both parameters < 0.05, so there is significant difference between exposed and control for them.

Chapter 5: Discussion:

The analysis for the chromosomal aberration test data showed a chromosomal aberrations increase in the test site, while the nonparametric tests revealed that there was no significant difference between exposed and control for PCS, Ctb and Dicentric data (P-value> 0.05), but there was significant difference between exposed and control for CA frequency, for % of cells that have CA and Csb (P-value < 0.05), (Table 7).

Gundy and Varga (1983) conducted a study about chromosomal aberrations in healthy persons which may be considered as a reference in monitoring studies for people exposed for radiation. Their study resulted in that: the chromosomal aberrations' frequencies occurred between 1 and 6% per cell (i.e. (counted # of CA /

scored # of metaphases for each subject) X100%), and the average of aberrations per person equaled 0.88% (i.e. the whole # of CA / the total # of the studied sample subjects). In our study, the chromosomal aberrations' frequencies of exposed samples were between 0.5 and 14% per cell, while the average of aberrations per person was about 8.07%. In comparison to their results, it appears that our exposed samples' chromosomal aberrations frequencies and average of aberrations per person exceed the average of the suggested values for healthy persons. Additionally, similar research was done by Kopjar, et al. (2006) resulted in that the range of the total number of chromosomal aberrations per 200 cells in healthy human volunteers was 0-5 (the counted # of CA out of the scored # of metaphases), while in our work the range was 1-28 for exposed sample, and 1-6 in control sample. Such a comparison indicates that our control sample range meets the expected range for healthy humans, while the exposed one exceeds the range of healthy persons that means their exposure to genotoxic agents. Their work showed another result, the range of total percentage of aberrant cells was 0-2.5% and its average was 0.48%, while in our work, the results obtained for the exposed sample were: a range of 1-12 with average 3.86%, and for control sample: a range of 0.5-4 with average 1.91%. It is clear that both our exposed and control samples' values exceed the expected values for healthy persons according to their study.

By comparing the detected types of chromosomal aberrations between exposed and control groups in our work, the percentage of Csb ((total # of Csb/ total # of CA) X100%) in control was 6.7% (2 Csb found in one person) while the percentage in exposed sample was 17.8% (43 Csb were found in exposed sample and found in 18 persons). Also, the percentage of dicentric chromosomes in control was 6.7% (only 2 dicentric were found in control sample and found in one person) while the percentage in exposed sample was 10.7% (26 dicentric were found in exposed sample and found in 16 persons). While, Pfeiffer et al.(2000) mentioned that naturally, the spontaneous occurrence of CA is low, that is about one dicentric chromosome per 1000 lymphocytes in humans. Therefore, such differences in CA occurrence, mainly Csb and dicentric chromosome is an indication of the exposure of Bruqeen residents to more dangerous pollutants than the control samples keeping in mind that Csb seemed to be the most important in the formation of CA (Natarajan, 1993; Pfeiffer et al., 2000) and that the dicentric chromosomes were considered a type of complex aberrations which were not found in the control group used in the work of Zeljezic and Garaj-Vrhovac (2001).

Studying the clastogenic effect of alkylating chemicals showed that these agents produced predominantly chromatid type aberrations and gaps. Alkylating agents which mainly form DNA adducts or crosslinks do not break the sugar-phosphate chain directly, instead, they can induce DNA-repair and then the misrepaired strand breaks can lead to the chromatid type aberrations (Major, 2000).

Ionizing radiation and a small number of chemical agents (e.g. streptonigrin, bleomycin, neocarzinosatin, cytosine arabinoside, and 8-methoxycaffeine) are capable of causing aberrations in all stages of the cell cycle (Major, 2000), this means chromosome-type aberrations in G1 and chromatid-type aberrations in S and G2 of the cell cycle (Pfeiffer, et al., 2000; Major, 2000). However, chromosomal aberrations caused by chemical treatments mostly occur during the S-phase, regardless of the treated stage of cell-cycle. Natarajan (1993) mentioned that the

types of CA induced by ionizing radiation depends on the stage of the cell cycle at the time of treatment, i.e. chromosome type aberrations in pre-replicative stages and chromatid type aberrations at S-phase and the post-replicative stages.

