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Cytogenetic Biomonitoring of the Mzamza Population Exposed to Untreated Wastewaters

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Abstract A cytogenetic biomonitoring study was performed on people from the Mzamza community near Settat, Morocco. These subjects live in and near the Bou Moussa valley where wastewaters from a great number of industries are discharged without any treatment. This wastewater is used as a source of drinking water for their cattle and irrigation of their land. The Mzamza population is therefore presumably exposed to continuous low doses of different kinds of pollutants. Our study demonstrated significant increases in micronucleated white blood cells indicating a considerable genetic risk in these subjects.

Keywords Cytogenetic biomonitoring · Micronucleus test · Mitotic index · Proliferation index · Mzamza population · Wastewater

Human and industrial activities result in discharge of multiple chemical substances in the environment which substantially contribute to environmental pollution. This is, for example, the case at the periphery of large cities in Morocco (Jemali and Kefati 2002). Seventy millions m^3 of wastewaters are approximately used each year in agriculture without any precaution to irrigate a surface of more than 7,000 ha of various cultures (fodder culture, market gardening, field culture, and arboriculture). In Morocco, irrigation of market gardening's with wastewater is banned but this prohibition is not always respected (Jemali and Kefati 2002).

Measuring the concentration of specific chemicals in the environment (water, air, soil, and food) is very often the only or principal approach to estimate the environmental pollution load and the resulting possible effects on human health. However, biological monitoring often allows a more precise risk assessment as it takes into account aspects of bioavailability and individual susceptibility. It furthermore also integrates all aspects of toxicokinetics: respiratory, digestive and cutaneous absorption, biotransformation, distribution and excretion, also of pollutants whose concentrations in the environment were not measured (EHC, 2001).

Numerous biomarkers exist to evaluate the various levels of toxicity, among which genetic effect biomarkers that are considered of uttermost importance. The investigation of the cellular kinetics and genic changes, and the evaluation of the frequency of target cells with structural chromosome aberrations, sister chromatid exchanges or micronuclei are well-known and often applied tools in biomonitoring studies. This is, among others, because there is experimental and epidemiological evidence for the association between specific structural or numerical chromosomal aberrations and carcinogenesis (Mitelman 1994; Rew 1994). Also, at the level of a (human) population, an increased frequency of structural aberration in peripheral blood lymphocytes is associated with an increased overall risk for cancer (Hagmar 1994; Bonassi et al. 1995). The same seems to be true for the micronucleus frequency (Bonassi et al. 2007).

In the present investigation a cytogenetic surveillance was conducted aimed at evaluating the impact of the existing wastewater pollution among the Mzamza population living in the province of Settat, Morocco.

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Materials and Methods

The town of Settat is located in the center of Morocco, 70 km South of Casablanca and has a surface of 30 km^2. Many industries are present in the South of the town. They are slaughter-houses and textile industries or are involved in glassmaking, tannery, ceramics, and the manufacture of margarine, surface treatments, the treatment of cereals, gut-dressing or printing works.

Wastewaters from above mentioned industries as well as from domestic origin are evacuated without any preliminary treatment into the Bou Moussa valley which became a discharge system with a flow of 9,632 m^3/day. Analyses performed by Kholtei et al. (2003) on the wastewater and water from neighboring wells revealed that the levels of heavy metals and organic matter exceeded those of national and international standards. The Bou Moussa valley crosses the community of Mzamza at a distance of 8 km (Fig. 1). Along its passage, the local population permanently uses the polluted water for the irrigation of their cereals and fodder and for watering their cattle.

