Cytogenetic Investigation of Workers Professionally Exposed to Phosphates and Their Derivatives

(micronucleus test / mitotic index / proliferation index / miners / production unit workers / phosphate)

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Abstract. The phosphor industry in Morocco employs a great number of workers that may be exposed to this essential but also toxic compound. In the present investigation, professionally exposed subjects from two different production lines (miners and production unit workers) were investigated for the presence of genetic damage in their peripheral blood lymphocytes. This was done with the well-known micronucleus assay. The proliferation and mitotic indices were also investigated. It was found that the micronucleus frequency was considerably increased compared to the frequency in non-exposed control subjects. Mitotic and proliferation indices were not increased, at least not in a statistically significant way. At the time being this investigation should be considered as a preliminary study in which the influence of potential confounders cannot be adequately assessed. However, our results are non-equivocal and clearly indicate a potential health risk in these workers.

In Morocco the exploitation of phosphates started in 1920 and is at present concentrated around six centers among which four are mines and two chemical transformation plants. Morocco holds 3/4 of the world's phosphate reserve and hence it is the world's first exporter of phosphates and phosphorous acid and second exporter of phosphor-based fertilizers. At the end of 2001 more than 22 500 persons worked in the phosphor industry in Morocco. This great number of workers further illustrates the importance of this industry in Morocco.

From the biological point of view, phosphate may be considered the 5th important element after carbon, oxygen, hydrogen and nitrogen. Biochemical investigations usually concern the orthophosphate ion PO_4^{-3}, which is a structural unit of biologically important molecules such as nucleic acids or the calcium phosphate of bones and teeth. Phosphorus is used by the body to help regulate the acid/base balance and strengthen cell walls. However, phosphorus and its components can be highly toxic, too. This was for example shown for inorganic phosphorus after inhalation, ingestion or percutaneous adsorption (Bretherick, 1981; Kettrup et al., 1988). Many other toxic effects have been reported in animals, as well as in humans (Moeschlin, 1962; Braker et al., 1977; Kettrup et al., 1988). Most information on the genotoxic potential of phosphorus and phosphor-containing chemicals comes from investigations on pesticides. This is for example the case for dichlorvos that was found highly mutagenic/genotoxic in many different test systems and organisms (e.g., EHC, 1988).

In the present investigation, professionally exposed subjects were investigated with the cytochalasin B micronucleus assay in order to assess the workers' possible genetic risk resulting from their professional activity. Besides the frequency of cells with or without micronuclei we also investigated the mitotic index and proliferation index that provide additional information on the subject's exposure and health risk. A non-exposed control population (students and employees from the "phosphate region") was of course investigated as well.

Material and Methods

Heparinized blood samples were obtained from healthy volunteers among which 12 were workers at a chemical transformation plant and six were miners. The blood from 10 donors, unrelated to the phosphorus industry, was used as a negative control. A questionnaire was filled in by each of the subjects. This was made in accordance with the ICPEMC recommendations (Carrano and Natarajan, 1988) and included, amongst others, information on the personal and professional history of the subjects, their smoking and eating habits and elements of their personal genetic predisposition to certain ailments or illnesses. The blood was cultivated during

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Abbreviations: MI – mitotic index, MN – micronuclei, PI – proliferation index.

72 h at 37°C using conventional methods (5 ml RPMI 1640 medium (Sigma, SOMAPROL Casablanca, Morocco) supplemented with 15% foetal calf serum (Sigma) and 1% phytohaemagglutinin (Sigma) and antibiotics (penicillin/streptomycine, Sigma)). Cytochalasin B (Sigma) was added after 44 h of cultivation in the concentration of 6 µg/ml. Following cultivation, cells were subjected to a hypotonic shock (0.075 M KCl) and fixed in methanol-acetic acid (3/1). Cells were spread on clean slides and stained with a 1/10 Giemsa solution.

