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Genotoxic potential of aluminum and fluoride on human peripheral blood lymphocytes

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Abstract: Aluminum (Al), the most abundant metal, is toxic especially in its trivalent form (Al^{+3}), which represents most soluble form at different physiological hydrogen ion concentrations (pH). Endemic fluorosis is a form of chronic fluoride intoxication resulting from ingestion of excessive quantities of fluoride through drinking water. The large quantity of drinking water consumed in hot arid climates is supposed to contribute to a higher daily intake of fluoride resulting in incidence of clinical fluorosis. The aluminum industry has hazardous waste by product of fluorides and this is also certified by Environment Protection Agency. Al is known to increase the intestinal absorption of fluoride at different physiological pH. In the present investigation, the genotoxicity studies of $AlCl_3$ and NaF independently and concurrently, was carried out on human peripheral blood lymphocyte *in vitro*. The study showed that NaF and $AlCl_3$ independently were significantly ($p<0.05$, $p<0.01$, $p<0.001$) toxic in the *in vitro* system. The combined treatment was more profoundly toxic ($p<0.01$, $p<0.001$) when compared to individual toxicity of the chemical on various parameters studied, without metabolic activation. The results suggest that NaF and $AlCl_3$ have a genotoxic and cytotoxic potential in human peripheral blood cultures *in vitro*.

Key words: Genotoxicity, Aluminum, Fluoride, Aberration, Micronuclei, Sister chromatid exchange

Introduction

Aluminum is the third most abundant element (8%) in the earth's crust, exceeded by oxygen (47%) and silicon (28%). The role aluminum plays in human physiology is not clearly known. However, the metal enters the body by intake of food and water. Aluminum has been detected in the brain cells of Alzheimer's disease patients (Munoz Garcia, 1986; Capper McLachlan *et al.,* 1989). The role of aluminum in cytotoxicity and DNA damage has also been explicated (Capper McLachlan and Farnell, 1986).

Genotoxicity tests indicate potential of fluoride to cause mutations, affect the structure of chromosomes and other genomic material; affect DNA replication, DNA repair, and the cell cycle; and/or transform cultured cell lines to enable them to cause tumors when implanted into host animals (EPA, 2006; Tsutsui *et al.,* 1984; Wu and Wu, 1995).

The aluminum industry is now worldwide and the major EPA-certified hazardous wastes by-products of fluorides have been established in these aluminum industries. In addition, one of the most toxic by-products of aluminum manufacture, sodium fluoride, is added to many public water supplies. Exposure of the central nervous system to aluminum salts notably fluoride and sulphate produces a progressive encephalopathy (Munoz Garcia *et al.,* 1986). Increased gut and intestinal fluoride absorption is noted in presence of aluminum at different physiological pH especially at acidic pH of stomach (Casarett *et al.,* 2001).

The present investigation was taken up to study the effects of aluminum and/or fluoride on peripheral blood lymphocyte cultures and thus understand the amount of DNA that can be caused indirectly. The degree of consistency of genotoxicity tests with the epidemiologic studies conducted, were evaluated.

Materials and Methods

The experiments were conducted by taking peripheral blood samples of five healthy individuals. A standard protocol was used to prepare mitotic chromosomes from peripheral blood lymphocyte culture (Hungerford, 1965). The metaphases thus obtained were stained and treated with the different techniques for different studies. The test compounds were added at '0' (zero) hr *i.e.* during the time of culture setting without metabolic activation. The doses were selected depending on the pilot dose finding studies. Sodium fluoride and/or aluminum chloride and positive control Mitomycin C was given at a concentration of 30 mg 10 ml^{-1} of culture and the cultures were exposed up to 69th hr after which the cultures were harvested using routine methods. The control included distilled water at the same volume as the treated cultures.

Plain giemsa staining and aneuploidy analysis: The slides were stained with 2% giemsa stain and were scanned for at least 100 well spread metaphases and were analyzed for different chromosomal aberrations *viz.*, chromosomal breaks and gaps, chromatid breaks and gaps and aneuploidy. One hundred and fifty cells per subject were blind scored for aneuploidy frequency.