Chromosome-type aberrations can be lethal to cells because of the loss of acentric fragments at division, or due to the mechanical interference of the aberration with division, or they can cause cancer because of their effect on oncogenes and tumor suppressor genes (Major, 2000). According to Natarajan (1993) the double strand breaks seem to be the critical lesions that lead to chromosomal aberrations.

Pfeiffer, et al. (2000) stated that it is thought currently that the chromosomal breaks are formed by double strand breaks. Besides, it could be said that the breaks seen as terminal or interstitial acentric fragments through metaphase occur by double strand breaks that have not been repaired completely or not repaired at all.

Premature centromere separation (PCD or PCS) which is defined as the separation of centromere early in the prometaphase-metaphase stage was found in various praeneoplastic and neoplastic diseases and in several experimental systems like in vitro pesticide-treated human PBLs . Additionally, PCS might have significant effect on aneuploidy induction and it appears to be a form of chromosome instability (Major, 2000).

Individuals suffering from severe infections or diseases, smokers and people occupationally exposed to genotoxins were excluded from our study, but the presence of high values in the cytogentic test results for some persons made an attention that additional criteria should be took under consideration, i.e. exposure to diagnostic X-ray (including dental X-ray) and magnetic resonance imaging as recommended by Kopjar, et al. (2006), since diagnostic X-ray exposure significantly increased the degree of genetic damage in their study. So, such difference between our results and the results of the previously mentioned two studies of healthy persons indicates either that: the exposure of some blood donors of Bruqeen residents to diagnostic X-ray that affected the results negatively, or the more alarming result that the residents of Bruqeen are actually exposed to genotoxic pollutants that induce damage to their genetic material.

Comet assay was the second technique used in this study since it is sensitive enough to detect DNA damage, although it is not specific (Valverde, et al., 1999). Comet assay results showed that there was significant difference between exposed and control groups for all parameters ((TL: NL), (TL: Total length) since P-value <0.05 (table 15). Such a result is compatible and consistent with our chromosomal aberration test results (as mentioned earlier: CA frequency, % of cells that have CA, number of Csb, chromosomal aberrations' frequencies per cell and average of aberrations per person).

Comet assay showed significant difference for all parameters while chromosomal aberration test did not show significant difference for all parameters. This difference is associated with the difference in detection principle of the two techniques. For one, the comet assay detects various forms of DNA damage including alkali-labile sites and single strand breaks that might be removed later on by DNA-repair system i.e. repairable damage. Therefore, comet assay detects short-term genotoxic damage i.e. is short lived which points to recent events of pollution (Wirzinger, et al., 2007).

On the contrary, chromosomal aberration test detects persistent mutations that remained at least after one mitotic cycle (Kassie, et al., 2000 cited in Kopjar, et al., 2006). This is because that DNA damage in the non-cycling lymphocytes will not be converted into aberrations until the stimulation of these cells to re-enter cell cycle and DNA replication *in vitro*. Therefore, it is not necessarily that the frequency of aberrations will be proportional to the amount of induced DNA damage, due to the fact that DNA damage repair might occur in G_0 cells and during the first *in vitro* G1 stage. So that, the aberrations' frequency will be proportional to the remained amount of DNA damage at the time of DNA replication (IPCS, 1985). Zeljezic and Garaj-Vrhovac (2001) reported the same idea too as follows: the lesions induced by chemicals have to pass through the S phase, so that they can be transformed into aberrations, such transformation occurs after stimulation by the mitogen *in vitro* which provides a suitable time for repairing of the damage. Therefore, IPCS (1985) and Wong, et al. (2005) even mentioned that cytogenetic studies are commonly used on a wide range in such studies, but since their principle provides a time (between stimulation of CA *in vitro* by a mitogen and fixation) which is normally the first DNA replication *in vitro* that enables repairing of many of the genetic damage occurred *in vivo*, they considered cytogenetic studies of limited sensitivities and that this feature is a drawback of cytogenetic assays.

