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CYTOLOGICAL EFFECTS OF SODIUM FLUORIDE ON MICE

by

A.H. Mohamed and M.E. Chandler
Kansas City, Missouri

SUMMARY: Inbred mice, fed a low-fluoride diet, 0.263 ±.028 ppm F^-, were given drinking water containing 0, 1, 5, 10, 50, 100, or 200 ppm F^- for 3 to 6 weeks. Cytological studies on bone marrow cell chromosomes and spermatocytes showed that 1-200 ppm F^- (as sodium fluoride) was able to induce chromosomal changes in a dose-dependent manner. The frequency of the induced chromosomal damage was significantly higher in each treatment than in the controls. The observed abnormalities included translocations, dicentrics, ring chromosomes, and bridges plus fragments, or fragments by themselves. There was a significant correlation between the amount of fluoride in the body ash and the frequency of the chromosomal abnormalities.

Introduction

According to Muller (1) an increasing number of toxic substances in the environment had been found to produce their primary damage by injuring the genetic material of the cell they enter. Extensive studies on the cytological effects of fluoride have been conducted mainly on plants. Aqueous sodium fluoride in a concentration of $1x10^{-2}$M was shown to produce chromosomal changes in onion root-tip chromosomes (2). Studies on the effects of HF (3 $\mu g/m^3$) on mitotic and meiotic chromosomes in tomato plants (3-4) and on meiotic chromosomes in maize (5) indicated a positive correlation between the frequency of chromosomal aberrations and treatment durations. The treatment, in vitro, of oocytes from mice, ewes and cows with sodium fluoride (6) induced chromosomal changes similar to those reported by other investigators (2.4). Cow oocytes exhibited the greatest sensitivity to the treatment (6). Attempts, however, to induce meiotic abnormalities in mouse oocytes, in vivo, by injecting sodium fluoride intravenously or subcutaneously produced only a small number of cells with abnormal chromosomes (6). Some investigators (7-9) have pointed out that fluoride, especially at a low concentration, failed to induce significant adverse chromosomal changes in mammalian cells. Others (10-11) however, who challenged this claim, showed that the addition of fluoride to human leukocyte cell cultures was able to induce chromosomal changes significantly higher than in the controls.

The present studies were undertaken with the purpose of determining the cytological effects of sodium fluoride on mitotic and meiotic chromosomes of mice in vivo.

From the Dept. of Biology and School of Medicine, University of Missouri Kansas City, Missouri. Presented at the 12th I.S.F.R. Conference, May 16-18, 1982, St. Petersburg Beach, Florida.

Materials and Methods

Seventy-two adult male mice (20-25g), BALB/c inbred strain obtained from Jackson Laboratories, Bar Harbor, Maine, were used. On arrival at the Laboratory Animal Center of the University, each mouse was kept in a separate cage, a shoe box white polypropylene with stainless steel wire lid. The first three days, the animals were given the Purina mouse standard chow and tap water. After the first day all cages were cleaned of nesting material because such material contains an uncontrollable amount of ingestable fluoride.

The mice were divided into nine groups (Table 1) of eight animals each. The first group consisted of the initial controls, the second and third groups were the controls for three and six weeks, respectively. The remaining six groups constituted the treated animals. All animals were given deionized drinking water and a low-fluoride diet (0.263 ± .028 ppm F) for one week before the start of the experiment. After the initial seven-day period, the eight initial controls were sacrificed for cytological studies and determination of the amount of fluoride in the body ash. Treated animals were given 1, 5, 10, 50, 100, or 200 ppm F , as sodium fluoride, through the drinking water. Four of the eight animals in each group were treated for three weeks and the other four for six weeks. Therefore, the experimental design consisted of 12 treatments. For treated animals, deionized water with NaF was provided freely in polypropylene water bottles with stainless steel drinking tubes. The same without NaF was provided for controls.

The mice were fed on a low-fluoride diet (12) during the entire experiment freely in stainless steel powder feeders. Fluoride content in the feed was determined by utilizing the Orion Specific Ion Meter (Model 401) with fluoride ion activity electrode (Model 94-09).

Each day one-third of the mice were transferred to clean cages. Water levels were recorded and drinking bottles refilled as necessary. Twenty-five grams of powdered diet food were weighed for each animal and placed in the feeder. Since one mouse eats from 3-5g/day, 25g allowed each mouse to have sufficient feed for 3 days without excessive waste.

Cytological studies were conducted on chromosomes of bone marrow cells (13) and on meiotic chromosomes from testes (14). The bone marrow cells were stained in Gurr's Giemsa stain 1:50 diluted in phosphate buffer, pH 6.8. The spermatocytes were stained for 30 min in 45% aceto-orcein. The preparations were mounted in euparal.

