

CYTOGENETIC ANALYSIS OF HUMAN LYMPHOCYTES OF FLUOROSIS-AFFECTED MEN FROM THE ENDEMIC FLUOROSIS REGION IN NALGONDA DISTRICT OF ANDHRA PRADESH, INDIA.

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SUMMARY: Cytogenetic analysis was carried out on human lymphocytes of 73 fluorosis-affected men from the endemic fluorosis region in Nalgonda district of Andhra Pradesh, India, who were drinking water with a mean concentration of fluoride (F) of 4.13 ± 0.55 mg/L, range 1.56–8.36 mg/L. Eighty healthy men, of a similar age living near the endemic fluorosis area, drinking water with <1 mg F/L were selected as controls. The conventional cytogenetic protocols for detecting chromosomal aberrations (CA) and sister chromatid exchanges (SCE) were performed. More CA were present in the subjects from the endemic fluorosis region, $3.57 \pm 0.27\%$, compared to the controls, $0.68 \pm 0.09\%$ ($p < 0.01$). Similarly, more SCE were present in the subjects from the endemic fluorosis region, mean 8.96 ± 0.68 SCE/cell, compared to the controls, mean 3.58 ± 0.67 SCE/cell ($p < 0.001$). This indicates that F acts as a mutagen inducing chromosomal damage in fluorosis-affected men.

Keywords: Chromosomal aberrations; Endemic fluorosis; Peripheral blood lymphocytes; Sister chromatid exchanges.

INTRODUCTION

Fluoride (F) is a ubiquitous element found naturally in food and water. Chronic exposure to F is known to cause toxicity and serious health problems in many parts of the world, where the drinking water contains more than 1–1.5 ppm of F.^{1,2} The World Health Organization recommends that drinking water should not contain more than 1.5 mg/L of F.

In India, high levels of both F and arsenic in ground water cause public health problems. Approximately 60–65 million people in the country drink water containing ≥ 1.5 mg F/L and it is estimated 2.5–3.0 million are affected by fluorosis.³ Chronic fluorosis can severely damage many systems of the human body, but its pathogenicity is poorly understood.⁴

The majority of carcinogenic or mutagenic compounds exert their activity directly on the DNA molecule. F is known to be cytotoxic⁵ and its genotoxic potential has been examined in a large number of studies using a variety of test systems.^{6–10} F is a mutagenic agent able to cause chromosomal and DNA damage, sister chromatid exchanges (SCE) and lipid peroxidation.^{11,12} A few investigations have found that F does not produce genotoxic effects.^{13–16} Because of the mixed results on the genotoxic nature of F in mammalian cell systems, a need was seen for more studies on the genotoxic nature of F. The present study was undertaken to study the possible damage induced by F to the genetic apparatus of the persons drinking water containing elevated levels of F by using standard

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cytogenetic end points such as chromosomal aberrations (CA) and SCE in peripheral blood lymphocytes (PBL).

MATERIALS AND METHODS

F in the drinking water was measured with a F ion selective electrode method¹⁷. One hundred and forty-two drinking water samples were collected in plastic bottles from wells in 2 different endemic fluorosis areas and 100 water samples were collected from the control area.

Study population: A total of 73 males with fluorosis were selected randomly from Nemmani and Mothukur villages in Nalgonda district of Andhra Pradesh, India. The two villages are located 55 km and are notified as severe endemic fluorosis-affected areas. As there was no significant difference in the mean F values from the two villages, the subjects selected from the two villages were considered as a single group (F-affected subjects). A formal control group of 80 healthy males of similar age were selected from Aleir village, located near the endemic fluorosis region.

Sampling was done in a randomized manner by using a standard questionnaire under the supervision of a local medical practitioner. All the subjects gave written informed consent and were interviewed for personal data, recent viral infections, vaccinations, and medical history. Alcoholics, smokers, and tobacco chewers were excluded since these confounding factors may alter F toxicity. Females were excluded from the study because the hormonal profiles and the medication for contraception may interfere markedly with the results. The local institutional ethical committee screened and approved the study. The work was carried in accordance with the ethical standards of the committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2000.