Blood sampling using sterile heparine containing tubes was performed in 40 individuals from the test population. The control group was made of 30 subjects. Their characteristics are summarized in Table 1. The target population, children, women, and men were native in the locality and lived there for more than 5 (children) or 10 (adults) years. They consume crops irrigated by polluted water and drink water from the wells at the neighborhood of the Bou Moussa valley. The control population also consisted of a rural community who present the same socio-economic level and live at the South of the town of Settat. They are not exposed to the polluted water from the Bou Moussa valley as they use water from unpolluted wells as source of drinking water as well as for irrigation of their crops (no excess levels of pollutants found after analyses; unpublished data). They are also not in contact with any other known source of pollution. As many factors may influence the level of DNA damage and the cellular kinetics (IAEA 1986) a personal questionnaire was used in accordance with the recommendations of the IC-PEMC (Carrano and Natarajan 1988). Each blood donor furthermore underwent a medical check-up. Data were collected on his/her personal and professional history, genetic predisposition to disease, smoking habit, food consumption and particular chemical exposures.

Routine cytogenetic methods were used to obtain respectively metaphase figures and cytochalasin B blocked binucleated cells. Briefly, 0.5 mL of whole blood was added to 5 mL RPMI 1640 culture medium supplemented by 15% of fetal bovine serum, 1% of phytohemagglutinine and 1% of antibiotics (penicilline/streptomycin). These cultures were incubated at 37°C. Colcemid was added 2 h before harvest. After 48 h of cultivation, cells were sub-

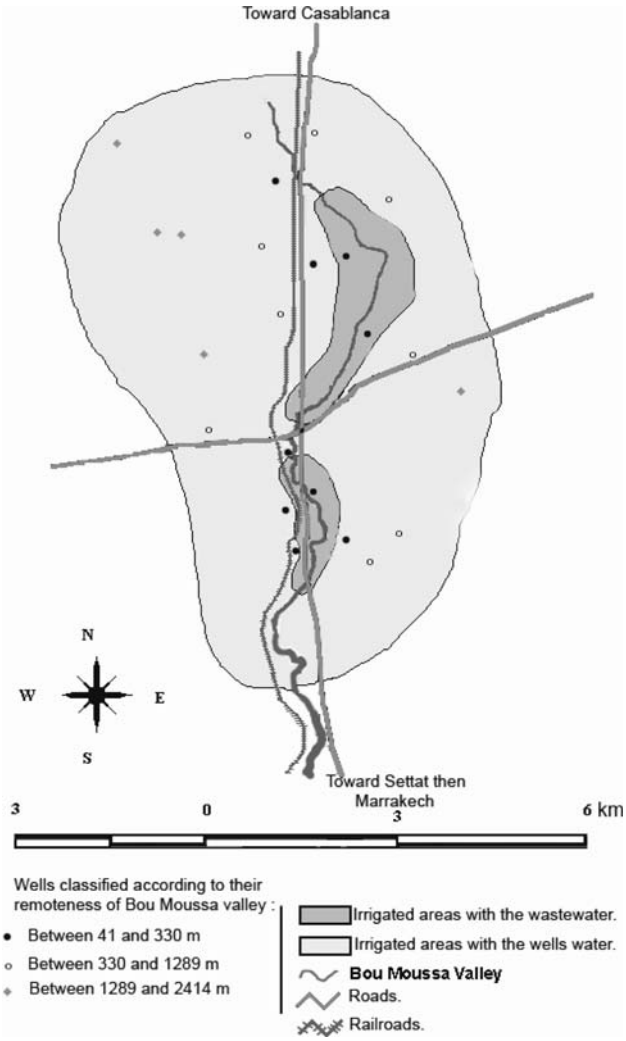

Fig. 1 Geographic localisation of the Mzamza localitie along the Bou Moussa valley (adapted from Laamari et al. 2004)

Table 1 Distribution of the age, sex and smoking habit in the test and control population

	Test population	Control population
Number of subjects	40	30
Age		
<20 years	17	11
20–39 years	12	10
40 years and more	11	9
Sexes		
Female	16	13
Male	24	17
Tobacco		
Non smokers	33	25
Smokers	7	5

