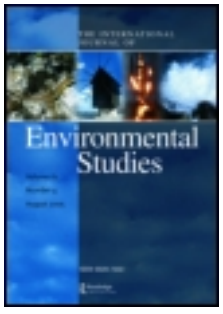

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Genotoxic effects of Israeli industrial pollutants on residents of Bruqeen village (Salfit district, Palestine)

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Many industrial byproducts are genotoxic agents that induce cytogenetic changes and DNA damage. Bruqeen, a Palestinian village in Salfit district in the northern West bank, is subject to industrial waste products coming from Barqan Israeli industrial settlement. We evaluated the extent of chromosomal breaks (Csb) and DNA damage induced to human cells using whole blood samples from both test and control sites. Csb were assessed by routine cytogenetic methods and DNA damage was assessed via the Comet assay which is also called single cell gel electrophoresis. Cytogenetic analysis for Bruqeen residents' sample ($n = 30$) showed 133 Premature centromere separation (PCS), 43 Csb, 40 chromatid breaks (Ctb) and 26 dicentrics. In total, the findings showed an average of 4.08% chromosomal aberrations (CA) and 3.81% cells with CA. The results of controls (from Bethlehem area $n = 8$) showed 21 PCS, 2 Csb, 5 Ctb and 2 dicentric, and in total the average showed 1.97 CA and 1.91% cells with CA. Statistical analysis showed that there were no significant differences between exposed and control subjects 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 the 25 Bruqeen residents showed that there was a significant difference from those of the control samples (p-value < 0.05).

Keywords: Pollution; DNA damage; Chromosomes; Israel

Introduction

Environmental pollutants are known carcinogens and mutagens and testing for chromosomal aberrations (CA) and DNA damage is the most common technique to assess their impact [1–3]. The CA 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 [4,5].

Such studies are now common in developed countries but much more remains to be done in developing countries [6]. Kamboj and Sambyal [7] reported chromosome aberrations in traffic policemen exposed to automobile exhaust through their daily work in Amritsar city, Punjab (India). The CA test was not applied in the occupied Palestinian territories except by Laqqan et al. [8] in Gaza Strip individuals who are occupationally exposed to hazardous material (farmers, plumbers, taxi drivers, paint factory workers and

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petrol station workers). They found that the occurrence of CA in the study group was significantly higher than in the control group [8].

The Comet assay (also known as Single Cell Gel Electrophoresis [SCGE]), especially its alkaline version, allows sensitive detection of DNA damage in various settings [9–15].

Accordingly, it was decided to use a combination of cytogenetics and Comet assay to examine the impact of the industrial waste on the human population directly. Bruqeen, a Palestinian village in Salfit District in the northern West bank, is subject to industrial waste products coming from Barqan Israeli industrial settlement [16,17]. We evaluated the extent of chromosomal breaks (Csb) and DNA damage in human cells using blood samples from both test and control sites.

Materials and methods

Study area

Bruqeen village is located 7 km to the south-west of Salfit City 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 affected by the industrial wastewater stream that is discharged from the industrial Park [16,17]. The Barqan residential settlement was established in 1981 in north-west of Salfit City on about 649 dunams (1 dunam = 1000 m^2) of land from Haris Palestinian village and has now expanded to an area of 1417 dunams to become the largest Israeli industrial park in the occupied Palestinian territories [18]. There are 73 factories in Barqan Industrial Park (plastics, paint, electroplating, metal, wood furniture, aluminium, electronics and surface coating). The assay of the water effluence from the area showed zinc, lead, cadmium, chromium, nickel, copper, volatile organic compounds, acids, cyanides, arsenic, mercury, nitrogen oxides and others [16,17]. Complaints by Palestinians about the environmental, economic, health and other problems were followed by a preliminary analysis of liquid output dumped into the nearby Palestinian village, which showed heavy metals, organic compounds and various phosphate and nitrate compounds [16].