Crebelli & Caiola (2009) in their commentary report stated that the chronic exposure to air pollutants can be discovered by peripheral blood circulating lymphocytes monitoring which are to some extent enough to show DNA damage. This is because the DNA repair ability is reduced because of the deficient intracellular deoxyribonucleosides pool and lower efficiency in nucleotide excision repair. On the other hand, they mentioned that it must be taken into consideration that although the use of cytogenetic analysis on human blood cells in biomonitoring studies is popular and widely used, these methods are of limited sensitivity to *ex vivo* chemical exposures relatively. This is because chromosomal aberrations, for example, require the stimulation of mitogen *in vitro* to be formed. Thereupon, there might be a repair of primary induced damage in the time between stimulation and fixation *in vivo* in circulating lymphocytes (i.e. the first DNA replication *in vitro*).

Regardless, comet assay gives an indication about the current genetic damage at the time of blood sampling pointing to the current exposure situation, while chromosomal aberration test gives further information about the degree of genetic damage at present and past exposures since it detects damage fixed after the action of cell repair system (Kopjar, et al., 2006).

It was pointed that lessening the exposure to the mutagen might return the CA to their accepted levels as expected control samples mentioning that the chromosomal alterations are reversible (Anwar, 1994). Similarly, according to Zeljezic and Garaj-Vrhovac (2001) spending particular time, i.e. 8 months away from the mutagen (pesticide exposure) after being exposed to it (for 8 months) decreased significantly the CA formation. Such an idea gives the hope for having again the normal degree of CA of Bruqeen residents if the source of pollution is removed. Moreover, it gives a hint for scientists for further studies about that region and others to make stronger decisions of exposure to clastognes.

For our control samples, the chromosomal aberrations' frequencies occurred between 0.5 and 4% per cell which does not contradict with Gundy and Varga

results, but average of aberrations per person equaled 3.75% which is higher than the result found in their study. Such a result for control sample might be due to the small size of the control sample (n=8) which might raise the value. If the small sample number might not affect the result, then this value is an alarm for another probable fact that even people who are not residing near the source of industrial waste water, they are exposed to other forms of genotoxins in their residence locations.

These results support our research hypothesis that Bruqeen residents are exposed to factors that induce DNA damage due to the flow of Barqan Industrial waste water.

From the descriptive table (4) it appears that the standard deviations' values for the exposed group are relatively high. This can be explained according to the work of Kopjar, et al. (2006) who pointed to the presence of inter-individual differences between persons that might be associated with different genome sensitivity, and mentioned that the idea of variability was reported by different researchers who conducted cytogenetic end points and comet assay.

From the descriptive table (4), it appears that the standard deviations of exposed data for cytogenetic analysis are much higher than that of control data. It seems that this is due to the difference in control and exposed samples' sizes. Another possible reason is that the exposed individuals varied in their residing locations from waste water flow from 10 meters to 1 kilometer, while all control sample participants are far away from industrial waste water supply.

Some biomonitoring studies included in addition to their major aim noticing whether the gender contributes to the degree of DNA damage or not. The results obtained about the effect of gender on the degree of genetic damage was contradictory since some studies found that there are differences between males and females while others found that the degree of genetic damage in males and females are similar (Kopjar, et al., 2006). Møller, et al. (2000) mentioned that the effect of gender is not clear since various reports showed that both men and women had high levels of DNA damage. In the work of Kopjar, et al. (2006) gender did not significantly affect the degree of genetic damage. Our study showed a similar result, it seemed from the table (9) that P-value for all parameters (except for Csb) > 0.05, so there is no significant difference between males and females for those parameters.