Sample collection and lymphocyte culture: Heparinized venous blood samples were drawn from each donor and brought back to the laboratory within a few hr after sampling. The blood samples were initiated for culture within 48 hr after the collection. Micro cultures were set up in duplicate for each sample to isolate the lymphocytes as per the standard protocol of Hungerford.¹⁸ A cell density of $100 \times 10^6/\text{mL}$ was added to 2 mL of RPMI-1640 culture medium, containing 20% fetal calf serum, 100 units/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin and 2% phytohaemagglutinin-P form. The cultures were incubated at 37°C for 72 hours.

Analysis for chromosomal aberrations and sister chromatid exchanges: For the CA assay, 0.1 mL of colchicine (0.1 $\mu\text{g}/\text{mL}$) was added to the cultures for mitotic arrest 4 hr before harvest and the hypotonic treatment was performed with prewarmed 0.075 M KCl for 15 min at 37°C. The culture was harvested by centrifuging at $400 \times g$ and the cell pellet was washed three times with 3:1 Carnoy's fixative (methanol: acetic acid v/v). The cells were prepared by air drying and stained with 10% Giemsa (pH 6.8).¹⁹ For the SCE assay, 5 bromodeoxyuridin was added to the culture and the culture was processed in the same way as was done for the CA assay. The cells were prepared from the fixed cells by air-drying and the slides were stained by the fluorescence-plus Giemsa

procedure.²⁰ For cell cycle studies, the first division (M_1) cells were identified as the cells containing chromosomes with both sister chromatids stained uniformly dark. Second division (M_2) cells were identified by their containing only differentially stained chromatids with one chromatid darkly stained and its sister chromatids lightly stained. The third or subsequent division (M_3) cells were identified by both sister chromatids being lightly stained.

Slide scoring: For cytogenetic analysis, preparations were coded and scored blindly. The analysis was conducted by two scorers, one per replicate. For each subject, using coded slides, 100 well spread metaphases with 46 chromosomes were analyzed for chromosomal aberrations and 30 metaphases were analysed for sister chromatid exchange frequencies. The cell replication index (CRI) or cell cycle proliferative index (CCPI) was calculated by calculating the percentage of cells in first (M_1), second (M_2), and third (M_3) cell divisions using the formula:

$$CRI = \frac{1 \times \% \text{ of } M_1 + 2 \times \% \text{ of } M_2 + 3 \times \% \text{ of } M_3}{n}$$

Statistical analysis: The results were tested for statistical significance by 2×2 contingency chi-square test for chromosomal aberrations and student t-test for sister chromatid exchange frequencies. Probability values of $p < 0.05$ were considered significant.

RESULTS

The concentration of F element in drinking water samples from F affected villages in Nalgonda District of Andhra Pradesh, India are shown in Table 1.

Table 1. Fluoride drinking water levels in the endemic fluoride villages of Nalgonda District (Values are mean ± SD)

Village	No. of samples	Fluoride in drinking water (mg/L)	
		Mean ± SD	Range
Aleir*	60	0.78 ± 0.02	0.12 – 0.90
Nemmani†	60	4.13 ± 0.55‡	1.56 – 8.36
Mothukur†	82	4.02 ± 0.45‡	1.62 – 8.14

*Control village; †High drinking water fluoride villages; Compared to the control village: ‡ $p < 0.001$

There was little difference in the mean F levels in the drinking water between the two endemic fluorosis villages but their levels were significantly higher than that in the control village ($p < 0.001$).

The average percentage of chromosome-type and chromatid-type aberrations and the total of aberrant cells were significantly higher ($p < 0.01$) in the fluorosis-affected subjects than in the controls (Table 2).

Table 2. Frequencies of lymphocytes with chromosomal aberrations among the subjects from the endemic fluorosis region
(Values in parentheses are % \pm SD, 100 metaphases were scored for each subject)

Group (F in water in mg/L)	No. of persons	No. of metaphases scored	No. of cells with chromosome- type aberrations (% \pm SD)	No. of cells with chromatid-type aberrations (% \pm SD)	Total no. of aberrant cells (% \pm SD)
Control (<1)	80	8000	27 (0.34 \pm 0.08)	28 (0.35 \pm 0.06)	55 (0.68 \pm 0.09)
F-affected subjects (1.56–8.36)	73	7300	148 (2.02 \pm 0.16)	113 (1.54 \pm 0.08)	261 (3.57 \pm 0.27)*

Compared to the control group: * $p < 0.01$.