Slides were analysed using an optic microscope (Motic digital microscope). Only binucleated cells were analysed for the presence or absence of micronuclei. Criteria to decide upon the presence or absence of a micronucleus were strictly defined and described by Fenech (1993; 2000); amongst others: the micronucleus should be independent from one of the main nuclei and similarly stained and its size should be between 1/16 and 1/4 of the smallest of the main nuclei. In each slide 1000 binucleated cells were investigated. The proliferation index (PI) was calculated for each slide as follows (Titenko-Holland, 1997):

$$PI = \frac{(1\times N_1)+(2\times N_2)+(3\times N_3)+(4\times N_4)}{1000 \text{ analysed cells}}$$

N_1: number of mononucleated cells
N_2: number of binucleated cells
N_3: number of trinucleated cells
N_4: number of tetranucleated cells

The mitotic index (MI) was analysed on microscope slides that were prepared for conventional chromosome aberration analyses (not discussed here). It is defined as the number of cells in metaphase amongst 1000 stimulated cells.

$$MI = \frac{\text{number of metaphase figures}}{1000 \text{ stimulated cells}}$$

Monofactorial analysis of variance (ANOVA) was used to compare findings in the exposed and unexposed (control) subjects. 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

Table 1 summarizes the results of the cytogenetic analyses in the phosphate workers (miners and production unit workers) and the control subjects. It shows that the mitotic indices, which reflect the cellular responses towards phytohaemagglutinin, do not substantially differ between the three groups of subjects (statistically non-significant differences). Yet, there exists quite an important variation between individuals, especially among the production unit workers. The PI can be seen as an indirect measure of the cell cycle duration as it concerns first, second and ≥ third cell division stages. The data in Table 1 show that the PI is lower in the workers compared to the controls but again, no statistically significant differences were found between controls and workers or between the two groups of workers separately. This is different for the frequencies of micronuclei as it appears that both populations of workers have significantly higher micronucleus frequencies in their blood lymphocytes compared to the controls. Table 2 shows that the micronucleus frequency was significantly different between the three groups of donors (a) as well as between the production unit workers (b) or miners (c) and the controls, or between both groups of workers compared to the controls (d). On the other hand, there was no significant difference in the micronucleus frequencies in lymphocytes of extraction miners compared to those of the production unit workers (e).

Production unit workers showed more variation in micronucleus frequencies than miners. Controls showed less variability. The increased micronucleus frequency and variability in workers compared to the controls and in production workers compared to miners may find an explanation in a number of factors or additional exposures to pollutants as indicated in Table 3. The Tables 4 and 5 give a synthesis of these life-style factors and further professional exposures. A number of "tendencies" are seen. For example, the age, duration of work and tea consumption seem to influence the micronucleus frequency in workers, whereas coffee and tobacco consumption negatively correlates with the yield of micronuclei. However, as none of the life-style factors of Table 4 show a statistically significant correlation with the measured cytogenetic endpoints, at least some of these apparent relationships may have no real biological significance. The same holds true for 'other' exposures in the production unit workers (Table 5). These workers are indeed exposed to quite a great number of industrial pollutants from which some seem to influence especially the micronucleus frequency and the mitotic index. The number of involved subjects was, of course, limited and this should further warrant against too hasty conclusions. It is well known that interindividual variability in sensitivity to genotoxins is dependent on the nature of the toxin and its interaction with DNA as well as on the exposure conditions and duration and/or other combined exposures and effects (Obe and Beek, 1984). It is often not possible to estimate which of the different factors have the highest impact on health, but the highest micronucleus frequency in the production workers may probably yet be put on account of other exposures, too. This was found in many other investigations. An increased chromosome aberration frequency was for example found with increasing duration of work in workers from fossil-fuelled and nuclear power plants, but tobacco consumption and the number of radiological investigations did not increase the chromosome aberration frequency