The effect of age was also studied in some biomonitoring studies. Again, according to Kopjar, et al. (2006) the data obtained about the presence of age-related increase in genetic damage using the two applied tests were contradictory too. Møller, et al. (2000) stated that it seems that the effect of age is of little impact. Some studies found that there is an age-related increase in genetic damage using the alkaline comet assay. Others found that age does not affect the degree of genetic damage significantly. Our study showed that there is no age associated effect noticed in our samples due to the ungraduality in values of the control and exposed samples (tables 10, 11).

Correlation between Chromosomal aberrations, DNA damage and cancer:

Although Albertini, et al. (2000) mentioned that most human carcinogens are genotoxic, but not all genotoxic agents have been shown to be carcinogenic in humans. Various reports showed an association between the extent of chromosomal damage and chromosomal aberrations (CA) and the risk of cancer.

The role of some chemicals and ionizing radiation in inducing DNA double-strand breaks, if not repaired, are transformed into CA during cell division is well established. Measuring the frequency of chromosomal damage in humans exposed to occupational and environmental clastogens has been a priority in public health studies for decades, and an increased level of CA in population groups is currently interpreted as evidence of genotoxic exposure and early biologic effects on DNA (MacGregor, et al., 2006).

Major (2000) suggested that there is relation between the increased CAs and the increased cancer risk.

Rossener, et al. (2005) conducted a study to find the relation between the risk of cancer and the chromosomal aberrations in lymphocytes of healthy persons. They found that there is a significant correlation between the presence of chromosome-type aberrations and the occurrence of cancer in general, mainly with stomach cancer. The study resulted in assuring that the presence of chromosomal aberration is a predictor of cancer, especially stomach one.

Crebelli & Caiola (2009) in their commentary report mentioned that using cytogenetic biomarkers - that structural chromosomal aberrations are examples of them - can predict cancer risk. Additionally, they can be used as valid tools in cancer surveillance through the prevention programmes in occupational and environmental health.

According to Anwar (1994) the chromosomal changes may be associated intrinsically to cancer development, and particular chromosomal aberrations seem to be associated with many types of cancer. It is thought that the abnormalities can activate oncogenes or result in the losing tumor suppressor genes.

In general, Pfeiffer, et al. (2000) divided CA into two types: part of them are lethal, while others might force the oncogenic transformation, e.g. the inactivation of tumer suppressor genes, or the activation of oncogenes through formation of new fusion proteins that are able of beginning carcinogenesis. Additionally, the increased levels of CA are usually associated with increased levels of cancer.

Chapter 6: Conclusions and Recommendations:

Our study showed that there is a significant effect on the genetic material of residents of the polluted area (Bruqeen village). The cytogenetic analysis showed that there was a statistically significant difference for CA frequency, for percent of cells that have CA and Csb between exposed and control group, while Comet assay showed that there was significant difference for all comet parameters between exposed and control group.

Additionally, the control sample showed higher values for most compared parameters than that found in literature for healthy subjects, that suggests another hypothesis: also the control is subjected to genotoxins but to a lower degree than the studied site (i.e. Bruqeen).

Conducting further biomonitoring studies on Bruqeen village is recommended in order to give justified judgments about the actual healthy and environmental situation in the region. This will help in protecting the affected area residents' by demanding stopping the flow of industrial waste water and all forms of industrial wastes to their lands and surroundings from the industrial settlements.

It is recommended to assess the degree of genotoxic effect using whole blood samples from grazing animals (e.g. sheep and cows), field animals (e.g. rodents) and plants in addition to residents' blood samples.

It is recommended too to increase the exposed and control samples' sizes, with including Palestinians residing in Salfit region in the control sample.

In order to examine whether the Palestinians are more prone to chromosomal aberrations and genetic damage, it is recommended to apply the same tests on Palestinians residing in other regions rather than Salfit Province (i.e. other regions inside and outside Palestine).

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Appendixes*:*

Appendix (a)

Summary of cases in Bruqeen for years: 2006, 2007 & 2008.

(According to Statistics & Information Dep. in Salfit, Ministry of Health, Palestinian National Authority).