Fluorescent-BrdU staining and analysis of SCE, CCPI/RI: The method of Perry and Wolf (1974) was followed and the slides were then analyzed for sister chromatid exchanges (SCE's) and cell cycle kinetics studies. The cell cycle proliferative index (CCPI) for each individual was calculated according to the formula:

$$CCPI = \frac{1 \times (\% M_1) + 2 \times (\% M_2) + 3 \times (\% M_{3+})}{100}$$

Binucleate induction and micronucleus analysis: Cytokinesis was inhibited in dividing lymphocyte by the addition of cytochalasin B to the cultures. Binucleate induction and micronucleus analysis was done following the method of Ford *et al.* (1988).

Nuclear organizing region (NOR)-banding and acrocentric chromosomes associations (ACA) scoring: NOR staining was done following the method of Goodpasture and Bloom (1975). The visual association was considered if two or more acrocentric chromosomes were in close proximity at their satellite end.

Giemsa-trypsin-giemsa (GTG-banding/G-banding) and scoring of telomere association: The technique of Sun-Chu-Chang (1973) was followed. Telomere to telomere associations were scored when single or both the chromatids of two different chromosomes were observed to be "sticky" to each other and found to be fused end to end.

All the above data was statistically analyzed using student's t-test with SPSS software.

Results

Frequency of aneuploidy: Of the 100-120 metaphases scored for each individual, two types of aneuploidy were scored *viz*., hyper-ploidy and hypo-ploidy. Statistical analysis showed non-significant change in NaF treated group. $AlCl_3$ treatment alone and in combination with NaF showed a significant ($p<0.001$) increase in aneuploid cells as compared to control.

Frequency of chromosomal aberrations: A 100-120 metaphase plates were scored and four different types of chromosomal aberrations were observed *viz*., chromatid breaks/gaps and chromosomal breaks/gaps. A significant change in the mean frequency of chromosomal aberrations in treated cultures was

Table - 1: Frequency of chromosomal aberrations in control and treated groups

Groups/ Individuals	Types of chromosomal aberrations					Mean ± S.E.
	Chr. Break	Ctd. Break	Chr. Gap	Ctd. Gap	Total	
			Group I (control)			
1	0	0	0	1	4	0.8± 0.20
2	0	0	0	1		
3	0	0	0	0		
4	1	0	0	0		
5	0	1	0	0		
			Group II (control + MMC)			
1	1	2	1	1	20	4 ± 0.55***
2	2	1	0	1		
3	1	0	1	2		
4	0	1	2	2		
5	1	0	0	1		
			Group III (NaF treated)			
1	2	0	0	1	9	1.8 ± 0.37**
2	0	1	1	0		
3	0	1	1	0		
4	0	1	0	0		
5	0	0	0	1		
			Group IV ($AlCl_3$ treated)			
1	0	0	1	1	10	2.0 ± 0.21***
2	1	0	0	1		
3	0	0	1	1		
4	0	1	1	1		
5	0	0	0	1		
			Group V ($NaF+AlCl_3$)			
1	0	0	0	2	14	2.8 ± 0.37***
2	1	0	0	2		
3	3	0	1	0		
4	1	0	2	0		
5	0	0	1	1		