The types of aberrations that showed a significant increase in fluorosis-affected subjects, compared to the controls, were dicentrics, acentric fragments and chromatid breaks (Table 3).

Table 3. Classification of various chromosomal aberration frequencies from endemic fluorosis region in Nalgonda district of Andhra Pradesh, India. (N = number of persons, values in parentheses are %, 100 metaphases were scored for each subject, gaps were excluded)

Group (F in water in mg/L)	N	Types of chromosomal aberrations							Total chromosomal aberrations	
		Chromosome-type				Chromatid-type				
		Dicentric	Acentric fragments	Acentric rings	Gaps	Total	Breaks	Exchanges		Total
Control (<1)	80	4 (0.05)	5 (0.06)	2 (0.02)	16 (0.20)	27 (0.33)	28 (0.35)	0 (0.00)	28 (0.35)	55 (0.68 \pm 0.0)
F-affected subjects (1.56– 8.36)	73	32 (0.43)	36 (0.49)	8 (0.10)	72 (0.98)	148 (2.02)	105 (1.43)	8 (0.10)	113 (1.54)	261 (3.57 \pm 0.27)*

Compared to the control group: * $p < 0.05$.

The data on SCE are shown in Table 4. A two-fold increase in SCE frequencies was observed in the fluorosis-affected subjects compared to the healthy control group ($p < 0.001$). The CRI or CCPI was increased in the fluorosis-affected subjects when compared to the controls. The percentage of M_1 and M_2 cells increased while that for the M_3 cells decreased in fluorosis-affected subjects compared to the controls. The calculated RI values are higher in fluorosis-affected persons

compared to the controls. These data show that the DNA of the fluorosis-affected males was seriously damaged.

Table 4. Comparison of SCE frequency and CRI between persons with endemic fluorosis and the control group. (30 metaphases were scored for each subject)

Group (F in water in mg/L)	No. of persons	No. of cells scored	No. of SCE	SCE rate/cell Mean±SD	CRI			
					M ₁	M ₂	M ₃	%
Control (<1)	50	1500	5382	3.58±0.67	32	32	28	1.80
F-affected subjects (1.56–8.36)	73	2190	19642	8.96±0.65*	49	42	22	2.06

Compared to the control group: *p<0.001.

DISCUSSION

Fluorine is not an essential trace element for human health and can cause significant toxicity.²¹ Several studies on experimental animals, both *in vivo* and *in vitro*, have shown that F is tumorigenic, can increase tumor growth rate, and can interfere with DNA repair.¹⁵ The majority of the studies carried out with sodium fluoride, showed major chromosomal anomalies, mainly of chromatid gaps and breaks,²⁰⁻²⁴ and some *in vivo* studies carried out in endemic fluorosis areas, showed an increase in sister chromatid exchanges.²⁵ In the present investigation, the total chromosome aberrations (148) were more than the total chromatid aberrations (113). The observations made by Nair et al.²⁶ and Podder et al.²⁴ support our findings.

The increased frequency of chromosomal aberrations in the present study may indicate an increased cancer risk.^{27,28} Furthermore, chromosomal aberrations are the initial events in carcinogenesis and constitute an early warning signal for the development of cancer. In addition, the higher frequency of dicentrics recorded in this study may originate from chromosomal aberrations. Dicentrics are known to be lethal in cell proliferation and their presence implies a higher cancer risk.²⁹ The exact reason for the higher dicentric frequency recorded in this study is not known, but it may be possibly due to chromosomal instability.