#	Health Problem	# of cases in years		
		2006	2007	2008
1	Intestinal Infectious Diseases(Diarrhoea & gastroenteritis of presumed infectious origin)	46	19	26
2	Sexually Transmitted Diseases	1		
3	Viral Diseases(Chicken pox)	7	1	10
4	Helminthiasis			39
	Taeniasis	1		
	Enterobias vermicularis	25	12	
5	Malignant neoplasm of endocrine		1	
6	Benign neoplasms	1		
7	Anemias	14	4	18
8	Diabetes mellitus	82	38	131
9	Diseases or Central Nervous System			
	Abscess	2		1
	Migraine & other headache Syndrome	1		
	Epilepsy			4
10	Diseases of eye & adnexa	88	57	98
11	Diseases of the ear & mastoid process	47	27	95
12	Diseases of the circulatory system			
	Acute rheumatic fever	3		24
	Chronic rheumatic heart disease	28	26	24
	Hypertensive disease	174	148	272
	Angina	12		
	Chronic ischaemic heart disease	75	42	106
	Heart failure	3		
	Haemorrhoids	33	16	47
	Non rheumatic valve disorders			1
13	Diseases of Nose & Nasal sinusitis			
	Rhinitis	2		25
14	Upper respiratory tract infections			
	Acute tonsillitis	225	200	344
	Acute pharyngitis	142	110	161
	Acute laryngitis & tracheitis	3		
	Chronic tonsillitis	7		6
15	Lower respiratory tract infections			
	Acute bronchitis & acute bronchiolitis	154	100	359
	Pneumonia	85	50	
	Chronic obstructive pulmonary disease(bronchitis, emphysema & asthma)		56	116
	Bronchiectasis & lung abscess		13	
	Influenza			6
16	Oral cavity, glands & jaws	34	43	93

17	Disease of oesophagus, stomach & duodenum	88	105	202
18	Diseases of Intestines	1	6	12
19	Diseases of rectum & anus			6
20	Diseases of gallbladder, liver & pancreas	2		
21	Skin & Subcutaneous tissue	65	57	122
22	Diseases of the musculoskeletal system & connective tissue	151	127	272
23	Diseases of the urinary system	35	29	60
24	Diseases of male genital organs	13		
25	Disorders of breast	8		
26	Inflammatory diseases of female pelivic organs	58	13	61
27	Non inflammatory disorders of female genital tract	2		
28	Pregnancy, childbirth & puerperium	33	3	5
29	Congenital malformations, deformations & chromosomal abnormalities			1
30	Symptoms, signs & abnormal clinical & laboratory findings, not elsewhere classified			3
31	Contact with venomous animals & plants			1
32	Factors influencing health status & contact with health services Persons encountering health services for examination & investigation Persons encountering health services in circumstances related to reproduction	249 175	158 155 1	407 401 1

Appendix (b)

جامعة بيت لحم

برنامج الدراسات العليا المشترك بين جامعة بيت لحم وجامعة بوليتكنك فلسطين

برنامج الماجستير في التكنولوجيا الحيوية

<u>نموذج موافقة</u>

صمم هذا البحث العلمي بهدف دراسة وتقييم مدى التأثيرات السلبية للمخلفات والفضلات الصناعية على صحة المواطنين. يتضرر الإنسان بوجود بعض المكونات الصناعية في مياه شرب المواطنين, أو في المياه التي تمتصها النباتات المزروعة وبالتالي تصل لجسم الإنسان سواء بتناول هذه النباتات أو الحيوانات التي ترعى هذه النباتات, بالإضافة للمواد السامة التي يستنشقها الإنسان عن طريق الهواء. التأثيرات الضارة لا تظهر فورا لكنها تعمل على إحداث تغيرات غير طبيعية و تدمير(بنسب متفاوتة) في الحمض النووي الوراثي والكروموسومات.

إن رصد وجود تغيرات غير طبيعية في المادة الوراثية هو مؤشر قوي لمدى احتمالية حدوث وانتشار أمراض مختلفة وخاصة السرطان.