Values are + S.E. **p<0.01, ***p<0.001

Table - 2: Cell cycle proliferative index

Groups / Individuals	Total metaphases	% M_1	%M_2	% M_3	CCPI
		Group I (control)			
1	100	30	55	15	1.85
2	100	28	57	15	1.87
3	100	29	61	10	1.84
4	100	31	54	15	1.84
5	100	32	55	13	1.81
Total	500				
Mean ± S.E.		30 ± 0.71	56.4 ± 1.25	13.6±0.98	1.84 ± 0.01
		Group II (control + MMC)			
1	100	61	35	4	1.43
2	100	54	38	8	1.54
3	120	62.5	29.16	8.33	1.46
4	100	57	38	6	1.51
5	107	61.68	31.78	6.54	1.45
Total	500				
Mean ± S.E.		59.24 ± 1.61	34.39 ± 1.74	6.57 ± 0.78	1.48 ± 0.02***
		Group III (NaF treated)			
1	100	42	51	7	1.65
2	100	40	51	9	1.69
3	102	39.22	50.98	9.8	1.74
4	100	37	55	8	1.71
5	100	40	50	10	1.7
Total	502				
Mean ± S.E.		39.8 ± 0.80	51.60 ± 0.87	8.76 ± 0.56	1.70 ± 0.01*
		Group IV ($AlCl_3$ treated)			
1	101	42.57	51.49	5.94	1.65
2	102	41.18	49.02	9.8	1.72
3	106	43.4	50	6.6	1.73
4	100	42	51	7	1.65
5	100	40	55	5	1.68
Total	509				
Mean ± S.E.		41.83 ± 0.58	51.30 ± 1.01	6.87 ± 0.81	1.68 ± 0.02*
		Group V ($NaF+AlCl_3$)			
1	100	52	48	2	1.5
2	106	52.83	41.51	5.66	1.62
3	101	52.48	42.57	4.95	1.54
4	100	49	46	5	1.56
5	100	52	44	4	1.52
Total	507				
Mean ± S.E.		51.66 ± 0.68	44.42 ± 1.17	4.32 ± 0.64	1.55 ± 0.02*

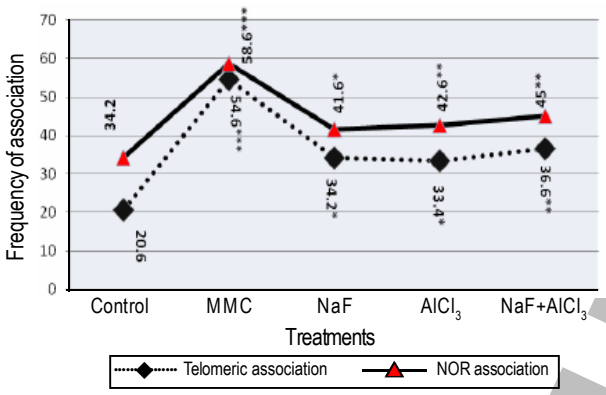
Values are ± S.E. * p<0.05, **p<0.01, ***p<0.001

noticed though some baseline aberrations were observed in control cultures.

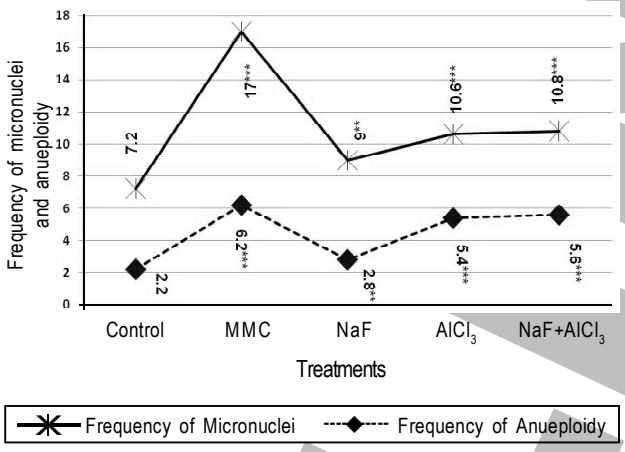
Sister chromatid exchange and cell cycle kinetics: The mean SCE showed a significant (p<0.001) increase in treated groups when compared to control. Cell from all individuals showed a higher incidence of total SCE and SCE/Cell after the treatment as compared to the control.

Cell cycle kinetics: The result of cell-cycle kinetics as analyzed by the differential staining technique showed significant decrease in the cell cycle proliferative index and increased percentage of M_1 cells in fluoride and/or aluminum treated samples while there was reduced percentage of M_2 and M_3+ plates due to treatments.