After collecting the cytological material, each mouse carcass was immediately placed in a plastic bag, sealed and stored in the freezer for determination of fluoride body ash. For the fluoride in the body assays, we used the Orion Specific Ion Meter (15-16).

Results

All mice appeared to adjust to the new environment equally well, adapting to the different type of cage, water and feed.

A. Water Intake

The mean values for water consumption based on ml/kg/day are given in Table 1. In the three week series, the treatments did not differ significantly from the control, except for the treatment with 5 ppm fluoride. When the different treatments were compared with each other, the t-tests indicated no significant increase in water consumption. In the six week series the mean water intake for the controls (219.92±5.64) differed significantly from that for the animals receiving 10 ppm (193.67±4.26), 50 ppm(186.16±3.63) and 200 ppm (176.46±4.85). It can be seen from Table 1 that the animals receiving 200 ppm (group 15) consumed the least amount of water. This amount differed significantly from all other groups except for those receiving 50 ppm (group 13) which had a mean value of water intake of 186.16±3.63.The difference was 9.7 ml/kg/day with a P value of .50-.10.

It would be safe to assume, at least in the six week series, that the treatment with 200 ppm significantly depressed the consumption of water, a conclusion reached previously by other investigators (12). Such depression in water intake might be attributed to plasma fluoride levels which act as a signal to regulate food consumption (17). In the same way, the plasma fluoride level might act as a signal to regulate the total fluoride intake.

B. Body Ash

The means and standard errors for the body ash fluoride as determined by the fluoride electrode are given in Table 1. The mean values for the different controls (groups, 1, 2 and 9) did not differ significantly from each other. Therefore it could be concluded that the body did not accumulate fluoride in appreciable amounts when the control animals were fed a low fluoride diet and given non-fluoridated water.

Table 1

Means and Standard Errors for Water Consumption in ml/kg/day ($\bar{X}_1$) and Body Ash Fluoride in PPM ($\bar{X}_2$)

Group	Treatment	$\bar{X}_1$±S.E.	$\bar{X}_2$±S.E.
1	Initial Control	Not recorded	145.29±5.31
	Three Weeks		
2	Control	205.14±7.30	148.38±5.07
3	1 ppm	214.81±9.00	158.25±3.94
4	5	240.68±11.30	187.25±4.07
5	10	202.40±8.71	244.50±21.94
6	50	195.44±7.66	447.75±10.47
7	100	192.01± .97	906.00±29.58
8	200	186.11±7.69	1237.50±43.28
	Six Weeks		
9	Control	219.92±5.64	156.88±4.33
10	1 ppm	221.22±5.26	164.25±3.57
11	5	216.30±6.37	207.50±4.80
12	10	193.67±4.26	273.25±9.06
13	50	186.16±3.63	892.50±25.29
14	100	212.08±4.24	1425.00±71.00
15	200	176.46±4.85	3010.00±66.45

The present data indicates, however, that the higher the concentration of fluoride in the drinking water, the higher will be the amount of fluoride in the body ash. This was evident with the intake of fluoridated water at doses above 1 ppm for either three weeks or six weeks. It can also be seen that the amount of fluoride in the body was dependent upon the duration of treatment.

C. Cytological Studies

Bone marrow cell studies showed that the control groups, including the initial group, did not differ significantly from each other with respect to the total frequency of chromosomal abnormalities (tables 2 and 3). The chi-square value was .53 with a P value of .80-.70 (d.f.2). On the other hand, each of the treatment groups differed significantly from the control for the three week series.

The treatment of 1 ppm for three weeks showed the total observed frequency of chromosomal abnormalities to be significantly less than those of each of the other treatments. Also the total chromosomal changes after treatment with either 5 or 10 ppm were significantly lower than those of 50, 100, or 200 ppm with P values of <.01. The frequency of the total aberrations induced by 50, 100, or 200 ppm did not differ significantly from each other. The chi-square value was 2.10 with a P value of .50-.30 (d.f.2).

Table 2

Chi-Square Values for the Test of Significance Among the Different Treatments for Chromosomal Abnormalities in Bone Marrow Cells

Treatments in ppm (3-week Series)

	1	5	10	50	100	200
N Ab	547 190	700 299	789 433	677 545	552 500	644 538
	X^2 P	X^2 P	X^2 P	X^2 P	X^2 P	X^2 P
Control N. Ab (1074-242)	15.19**	42.31 **	94.19**	203.31**	232.04**	219.18**
1 ppm		3.97**	20.91**	71.09**	89.31**	79.64**
5			7.48**	50.08**	67.40**	58.30**
10				21.39**	34.82**	27.52**
50					2.11**	.55*
100						.67*

Treatments in ppm (6-week Series)