كافة النتائج ستبقى سرية, ويبقى لكم خيار المشاركة في هذا البحث.

في حال الموافقة ستؤخذ عينة دم ليتم فحصها في مختبر جامعة بيت لحم.

مع تقديرنا لتعاونكم ومشاركتكم لإنجاح البحث.

أنا الموقع/الموقعة أدناه أوافق على المشاركة:

الاسم: _____

التوقيع: _____

التاريخ: _____

Appendix (c)

Biotechnology Master Program

Bethlehem University

Joint Master Program between Bethlehem University and Palestine Polytechnic University

Biotechnology Master Program

Form of specific information about each participant to ensure the relatedness of results to the effect of the industrial pollutants released in Bruqeen area.

I- General Information:

Date:_____

1- Sex: 1) Male 2) Female

Name: _____

2 -Place of residence: 1) Bruqeen

2) Other (for control samples)

Name of place: _____

3- Age: _____

4- Marital Status: 1) Single 2) Married 3) Other.

II- Healthy status:

5- Suffering from diseases, if yes, explain

Type of sickness: _____

Date of sickness (Period of sickness): _____

Frequency of sickness: _____

6- Presence of hereditary diseases in the family: 1) Yes 2) No

If yes, explain: _____

III- Occupation:

7- Occupation/s: _____

8- Place of work: In Bruqeen: 1) Yes 2) No

If yes, specify: _____

In Salfit District: 1)Yes 2) No. If yes, specify:_____

In other district: 1) Yes 2) No. If yes, specify:_____

In Israel: 1) Yes 2) No. If yes, specify: _____

In an Israeli settlement: 1) Yes 2) No. If yes, specify:_____

In Barqan Israeli industrial park: 1) Yes 2) No. If yes, specify:_____

9- Number of hours per day of working: _____

10- Number of days per month of working: _____

VI- Sources of water & food:

11- Source of drinking water: _____

12- % of eating plants grown in Broqeen: _____

13- % of consuming products of animals grazing in Broqueen: _____

VII- Smoking :