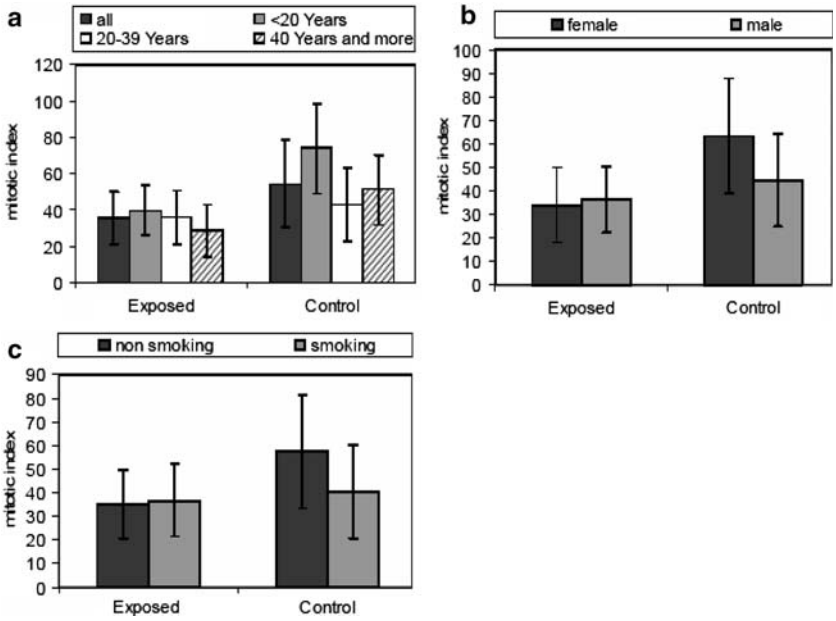

Fig. 2 **a** MI according to age, **b** MI according to gender, **c** MI according to smoking behavior

jected to a hypotonic shock using 0.075 M KCl, fixed in 1:3 acetic-acid/methanol and stained with Giemsa. Another culture was incubated 44 h after which cytochalasine-B was added and cultivation continued up to 72 h. Hypotonic treatment, fixation and staining followed as before.

Micronuclei (MN) are small, extranuclear bodies that originate from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division. An increased frequency of micronucleated cells is considered a biomarker of genotoxic effects that can reflect exposure to agents with clastogenic (chromosome breaking) or aneugenic (effect on chromosome number) modes of action (Albertini et al. 2000). They were scored in binucleated white blood cells according to well defined criteria (e.g., Fenech 2000): a micronucleus is defined as a small extra nucleus separated from the main nuclei, not exceeding 1/3 of the main nucleus diameter and with distinct borders and the same color as the nucleus. The frequency of MN was scored in 1,000 binucleated lymphocytes per individual.

The cellular proliferation index (PI) was calculated by the following formula:

$$\mathrm{PI} = \frac{(1 \times \mathrm{N}_1) + (2 \times \mathrm{N}_2) + (3 \times \mathrm{N}_3) + (4 \times \mathrm{N}_4)}{1,000 \text{ cells analyzed}},$$

where N1 is the number of mononucleated cells; N_2 the number of binucleated cells, N_3 the number of trinucleated cells and N_4 the number of tetranucleated cells.

The mitotic index (MI) was calculated as the number of metaphase figures per 1,000 phytohemagglutinin stimulated cells.

Monofactorial analysis of variance (ANOVA) was used to compare findings in the test and control population. All calculations were performed using Microsoft Office Excel 2003 software. Statistical data are shown only when differences between the compared populations were significant.

Results and Discussion

In this cytogenetic investigation we used the cytochalasin B blocked micronucleus test as this test allows the detection of a clastogenic and/or aneugenic event (chromosome breakage or chromosome loss). The proliferation index provides information on the cell proliferation, along with the mitotic index that especially measures the cellular response towards the mitogen phytohemagglutinin.