Our finding of an increased SCE frequency in the subjects with fluorosis indicates the genotoxic nature of F and this is supported by similar results found by others in humans, exposed to high drinking water F.^{16,25,30}

During this study the SCE analysis has been considered to be a sensitive tool for detecting DNA damage and there is a clear relationship between the ability of a substance to induce DNA damage and to mutate chromosomes and cause cancer. Any increase in SCE frequency is primarily considered as evidence of chromosomal damage. The decrease in M₃ divisions in the fluorosis-affected subjects suggests a possible paradoxical dose-response, as discussed by Burgstahler, with a greater M₃ division rate at a lower F level.³¹

The exact mechanism for F genotoxicity is quite complex. F may not induce DNA damage directly, but do so indirectly by causing an alteration in the normal DNA replication process by establishing a strong hydrogen bond with both purines and pyrimidines.³² F affects many enzymatic activities³³ and possibly these effects may delay both the mitotic and meiotic cycles of the cell leading to chromosomal breakages. Such chromosomal aberrations may eventually lead to the structural changes and fragmentation observed in the present investigation. Furthermore, F can inhibit nucleic acid synthesis,³⁴ and the aberrations reported in the present study could be the result of an interaction of F with the enzymes responsible for DNA synthesis or repair rather than F interacting directly with DNA.

In conclusion, the results indicate that the fluorosis-affected humans from the endemic fluorosis area in Nalgonda district of Andhra Pradesh, India, have experienced the mutagenic nature of F, as manifested by an increase in the frequencies of chromosomal aberrations and sister chromatid exchanges in peripheral blood lymphocytes. There is a need to monitor the health effects of exposure to high levels of drinking water F as well as a need to prevent fluorosis developing through the provision of safe drinking water, with defluoridation units if alternative safe water cannot be found, together with an appropriate nutritious diet including adequate antioxidant-containing fresh fruit and vegetables.

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REFERENCES

- 1 Das AA. Fluorosis. In: Bamji MS, Rao NP, Reddy V, editors. Text book of human nutrition. New Delhi: Oxford & IBH publishing;1996. pp. 424-40.
- 2 Susheela AK. Fluorosis management programme in India. *Curr Sci* 1999;77:1250-6.
- 3 Chand D. Proceedings of the international workshop on fluorosis in drinking water; strategies, management and mitigation; Bhopal: 2001. pp.13-27.
- 4 Krishnamachari KAVR. Skeletal fluorosis in humans: a review of recent progress in the understanding of the disease. *Prog Food Nutr Sci* 1986;10:279-314.
- 5 Jacobson-Kram D, Montalbano D. The reproductive effects of assessment group's report on the mutagenicity of inorganic arsenic. *Environ Mutagen* 1985; 7: 787-804.
- 6 Tao MS, Gao WHH, Zao ZL. A cytogenetic study on farmers who drink contaminated well water. *Environ and Health* 1992;9(1):24-5.
- 7 Meng ZQ, Zhang LZ. Cytogenetic damage induced in human lymphocytes by sodium bisulfite. *Mutat Res* 1992;298:63-9.
- 8 Meng ZQ, Zhang B. Chromosomal aberrations and micronuclei in lymphocytes of workers at a phosphate fertilizer factory. *Mutat Res* 1997;393:283-8.
- 9 Gadhia PK, Joseph S. Sister chromatid exchange frequency and chromosomal aberrations in residents of fluoride endemic regions of South Gujarat. *Fluoride* 2000;33(4): 154-8.
- 10 Bassin EB. Association between fluoride in drinking water during growth and development and the incidence of osteosarcoma for children and adolescents [dissertation for the degree of Doctor of Medical Sciences]. Boston (MA); Harvard School of Dental Medicine; 2001.