Are you smoker or not? _____

If yes, from what time? _____

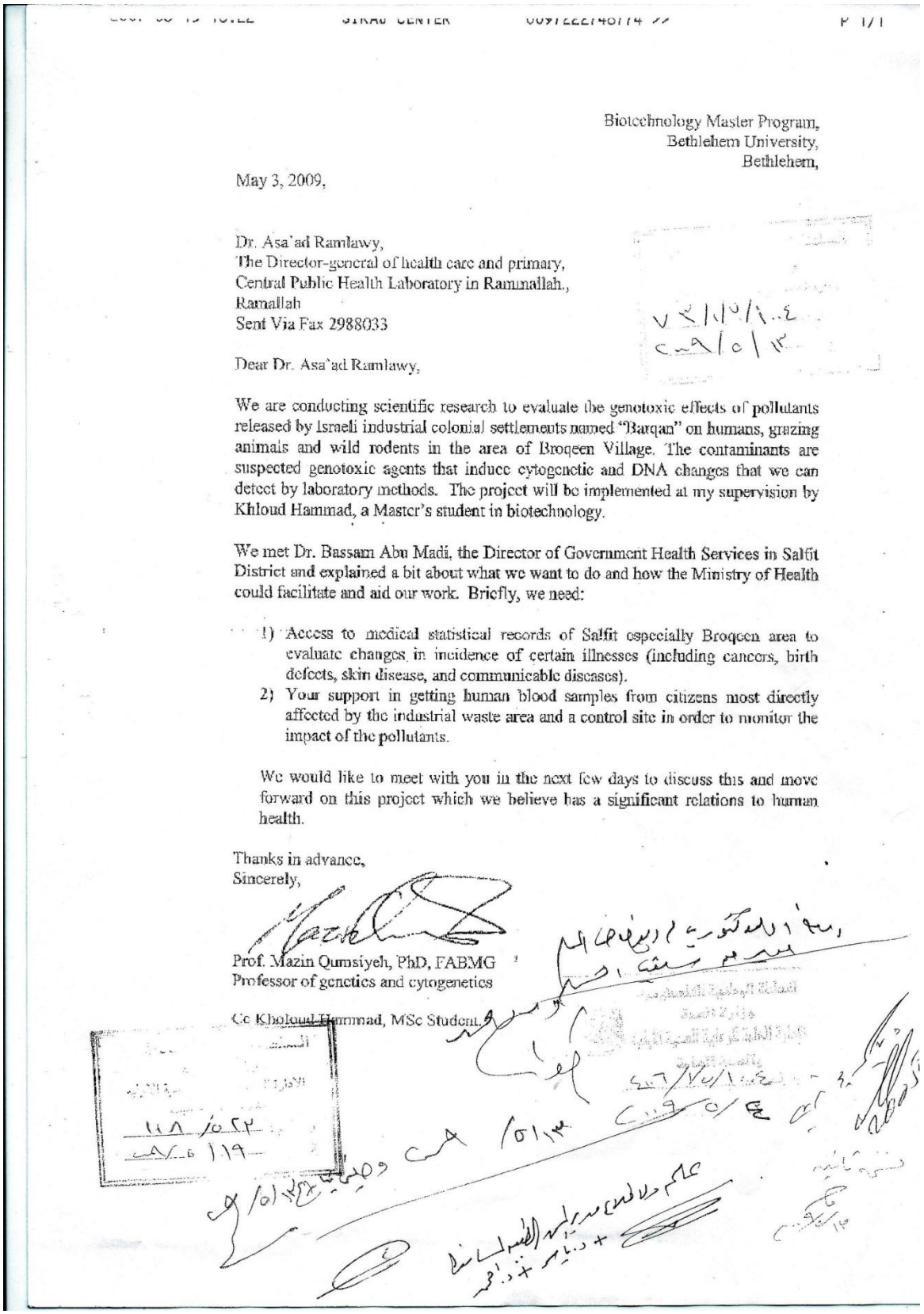
Appendix (d)

Table showing an overview of comet assay versions (Wong et al., 2005).

Version	Description/Characteristics	Advantages/Application	Reference
'Neutral' comet assay	Lysis and electrophoresis performed at pH 9.5; less DNA unwinding and less pronounced comet tails; detects single strand and double strand breaks/similar limit of detection but less sensitive than alkaline version	Useful in situations where less sensitivity is needed, e.g., when background damage or induced damage is high	Ostling & Johanson 1984; Singh, 1988; Angelis et al., 1999; Collins 2004
Alkaline comet assay	Lysis/unwinding/electrophoresis performed at more strongly alkaline conditions; at pH >13, alkaline labile sites are converted to single strand breaks; altering pH over the range 9.5-13 alters sensitivity	Clearer comet images are obtained; greater response to damage seen compared to neutral version; commonly used 'standard' comet assay model; usually adopted when investigating possible protection against damage inducers, e.g. H_2O_2	Singh, 1988; Olive et al., 1990; Angelis et al., 1999 ;Tice et al., 2000
Enzyme assisted comet assay	Specific enzymes used immediately after lysis to transform susceptible sites to single strand breaks; enzymes used include Endo III (reveals oxidized pyrimidines), FPG (reveals oxidized purines), uvrABC (reveals UV damage); assay has increased sensitivity, and enhanced specificity for particular types of DNA lesions	Can detect specific types of damage; has enhanced sensitivity; is useful for looking at differences in basal damage after supplementation	Collins et al., 1993; Dušinská & Collins, 1999
Proteinase K-assisted unwinding	Enhances unwinding of densely compacted DNA, allowing damage to be detected	Can enable DNA damage in sperm to be assessed	Duty et al., 2002
Fluorescent in situ hybridization comet assay (FISH comet)	Uses gene 'tagging' with fluorescent markers and so can be used to visualize a specific gene in the three-dimensional chromosomal structure	Enables domain-specific DNA repair to be investigated; a specific gene in the three-dimensional chromosomal structure can be located; different rates of repair of damage in specific genes relative to the genome overall can be measured	Santos et al. 1997; McKelvey-Martin et al. 1998; McKenna et al. 2003; Horváthová et al. 2004
Lysed cell or partially lysed (nucleus intact) comet assay	Lysis of cell and nuclear membranes or cell membrane only (leaving nucleus intact) prior to exposure to test agent, thus exposing 'naked' DNA or nucleus to the agent	In conjunction with the whole cell, 'standard' version, this version can be applied in assessment of direct versus indirect mechanisms of action of genoprotective agents	Kasamatsu et al., 1996; Thomas et al., 1998; Collins et al., 2001a ; Szeto et al., 2002