Micronuclei in cytochalasin-B induced binucleates: A total of 5000 binucleates were screened for control and treated groups (1000 binucleates/ individual-N=5) to determine the frequency of binucleates with micronuclei. In some cells, the binucleates had more than one micronucleus. The mean frequency of micronuclei was observed to be 7.2 ± 0.37 in control group. The values for NaF and $AlCl_3$ treatments alone or in combination were 9.0 ± 0.45,



Graph - 1: Frequency of telomeric and satellite associations in control and treated groups : *p<0.05, **p<0.01, ***p<0.001, Values are ± S.E.



Graph - 2: frequency of micronuclei and aneuploidy in control and treated groups : *p<0.05, **p<0.01, ***p<0.001, Values are ± S.E.

10.6 ± 0.67 and 10.8 ± 0.58 respectively. Analysis showed a significant (p<0.001) increase in the mean frequency of micronuclei induction in treated groups compared to baseline of control.

Studies on acrocentric chromosome association: The frequency of satellite association in metaphase spread varied in different individuals. About 100-120 metaphases were scored in each individual. Increased frequency of satellite association was found due to treatment and the values were significant (p<0.01, p<0.001) when statistically evaluated. The percentage association was 34.40% in control while that of treated increased to 41.43%, 41.84% and 44.37% respectively.

Frequency of telomeric association: The frequency of telomeric association did not show a exceedingly significant (p<0.05) increase in cultures treated with NaF and $AlCl^{-3}$ individually. Combined treatment of fluoride and aluminium however, caused more telomeric damage and hence showed a significant (p<0.01) increase in the mean frequency of telomeric association.

Discussion

In vitro sister chromatid exchanges and chromosomal aberrations in metaphase plates are two most reliable methods to study the genotoxic effects of any chemicals or compounds. Manna and Das (1972) injected mice intraperitoneally with 0.01, 0.05 or 0.1 molar $AlCl_3$ and observed a significant increase in chromatid type aberrations over the control. Chromosomal aberrations were also induced by aluminium in peritoneal cells from rats, mice and Chinese hamsters (Bhamra and Costa, 1992). In human leucocytes cultures also, there was increased aberrations post aluminum treatments (Roy *et al.,* 1990,1991). Aluminium is known to have nuclear effects such as binding to DNA phosphates, increasing the DNA-Histone binding, consequently altering the frequency of sister chromatid exchanges and cell cycle proliferative index. Aluminium may also be responsible for increased chromosomal aberrations and change the frequency of SCEs since it binds to molecules of nuclear compartments and alter DNA-protein interactions (Capper McLachlan and Farnell, 1986; Capper-McLachlan, 1989) leading to genotoxic impact. Neurotoxic effects of aluminium are well studied and it has been partially attributed to its subcellular adverse effects on DNA which may finally lead to genotoxic changes in the tissue. In the present study there was a significant increase in the SCEs and to a lesser extent chromosomal aberrations were seen as could be understood by the above discussions.

Mohammed and Chandler (1982) reported that fluoride was mutagenic and caused chromosomal damage even at a concentration of 1 ppm NaF in mice bone-marrow. Jachimczak and Skotarozak (1978) reported an *in vitro* study in which fluoride at 0.6, 6.0 and 60 ppm caused an increase in chromosome aberrations in human leukocytes when compared to control values. Sheth *et al.* (1994) reported an increase in the frequency of SCEs in endemic human population of north Gujarat, India as compared to the control population. Similar population studies by Wu and Wu (1995) in China showed an increased frequency of SCEs in PBLC at a concentration of 4-15 mg L^{-1} of fluoride in drinking water. Tsutsui *et al.* (1984) also found increased level of unscheduled DNA synthesis by 100-300 ppm fluoride in cultured human oral keratinocytes suggesting interference of fluoride with DNA synthesis. Though the underlying mechanism of fluoride toxicity is unclear, fluoride is known to interfere with DNA production and protein synthesis rather than inhibiting DNA synthesis (Imai *et al.,* 1983). The disturbed balance of DNA and protein concentration in different tissues of female and male albino mouse studies provide evidence that fluoride indirectly is responsible for multiple genotoxic effects (Chinoy *et al.,* 2003).