- 11 Riberio DA, Marques MEA, Salvadori DMF. Lack of effect of prior treatment with fluoride on genotoxicity of two chemical agents *in vitro*. *Caries Res* 2007;41:239-43.
- 12 He LF, Chen JG. DNA damage, apoptosis and cell cycle changes induced by fluoride in rat oral mucosal cells and hepatocytes. *World J Gastroenterol* 2006;12(7):1144-8.
- 13 Meng ZQ, Meng HQ, Cao XL. Sister-chromatid exchanges in lymphocytes of workers at a phosphate fertilizer factory. *Mutat Res* 1995;334:243-6.
- 14 Slamenova S, Gabelova A, Ruppova K. Cytotoxicity and genotoxicity testing of sodium fluoride on Chinese hamster V79 cells and human EUE cells. *Mutat Res* 1992;279:109-115.
- 15 Mihashi M, Tsutsui T. Clastogenetic activity of sodium fluoride to rat vertebral body-derived cells in culture. *Mutat Res* 1996;368:7-13.
- 16 Li Y, Liang CK, Katz BP, Brizendine EJ, Stookey GK. Long-term exposure to fluoride in drinking water and sister chromatid exchange frequency in human blood lymphocytes. *J Dent Res* 1995;74(8):1468-74.
- 17 Clesceri LS, Greenberg AE, Eaton AP, editors. Standard methods for examination of water and waste water. 20th ed. Washington, DC: American Public Health Association, American Water Works Association, Water Environment Federation; 1998.
- 18 Hungerford DA. Leucocytes cultured from inocula of whole blood and the preparation of metaphase chromosomes by treatment with hypotonic KCl. *Stain Technol* 1965;40:333-8.
- 19 Moorehead PS, Nowell PC, Mellman WJ, Battips DM, Hungerford DA. Chromosomal preparations of leucocytes cultured from human peripheral blood. *Exp Cell Res* 1960;20:613-6.
- 20 Perry PF, Wolf S. New Giemsa method for differential staining of sister chromatids. *Nature* 1974;251:156-8.
- 21 Zeiger E, Shelby MD, Witt KL. Genotoxicity of fluoride. *Environ Mol Mutagen* 1993;21:309-18.
- 22 Tsutsui TN, Suzuki M, Ohmori M, Maizumi H. Cytotoxicity, chromosome aberrations and unscheduled DNA synthesis in cultured human diploid fibroblasts induced by sodium fluoride. *Mutat Res* 1984;139:193-8.
- 23 Thomson EJ, Kilanowski FM, Perry PE. The effect of fluoride on chromosome aberration and sister-chromatid exchange frequencies in cultured human lymphocytes. *Mutat Res* 1985;144:89-92.
- 24 Podder S, Chattopadhyay A, Bhattacharya S, Ray MR. Differential *in vivo* genotoxic effects of lower and higher concentrations of fluoride in mouse bone marrow cells. *Fluoride* 2008;41(4):301-7.
- 25 Wu DQ, Wu Y. Micronucleus and sister chromatid exchange frequency in endemic fluorosis. *Fluoride* 1995;28(3):125-7.
- 26 Nair SB, Jhala DD, Chinoy NJ. Mitigation of genotoxic effects of fluoride and arsenic by ascorbic acid in human lymphocyte cultures. *Fluoride* 2004;37(4):249-56.
- 27 Au WW, Cantelli-Forti G, Hrelia P, Legator MS. Cytogenetic assays in genotoxic studies: somatic cell effects of benzene and germinal cell effects of dibromochloropropane [review]. *Teratog Carcinog Mutagen* 1990;10:125-34.
- 28 Santos-Mello R, Silva JC, Nunes MH, Braga MA. Cytogenetic study on coke oven workers with abnormal blood counts. *Mutat Res* 1992; 280:261-9.
- 29 Awa AA. Chromosome damage in atomic bomb survivors and their offspring—Hiroshima and Nagasaki. In: Ishihara T, Sasaki MS, editors. Radiation-induced chromosomal damage in man. New York: Alan R Liss Inc.; 1983.
- 30 Sheth FJ, Multani AS, Chinoy NJ. Sister chromatid exchanges: a study in fluorotic individuals of North Gujarat. *Fluoride* 1994;27(4):215-9.
- 31 Burgstahler AW. Paradoxical dose-response effects of fluoride [editorial]. *Fluoride* 2002; 35(3):143-7. Comment in: *Fluoride* 2002;35(4):230.
- 32 Srivastava SK, Gillerman E, Modak MJ. The artifactual nature of fluoride inhibition of reverse transcriptase and associated ribonuclease H. *Biochem Biophys Res Commun* 1981;101:183-8.
- 33 Bogin EM, Abrams M, Avidar Y, Israelli B. Effect of fluoride on enzymes from serum, liver, kidney, skeletal and heart muscles of mice. *Fluoride* 1976;9(1):42-6.
- 34 Bempong MA, Tower EC. Sensitivity of rat testis to inhibitors of nucleic acid synthesis. II. mutagenic action of mytomyacin. *J Hered* 1973;64(6):324-28.