Table 1. Results of the cytogenetic analyses in the different groups of subjects

	Subject number	MI	PI	Number of binucleated cells	Binucleated cells with one micronucleus	Binucleated cells with two micronuclei	Binucleated cells with three micronuclei	Number of micronuclei
Chemical treatment plant (production unit workers)	1	82	2.06	1000	05	02	00	09
	2	69	1.61	1000	08	00	00	08
	3	58	1.77	1000	12	03	00	18
	4	78	1.84	1000	05	00	01	08
	5	56	1.78	1000	08	00	00	08
	6	85	1.45	1000	06	00	00	06
	7	101	1.60	1000	16	01	00	18
	8	115	2.00	1000	19	04	00	27
	9	97	1.83	1000	12	01	01	17
	10	90	1.69	1000	20	01	01	22
	11	115	1.61	1000	13	00	00	13
	12	102	1.61	1000	18	02	00	22
Sum					142	14	03	176
Average		87	1.71		11.83	1.17	0.25	14.67
Miners	13	73	1.88	1000	14	00	00	14
	14	78	1.70	1000	08	04	00	16
	15	68	1.58	1000	07	02	00	11
	16	72	1.82	1000	10	00	00	10
	17	81	1.77	1000	09	02	00	13
	18	96	1.88	1000	08	03	00	14
Sum					56	11	00	78
Average		78	1.78		9.33	1.83	00	13
Controls	19	78	1.90	1000	1	0	0	1
	20	85	1.86	1000	3	0	0	3
	21	82	1.89	1000	2	0	0	2
	22	76	1.76	1000	2	0	0	2
	23	84	1.75	1000	2	0	0	2
	24	76	1.82	1000	3	0	0	3
	26	88	1.90	1000	2	0	0	2
	27	75	1.92	1000	1	0	0	1
	28	73	1.88	1000	3	0	0	3
		80	1.80	1000	2	0	0	2
Sum					21	0	0	21
Average		80	1.85		2.1	0	0	2.1

MI – mitotic index, PI – proliferation index

Table 2. Results of the statistical analyses of micronucleus frequencies in phosphate workers and control subjects (ANOVA)

	Comparisons	F obs	F th	interpretation
a	Production unit x extraction unit x controls	20.95	9.22 (1‰)	Statisticaly highly significant difference (P = 4.5067E-06)
b	Production unit x controls	32.17	14.82 (1‰)	Statisticaly highly significant difference (P = 1.4988E-05)
c	Extraction unit x controls	215.83	17.14 (1‰)	Statisticaly highly significant difference (P = 6.6998E-10)
d	(Production unit + extraction unit) x controls	42.25	13.73 (1‰)	Statisticaly highly significant difference (P = 6.8601E-07)
e	Production unit x extraction unit	0.32	4.49 (1‰)	Statistically not significant difference (P = 0.579)

F obs – observed, F th – expected

Table 3. Selected characteristics of the different groups of subjects (X = no exposure; .. = data not available)

N° subject	Group[a]	Age (years)	Years of present work	Exposure time[b]	Exposures to							
					Tobacco (consumption)	Alcohol (consumption)	Tea (times/days)	Coffee (times/day)	Asbestos	Radiation	Coal	Dust
1	A	42	17	d4	Yes	Little	2	2	No	Little	No	Yes
2	A	36	11	d3	Yes	No	2	2	No	Little	No	Yes
3	A	35	10	d2	Yes	..	..	..	..	..	..	..
4	A	38	10	d2	Yes	..	..	..	..	..	..	..
5	A	42	18	d4	No	No	2	2	Yes	Little	No	Yes
6	A	35	10	d2	Yes	..	..	..	..	..	..	..
7	A	45	18	d4	Yes	Little	2	1.5	No	Little	Yes	No
8	A	41	21	d5	No	No	3	2	No	Little	No	No
9	A	49	25	d6	No	No	3	1	No	No	No	No
10	A	28	4	d1	No	No	3	0	No	No	Yes	Yes
11	A	39	16	d4	Stopped smoking	No	3	2	Yes	Yes	No	Yes
12	A	43	19	d4	No	No	4	0	Yes	Yes	Yes	No
13	B	57	28	d6	No	Little	2	1	X	X	X	X
14	B	52	25	d6	Stopped smoking	Stopped drinking alcohol	3	0.5	X	X	X	X
15	B	50	30	d6	Stopped smoking	No	2	2	X	X	X	X
16	B	44	19	d4	No	No	2	1	X	X	X	X
17	B	50	25	d6	No	No	2	0.5	X	X	X	X
18	B	54	28	d6	Stopped smoking	No	2	0.5	X	X	X	X
19	C	26	0	d0	No	No	2	1.5	X	X	X	X
20	C	50	0	d0	Stopped smoking	No	2	0.5	X	X	X	X
21	C	24	0	d0	No	No	2	1	X	X	X	X
22	C	24	0	d0	No	No	3	0	X	X	X	X
23	C	25	0	d0	No	No	2	1	X	X	X	X
24	C	48	3	d0	No	No	No	1	0.5	X	X	X
25	C	37	2	d0	No	No	No	2	1	X	X	X
26	C	31	2	d0	No	No	No	2	2	X	X	X
27	C	57	4	d0	No	No	No	4	0	X	X	X
28	C	42	3	d0	No	No	No	2	0.5	X	X	X