Though the mechanism of action of aluminium and fluoride individually are to some extent elucidated, their effect as aluminofluoride complex is particularly unclear. Combined complexes of aluminium and fluoride have been studied to induce toxic effects in various tissues *in vitro* (Strunecká and Patocka, 1999). The effect of aluminium and fluoride as in experiment by Tarkka *et al.* (1993) suggest that AlF^{4-} a phosphate analog binds to the nucleotide-binding site of H_1 histones. This adduct may abolish the nucleoside tri-phosphate hydrolysis and interfere with the nucleotide modulation of H^{-}_1 DNA binding. This affects the expression of certain cell-specific genes, leading to impaired chromatin function and thus, may increase

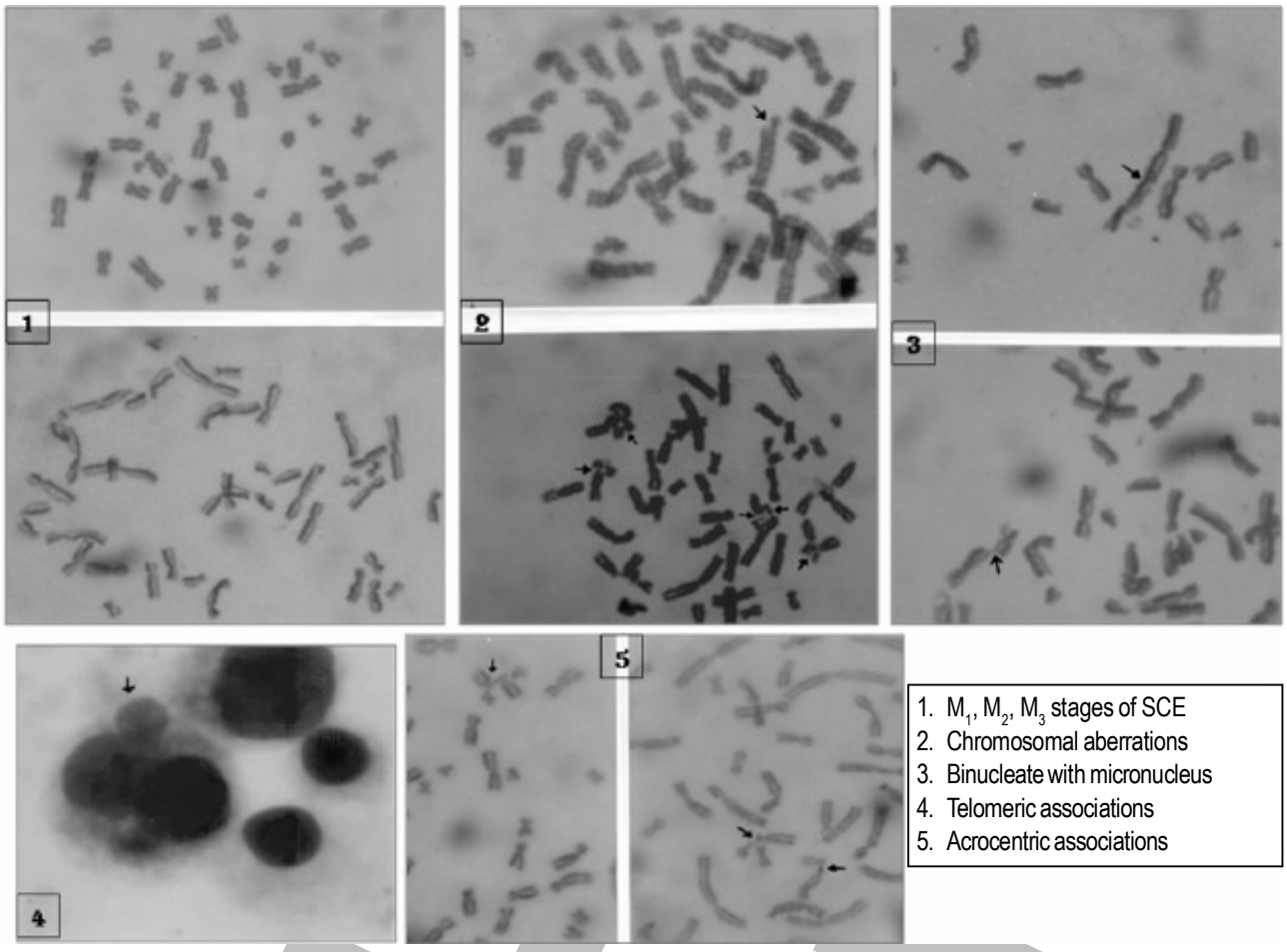


Fig. 1: Showing the different types of toxicological indexes studied

chromosomal aberrations and alter the frequency of sister chromatid exchange in the cultures.

AlF^{-4}, a phosphate analog, slows down microtubule turnover resulting in its destabilization which may result into non-disjunction and aneuploidy (Carlier *et al.,* 1988). Chromosome displacement is a function of spindle tubule dysfunction and leads to form micronucleus or it might randomly incorporate into one of the two daughter cells resulting in trisomy or monosomy. The frequencies of micronuclei and binucleates with MN in PBLC of workers exposed to fluoride showed a significant increase than in control population (Zhang and Meng, 1999). This may suggest that an increase MN may cause a higher chromosome malformation frequency (Lu Wenqing, 1999). Mohamed and co-workers (Mohamed, 1970) reported that NaF, at a concentration of 10^2 (190 ppm), had mutagenic action on the mitotic chromosomes of onion root tips, causing anaphase lags and bridging, tetraploid nuclei and multipolar anaphases which is a result of non-disjunction and can further lead to increase in micronuclei frequency. Aluminium at concentration as low as 0.1 mM inhibited microtubule formation in vivo and in vitro in protozoa (Bonhaus *et al.,* 1980). Micromolar aluminium levels have also been shown to reduce ^{3}H-thymidine