(Wong, et al., 2005)

Appendix (e)



Biotechnology Master Program,
Bethlehem University,
Bethlehem,

May 3, 2009,

Dr. Asa'ad Ramlawy,
The Director-general of health care and primary,
Central Public Health Laboratory in Ramallah.,
Ramallah
Sent Via Fax 2988033

Dear Dr. Asa'ad Ramlawy,

We are conducting scientific research to evaluate the genotoxic effects of pollutants released by Israeli industrial colonial settlements named "Barqan" on humans, grazing animals and wild rodents in the area of Broqeen Village. The contaminants are suspected genotoxic agents that induce cytogenetic and DNA changes that we can detect by laboratory methods. The project will be implemented at my supervision by Khloud Hammad, a Master's student in biotechnology.

We met Dr. Bassam Abu Madi, the Director of Government Health Services in Salfit District and explained a bit about what we want to do and how the Ministry of Health could facilitate and aid our work. Briefly, we need:

1) Access to medical statistical records of Salfit especially Broqeen area to evaluate changes in incidence of certain illnesses (including cancers, birth defects, skin disease, and communicable diseases).
2) Your support in getting human blood samples from citizens most directly affected by the industrial waste area and a control site in order to monitor the impact of the pollutants.

We would like to meet with you in the next few days to discuss this and move forward on this project which we believe has a significant relations to human health.

Thanks in advance,
Sincerely,

Prof. Mazin Qumsiyeh, PhD, FABMG
Professor of genetics and cytogenetics

Cc Khloud Hammad, MSc Student

Appendix (f)

FAX NO. :092385956 Jul. 06 2009 12:42AM P1

JUL-09 THU 10:58 FUTURE_OFFICE_TECHNIEOT) 2776711

Biotechnology Master Program
Bethlehem University
Bethlehem

July 1, 2009.

Dr. Nairn Sabra,
The Director-General hospitals,
General Directorate of Hospitals,
Ramallah.

Dear Dr. Nairn Sabra,

We are conducting scientific research to evaluate the genotoxic effects of pollutants released by Israeli industrial colonial settlements named "Barqan" on humans, grazing animals and wild rodents in the area of Bruqeen village. The contammants are suspected genotoxic agents that induce cytogenetic and DNA changes that we can detect by laboratory methods. The project will be implemented at my supervision by Kholoud Hammad, a Master's student in biotechnology.

We met Dr. Bassam Abu Madi, the Director of Government Health Services in Salfeet District and explained a bit about what we want to do and how the Ministry of Health could facilitate and aid our work. We got access to medical statistical records of Salfeet area from Dr. Asa'ad Ramlawy, the Director-General of health care and primary Central Public Health Laboratory in Ramallah.

Since the cancer and malignancy cases are not included, we need access to medical statistical records in The National Cancer Registry(Beit Jala), The National Hospital (Nablus) and the Government Hospital in Ramallah to get data about Bruqeen and the surrounding areas(control areas).

We believe that such data is significant for our research, which has its relation to human health.

Thanks in advance,
Sincerely,

Prof. Mazin Qumsiyeh, PhD, FABMG
Professor of genetics and cytogenetics

Ce Kholoud Hammad, MSc Student.

السلطة الوطنية الفلسطينية
وزارة الصحة
الإدارة العامة للمستشفيات