[a]A – production unit workers, B – extraction miners, C – control subjects
[b]d0 – unexposed
d1 < 5 years; d2 < 10 years; d3 < 15 years; d4 < 20 years; d5 < 25 years; d6 >25 years

(Leonard et al., 1984). Peripheral blood lymphocytes from agricultural workers who were exposed to pesticides, insecticides, herbicides, and fungicides showed a highly significant increase in the mitotic index and micronucleus frequency but no significant variation in the proliferation index compared to the control population (Sandra et al., 2000). In a large-scale biomonitoring study of adolescents in Flanders (Belgium) it was found that sexual maturity was terminated at an older age in children who lived near a waste incinerator and testicular volume was smaller in boys from the suburbs than in rural areas. Biomarkers of glomerular or tubular renal dysfunction in individuals were positively correlated with blood lead and biomarkers of DNA damage were positively correlated with urinary metabolites of polycyclic aromatic hydrocarbons and volatile organic compounds (Staessen et al., 2001). Interaction between different factors or pollutants results in complicated sta-

Table 4. Result of the cytogenetic analyses compared to a number of life-style factors of the investigated subjects

		Workers			Controls		
Biomarker		**MN**	**MI**	**PI**	**MN**	**MI**	**PI**
Average (total)		14.11	84.22	1.75	2	79.7	1.85
Age	> 40 years	14.91	85.08	1.78	2.75	78.5	1.83
	< 40 years	12.5	82.50	1.659	1.66	80.5	1.85
Duration of work	> 15 years	14.76	87.38	1.77	*	*	*
	< 15 years	12.40	76	1.670	*	*	*
Tobacco consumption	Yes	11.16	78.83	1.72	*	*	*
	Stopped	13.50	89.25	1.69	3	85	1.860
	No	16.62	85.75	1.79	2	79.11	1.85
Alcohol consumption	Yes	14.25	83.5	1.80	*	*	*
	No	15	87.36	1.74	2	79.7	1.85
Tea consumption	2 times/day	11.66	77.56	1.77	1.86	81.71	1.86
	≥ 3 times/day	16.56	90.88	1.72	2.66	75	1.82
Coffee consumption	0	22.00	96	1.648	2.5	74.5	1.82
	< 2 times/day	14.57	85.43	1.78	2.14	81.6	1.84
	> 2 times/day	12.66	84.16	1.77	1	75	1.92

Table 5. Result of the cytogenetic analyses compared to a number of different professional exposures in production unit workers

	MN		**MI**		**PI**	
	Exposed	Non- exposed	Exposed	Non- exposed	Exposed	Non- exposed
Asbestos	14.33	16.83	91	92.33	1.664	1.795
Radiation	16.16	15.60	97.33	81	1.74	1.764
Coal	20.60	13.66	97.66	89	1.630	1.812
Dust	12	21	82.4	103.75	1.747	1.757
Pesticides/ Herbicides	22	15.25	102	90.66	1.611	1.769
Petroleum products	16.8	15	88.8	95.75	1.821	1.664
Dyes	12.66	17.66	71.66	102	1.689	1.782
Solvents	15	16.28	79.5	95.42	1.646	1.781
NH_3	18.14	0	96.57	*	1.628	*
Fluor	18.14	8.50	96.57	75.5	1.728	1.834
SO_2	18.14	8.50	96.57	75.5	1.728	1.834
SO_3	15.25	22	90.62	102	1.769	1.611
NaOH	16.80	15	98	84.25	1.711	1.802
H_2S	19	10	93.5	88.66	1.748	1.758
Ether	17.50	13	97.66	80.33	1.735	1.784
H_2SO_4	16.8	15	98	84.25	1.711	1.802
NaOCI	20	14.85	115	85.28	1.799	1.738
HNO_3	13	16.37	115	89	1.606	1.769
NO_3	22	15.25	102	90.62	1.661	1.769

tistical analyses (Diana, 1999), which should be carefully conducted in studies that comprise a sufficient number of subjects. In the present study we did not want to go that far; however, the present investigation clearly demonstrated that the phosphate workers have significantly more genetic damage (here in terms of the micronucleus frequency), but no alterations in cell division or response to the mitogen phytohaemagglutin. This indicates a potential health risk that should further be investigated and urges for measures to reduce the individuals' exposures.

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