incorporation in a transformed cell line, which indicates that aluminium may impede cell cycle progression (Blair *et al.,* 1989). Increased micronuclei in the present study may be result of increased chromosomal displacement and altered cell cycle progression by fluoride, aluminum and combined treatments thus lagging of chromatid at anaphase. An increase in aneuploidy in the present study may also be attributed to microtubular malfunctioning leading to arbitrary incorporation in daughter cells.

An increase in the frequency of NOR association is due to increased transcriptional activity and nucleolar dispersion of ribosomal RNA in the satellite region of chromosomes. This has recently attracted much attention because of claims that their frequency within the nuclei is significantly higher in malignant cells than in normal ones (Crocker and Paramjit Nar, 1987; Underwood and Giri, 1988). The interactions of NOR may also be etiologically involved in the process of non-disjunction of acrocentric chromosomes leading to aneuploidy (Schmickel *et al.,* 1985). This is reason for considering the parameter for the study and it showed an increased association in the treated cultures to the normal cultures.

The loss of telomeres and increased incidence of "telomeric associations" have been related to senescence and rapid aging.

Molecular studies by Hastie *et al*. (1990) showed that end-to-end chromosome fusions, observed in some tumors play a part in genetic instability associated with tumorigenesis. Telomere damage leading to instability in chromosomes causing cell apoptosis by toxic compounds has been known. In the present study the increased telomere association was found in treated cultures than in the normal controls. This may indicate that the chemicals have a specific affect on cell senescence and chromosome instability due to telomeric damage under culture conditions.

In the present study it can be derived that the exposure to aluminum or fluoride or their combination through air or water for extended term may have DNA damaging effects and may be contributing to tumorigenesis.

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