Samples

Subjects ranged in age from 15 to 40 years old. They were chosen to meet the following criteria: non-smokers, residing in Bruqeen within a 1 km radius of the toxic dump area (our control site is more than 50 km away from any known toxic dump area), and their occupations do not require dealing with genotoxic materials. They included persons of various occupations (students, housewives, government employees, workers, taxi drivers and unemployed). The study was cleared by both Bethlehem University's institutional ethics committee and by the Palestinian Ministry of Health. Signed informed consent forms were obtained from all participating individuals prior to the blood donation with a full explanation given about the intent and extent of study and its potential implications. Each donor completed a standardized questionnaire in order to obtain relevant details of current health status, health history and lifestyle. 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 the 5th of April 2009 (14 samples), while the second one was the 24th of February 2010 (29 samples).

CA test

Peripheral blood samples (volume 5 ml) were collected by venipuncture into heparinized tubes. After collection, all blood samples were coded, transported in a dark cool container to Bethlehem University laboratory and processed as quickly as possible. The CA test was possible on 12 samples from the first sampling and 18 from the second sampling (20 males and 10 females in general). The Comet assay was conducted using 6 samples from the first sampling and 19 from the second sampling (15 males and 10 females in general). The control group for CA test comprised 8 samples (4 males and 4 females). The control sample for Comet assay comprised 5 samples (2 males and 3 females).

A quantity of 0.5 ml whole blood was incubated for at 37 °C for 72 h with 5 ml RPMI blood culture medium with a Phytohemagglutinin (Biological Industries, Cat.# 01-201-1A). To arrest dividing lymphocytes in metaphase, 40 µl Colcemid solution (Biological Industries, 10 µg/ml in DPBS, Cat.# 12-004-1D) was added 1 h prior to the harvest. Cells were then harvested by the usual cytogenetic methods. Rough handling and exposure to light were avoided as much as possible during the process.

Stained slides were coded and scored without reference to source (test or control). Two hundred metaphases per subject were analysed for CA using a light microscope with 100× magnification. Only metaphases containing 45–47 centromeres were analysed. For each individual, the control group ($n = 8$) and test group ($n = 30$), the following parameters were examined for premature centromere separation (PCS): Csb, Chromatid break (Ctb) and Dicentrics. From each individual, 200 cells were scored and we calculated for each case the average frequency of CA (CA frequency is number of aberrations detected in all cells divided by total number of cells or 200) and percentage of cells that have CA (number of cells showing any abnormality divided by 200 scored cells as a percentage).

Comet assay (SCGE)

The Alkaline Comet assay was performed according to published methods [19,20] with minor modifications at Bethlehem University laboratory: (1) we used 0.8% Low Melting Point Agarose (Sigma, Cat.# A9414-5G) mixed with 15 µl of whole blood sample (after letting the vacutainer tube settle to increase lymphocytes' collection potential from the top part) and (2) all preparations and solutions except when coating slides with Agarose were done on ice or in a cold room. The electrophoresis was at 15 V (∼0.74 V/cm) for 30 min.

Slides were stained with Ethidium Bromide and scored immediately using a fluorescent microscope (Olympus BX41). If scoring was not done immediately, slides were first dried as follows: slides were drained and kept for 20 min in cold 100% methanol for dehydration. The slides were air dried and placed at 50 °C for 30 min, then stored in a dry area. Before examination, they were rehydrated with chilled distilled water for 30 min and stained with Ethidium Bromide, then covered with a fresh coverslip.

Observations were made of stained DNA using a 10× and 20× objectives on the fluorescent microscope. Infinity Digital Microscopy Camera and software (Lumenera Corporation, Ottawa, Canada) was used for taking pictures and measuring tail and nucleus lengths. One hundred randomly selected cells were analysed per sample. All statistical analyses were done using SPSS 13.0 for Windows (SPSS Inc.).

Results

Chromosome aberrations

We obtained data from 30 subjects (20 males, 10 females, ages 15–39) from the test site and scored 200 metaphase each. Total PCS observed in these 6000 cells was 133, Csb 43, Ctb 40 and Dicentrics 242. Total aberration frequency (taken as total aberration in 200 metaphases divided by 2) averaged 4.08%, while per cent of cells with any aberration (number of cells with any aberrations in 200 metaphases divided by 2) averaged 3.81%. Corresponding data were obtained for 8 control subjects from Bethlehem and Hebron (4 females, 4 males, ages 19–38). Total PCS observed in these 1600 control cells was 21, Csb 2, Ctb 5, Dicentrics 30. Total aberration frequency averaged 1.97%, while per cent of cells with any aberration averaged 1.91%.