Results are presented in Figs. 2, 3 and 4. The following observations were made:

- A lower average MI was seen in the blood from the Mzamza population ("exposed" subjects) compared to the average observed in the controls. As a matter of fact, 36 out of 40 individuals from the test group had lower MI's compared to the average in the controls. The difference in MI's between both groups is highly significant ($p < 0.001$; Fig. 2a).
- The MI decreased with age (Fig. 2a) and is significantly higher in non exposed females compared to males, but this was not found in the exposed group (Fig. 2b). The smoking behavior did not influence the MI (Fig. 2c).
- Proliferation indices were slightly but significantly lower in the test group compared to the controls

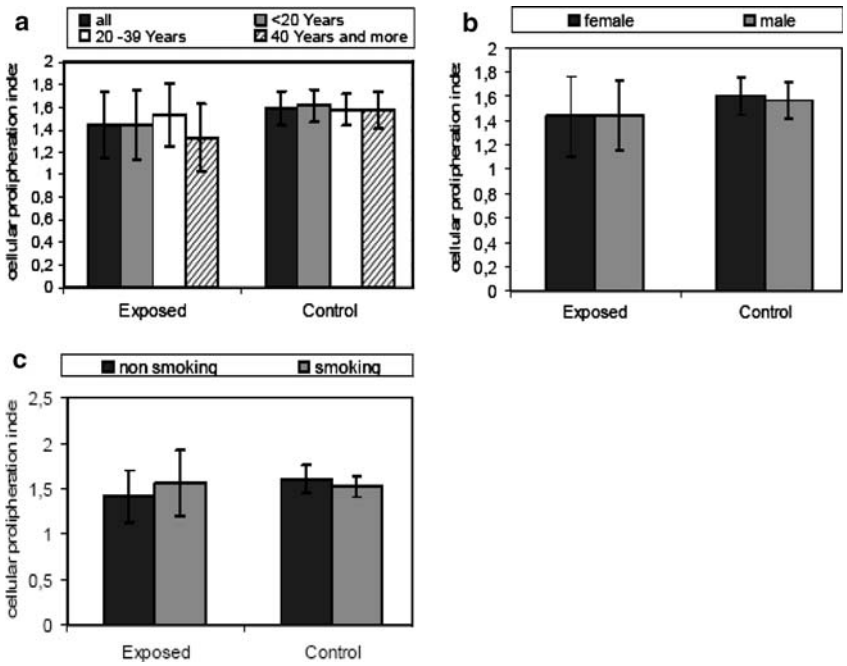

Fig. 3 **a** PI according to age, **b** PI according to gender, **c** PI according to smoking behavior

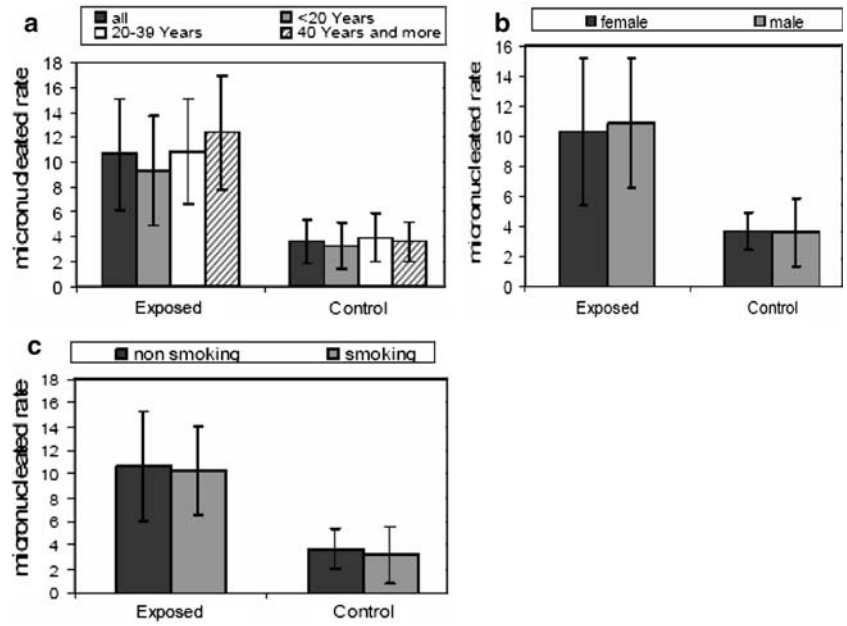

Fig. 4 **a** MN rate according to age, **b** MN rate according to gender, **c** MN rate according to smoking behavior

($p < 0.02$) but there was no difference with age (Fig. 3a).