To establish whether these differences in CA between the two groups are significant or not, data normality was first tested considering the null hypothesis that the data follow normal distribution and normality was rejected (table 1).

The Mann–Whitney Test was used for comparisons between the exposed and control data for all variables. The null hypothesis was that the mean ranks for exposed and control samples are equal. Statistical analysis showed that there were no significant differences between exposed and control subjects 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 and for total frequency of aberrations between test and control sites (table 2). Neither gender nor age had any significant effect on the CA data (all $p > .05$; data not shown).

Comet analysis for DNA damage

The data are divided into two groups: exposed group ($n = 25$) and control group ($n = 5$). With each we scored 100 nuclei (so total 2500 test and 500 control nuclei). For each, two measurements were determined: the ratio of tail length: nucleus length (TL:NL); and the ratio of tail length: total length (TL/(TL + NL)). Means and standard deviation for these

Table 1. 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).

		Kolmogorov-Smimov[a]			Shapiro-Wilk		
1: Control group, 2: Exposed group		Statistic	df	Sig.	Statistic	df	Sig.
Premature centrome separation	Control group	0.168	8	0.200*	0.966	8	0.862
	Exposed group	0.278	30	0.000	0.723	30	0.000
Chromosomal breaks	Control group	0.513	8	0.000	0.418	8	0.000
	Exposed group	0.233	30	0.000	0.806	30	0.000
Chromatid breaks	Control group	0.300	8	0.033	0.798	8	0.027
	Exposed group	0.265	30	0.000	0.709	30	0.000
Dicentric	Control group	0.513	8	0.000	0.418	8	0.000
	Exposed group	0.289	30	0.000	0.797	30	0.000
Frequency of chomosomal aberration	Control group	0.183	8	0.200*	0.945	8	0.661
	Exposed group	0.140	30	0.140	0.862	30	0.001
Percentage of cells that have chromosomal	Control group	0.170	8	0.200*	0.950	8	0.707
	Exposed group	0.149	30	0.086	0.882	30	0.003

*This is a lower bound of the true significance.
[a]Lilliefors significance correction.

Table 2. Results of applying 2-independent samples test (Mann–Whitney Test) and Wilcoxon test on exposed and control CA data.

	PCS	Csb	Ctb	Dicentric	CA Freq	% Cells w/ CA
Exposed mean	*4.433*	*1.433*	*1.333*	*0.867*	*4.201*	*3.867*
Control mean	2.635	0.250	0.625	0.250	1.970	1.907
Exposed St. D.	*3.287*	*1.716*	*2.023*	*0.937*	*3.133*	*2.666*
Control St. D.	1.685	0.707	0.744	0.707	1.055	1.100
Mann–Whitney U	86	63	107	74	62	59.5
Wilcoxon W	122	99	143	110	98	95.5
Z	−1.239	−2.193	−.502	−1.828	−2.083	−2.176
Sig. (2-tailed)	0.215	0.028	0.616	0.068	0.037	0.030
Sig. (1-tailed)	0.235	0.041	0.661	0.104	0.038	0.028

Table 3. Results of applying normality tests on exposed and control Comet data: TL:NL and TL/(TL + NL).

		Kolmogorov-Smimov (Lillifors significance correlation)			Shapiro-Wilk		
		Statistic	df	Significance	Statistic	df	Significance
TL:NL	Exposed	0.166	2500	0.000	0.732	2500	0.000
–	control	0.284	500	0.000	0.561	500	0.000
TL:(TL + NL)	Exposed	0.054	2500	0.000	0.970	2500	0.000
–	control	0.315	500	0.000	0.916	500	0.000

Table 4. Statistical analysis for the difference between the test group (2500 nuclei) and control group (500 nuclei) in tail length (TL) relative to nucleus length (NL) and relative to both TL and NL in the Comet assay.