In both groups males and females did not differ with respect to the PI's (Fig. 3b). There was also no difference between smokers and non smokers in both groups (Fig. 3 c).

The micronucleus test showed that there are significantly more micronucleated cells in the Mzamza population compared to the controls ($p < 0.001$) (Fig. 4a). The micronucleus frequency increased with age in the exposed group but not in the controls (Fig. 4a). Tobacco consumption and gender did not influence the MN significantly (Fig. 4b, c). Overall it thus appears that exposed subjects have significantly more micronucleated cells than non-exposed subjects and that the cellular kinetics decreases with increasing DNA damage.

No correlation was found between the micronucleus frequency and the PI, although PI and MI were significantly correlated ($p < 0.05$).

The Mzamza population uses the wastewater as a source of drinking water for their cattle and irrigation of their land

to produce the cereals and fodders cultures. Human population exposure to potential hazardous substances thus occurs directly by physical contact following irrigation practice, either indirectly via their food and drinking water polluted by infiltration of the wastewater's pollutants into the water table. The control population uses the waters of unpolluted wells (unrelated to the Bou Moussa valley) for irrigation and as their source of drinking water. This investigation revealed that this, merely indirect exposure to presumably continuous low doses of pollutants in untreated wastewaters constitutes a genetic risk. Subjects do respond differently to the exposure.

This was shown by the relative high variability in especially the mitotic index (this ranged actually from 10 to 60) and micronucleus frequency (4 up to 21 micronuclei/1,000 cells). The inter-individual variability in these biomarkers is obviously due to inter-individual differences in genetic susceptibility (Obe and Beek 1982, 1984; Anderson 1999) or to different exposures to different pollutants. This is also the reason why attention was paid to tobacco consumption, gender and age but many others factors may contribute to the overall picture. Some of them were taken into account (e.g., medical history) but did not reveal of particular significance in this study. Our results, although preliminary, are unequivocal and in agreement with many other investigations on human exposure to chemical or physical agents. Various studies showed for example that exposure to ionizing radiations decrease the mitotic index and increases DNA damage. This was shown by investigations of the micronucleus frequency in human blood cells, but also by investigations of other genetic endpoints as, e.g., sister chromatid exchanges and chromosomal aberrations.

In these studies the mitotic index decreased with increasing exposure level (Léonard et al. 1998; Hung et al. 1995). Another example is given by a cytogenetic investigation of inhabitants from the village of Mellery in Belgium where exposures to environmental pollutants (benzene, toluene, ethylbenzene, meta-paraxylene, *O*-xylene, *n*-propylbenzene 1,3,5-trymethylbenzene, 2-ethyltoluene, 1,2,4-trimethylbenzene, trichloroethylene and tetrachlorethylene; probably originating from a neighboring chemical waste disposal site) revealed high SCE and micronucleus frequencies in cells from exposed subjects whereas the PI was not altered by the exposures. Smoking habits also did not influence the results (Klemans et al. 1995). Human environmental exposure to arsenic in the Antafasta area (Northern Chili), where levels in drinking water as high as 0.75 mg/L were observed, significantly increased the micronucleus frequency whereas it decreased the proliferation index (Martinez et al. 2004). Sang and Li (2005) recently investigated the induction of chromosomal aberrations in mouse bone marrow cells from animals drinking municipal landfill leachates of different concentration for a given period of time. They found a dose-dependent decrease in MI along with an increased chromosome aberration frequency. Their results were in accordance with those obtained on plant cells (Sang and Li 2004). Increased cytogenetic damage was furthermore also found in cattle living in the localities of Dladla and Boukallou near Settat (Meftah El Kadmiri et al. 2006).

These few examples clearly demonstrate, together with our present study, that such biomonitoring studies in apparently healthy subjects who were exposed to small doses of many different pollutants, are able to detect cytogenetic damage that may give rise to important adverse health consequences. Chemical analyses of only a few pollutants out of an unknown mixture are not able to give such an overall risk assessment at the population and individual level.

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