	TL:NL	TL:(NL + TL)
Exposed mean	*1.226*	*0.452*
Control mean	0.736	0.280
Exposed St. D.	*1.263*	*0.219*
Control St. D.	1.290	0.245
Mann–Whitney U	368774.5	368774.5
Wilcoxon W	494024.5	494024.5
Z	−14.5	−14.5
Sig. (2-tailed)	0.000	0.000

two measurements for test site were 1.2256 + 1.26258 and 0.4523 + 0.21931. The corresponding means with SD for the control site were 0.7356 + 1.29012 and 0.2801 + 0.24519. Both mean and median data showed increased DNA damage in the test vs. control group. To evaluate whether these differences in the two parameters between the two groups are significant or not, the data were tested for normality (null hypothesis) and normality was rejected (table 3).

Comparison between test and control nuclei via 2-independent samples test (Mann–Whitney Test) showed high significance (table 4).

Discussion

The analysis for the CA test data showed a CA increase in the test site. The non-parametric 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 3).

Previous studies in healthy individuals showed average CA of 0.88% (i.e. the whole # of CA/the total # of the studied sample subjects) [21]. In our study, frequencies of CA in exposed samples were between 0.5 and 14% per cell, while the average of aberrations per person was about 8.07%. Kopjar et al. [22] showed that the range of the total number of CA per 200 cells in healthy human volunteers was 0–5 (the counted # of CA out of the scored # of metaphases) [22]. In our work, the range was 1–28 for the exposed sample, and 1–6 in the control sample. Kopjar et al. [22] showed that the percentage of aberrant cells in healthy individuals averaged 0.48% (range 0–2.5%). Variations (wide standard variation also noted in our samples) are usually explained as being due to different genome sensitivities or technical variations between samples [22,23]. In our control site, the average was 1.91% (range 0.5–4%) while in our test site, the average was 3.86% (range of 1–12%). There was statistical difference between our test site and the control site, but the increase over previous studies in the control data is very curious. It can be explained either by technical aspects (e.g. culture conditions for blood or the small size of the control sample) or biological (our control areas also were affected by pollution but at a lower level than the test site).

The percentage of Csb ((total # of Csb/ total # of CA) $\times$ 100%) in the control was 6.7% (2 Csb found in one person), while the percentage in the exposed sample was 17.8% (43 Csb were found in exposed sample; found in 18 persons). The percentage of dicentric chromosomes in the control was 6.7% (only 2 dicentric were found in the control sample and were found in one person), while the percentage in the exposed sample was 10.7% (26 dicentric were found in 16 exposed subjects). Pfeiffer et al. [24] mentioned that the spontaneous occurrence of dicentrics is low at about one per 1000 lymphocytes in normal individuals [24]. Double-strand breaks lead to CA which 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 tumour suppressor genes [2,24–26].

The 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 3). Such a result is compatible and consistent with our CA test results (as mentioned earlier: CA frequency, % of cells that have CA, number of Csb, CAs' frequencies per cell and average of aberrations per person). Our data (table 4) agree with previous studies that showed that different scoring methods give similar results in Comet assays [27].

The Comet assay detects various forms of DNA damage especially reparable damage and is thus useful to assay short-term genotoxic damage [9–15]. The CA test detects persistent mutations that remained through to mitosis. Therefore, the frequency of CA will not be proportional to the frequency of DNA breaks; many get repaired before the cell is allowed to proceed to mitosis [26].

These results suggest that Bruqeen residents exposed to waste from the industrial settlement sustain higher Csb and DNA damage than in control sites. The cytogenetic analysis showed that there was a statistically significant difference for CA frequency and for per cent of cells that have CA and Csb in favour of the exposed rather than the control group, while Comet assay showed that there was significant difference for all Comet parameters in the exposed from those in the control group.

We did not bring up to date the laboratory studies of the chemicals/organics in the discharged waters done by ARIJ 7 years prior to our study of genotoxicity, because such a study is not required to look for differences in genomic damage, which happens in any case over a long period of exposure. We do know that the factories in that area did not change (except that there are now more of them); but even if miraculously they discharged pure water at the time of our study, it still would not change the conclusion. In future studies, we do need to examine directly levels of pollutants in human (and perhaps animal) blood in addition to further environmental work. We plan future studies to confirm and expand these data in our area to assess the degree of genotoxic effect using additional samples and control sites outside the occupied Palestinian territories, to screen grazing animals and field animals (e.g. rodents [28]), and to check human sperms from these sites via SSGE. *In vitro* studies of selected pollutants showed even more damage in spermatozoa than in lymphocytes [29].

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