	1	5	10	50	100	200
N Ab	593 280	594 417	651 555	611 544	623 574	596 552
	X^2 P	X^2 P	X^2 P	X^2 P	X^2 P	X^2 P
Control N Ab (1086-259)	47.27**	135.90**	208.14**	220.22**	442.90**	473.99**
1 ppm		17.28**	41.07**	46.52**	52.68**	59.45**
5			4.89**	7.39**	9.67**	13.60**
10				.47*	.90*	2.59*
50					.38*	1.07*
100						.54*

N = Number of scored cells with normal chromosomes; Ab = Number of scored cells with chromosomal aberrations; * = Not significant at 5% level of significance; ** = Significant difference at 5%; X^2 value of 3.841 has a P value of .05; X^2 value of 6.635 has a P value of .01.

Table 3

Frequency Distribution of Chromosomal Changes in Bone Marrow Cells

Treatment	No. Cells Studied		Fragments	Bridges	Rings	Ball Metaphase	Total Aberrations
Initial Control	1344	No.	118	7	32	89	246
		%	8.8	0.5	2.4	6.6	18.3
Three Weeks							
Control	1316	No	134	4	32	72	242
		%	10.2	0.3	2.4	5.5	18.4
1 ppm	737	No.	104	1	21	64	190
		%	14.1	0.1	2.8	8.7	25.7
5	999	No.	172	5	45	77	299
		%	17.2	0.5	4.5	7.7	29.9
10	1222	No.	244	6	55	128	433
		%	20.0	0.5	4.5	10.5	35.5
50	1222	No.	257	13	81	194	545
		%	21.0	1.1	6.6	15.9	44.6
100	1052	No.	223	8	72	197	500
		%	21.2	0.8	6.8	18.7	47.5
200	1182	No.	220	14	100	204	538
		%	18.6	1.2	8.5	17.3	45.6
Six Weeks							
Control	1345	No.	118	7	40	94	259
		%	8.8	0.5	3.0	7.0	19.3
1 ppm	873	No.	155	7	30	88	280
		%	17.8	0.8	3.4	10.1	32.1
5	1011	No.	224	6	52	135	417
		%	22.4	0.6	5.1	13.4	41.3
10	1206	No.	276	18	74	187	555
		%	22.9	1.5	6.1	15.5	46.0
50	1155	No.	245	15	76	208	544
		%	21.2	1.3	6.6	18.0	47.1
100	1197	No.	235	20	101	218	574
		%	19.6	1.7	8.4	18.2	47.9
200	1121	No.	203	27	81	241	552
		%	18.1	2.4	7.2	21.5	49.2

In the six weeks series, all treatments showed a significantly higher frequency of chromosomal changes than the controls for this duration. The treatment of 1 ppm and 5 ppm showed the total observed frequency of chromosomal abnormalities to be significantly lower than those of the other treatments. However, in this duration the frequency of chromosomal aberrations observed after treatments with 10, 50, 100, or 200 ppm did not differ significantly from each other.

The observed total frequencies of the chromosomal aberrations in spermatocytes (Tables 4 and 5) for the initial group and for the control groups did not differ significantly from each other. The chi-square value was .04 with a P value of .98-.95 (d.f.2). The obtained results also indicated that each of the treatments had a significantly higher frequency of spermatocyte chromosomal abnormalities than the controls for either the three or six week groups. In both series the frequency of aberrations observed at 1 ppm did not differ significantly from that of 5 ppm for the same duration (chi-square values .22 and .26 with a P value of .70-.50 for both), but it differed significantly from other treatments. It can be seen that the frequency of aberrations observed after treatments with 5 ppm for three weeks did not differ significantly from 10 ppm (chi-square value 3.78 with a P value

Table 4

Chi-Square Values for the Test of Significance Among the Different Treatments for Chromosomal Abnormalities in Spermatocytes

Treatments in ppm (3-week Series)

	1	5	10	50	100	200
	N Ab	N Ab	N Ab	N Ab	N Ab	N Ab
	188 51	173 52	139 61	145 76	120 81	105 88
	X^2 P	X^2 P	X^2 P	X^2 P	X^2 P	X^2 P
Control						
N Ab (411-79)	4.93**	4.32**	19.74**	26.48**	26.48**	65.53**
1 ppm		.22*	6.18**	8.47**	18.45**	29.33**
5			3.78*	5.96**	14.66**	29.36**
10				.23*	3.37*	8.78**
50					2.04*	6.92**
100						1.37*

Treatments in ppm (6-week Series)

	1	5	10	50	100	200
	N Ab	N Ab	N Ab	N Ab	N Ab	N Ab
	193 52	167 49	156 66	124 87	93 87	100 101
	X^2 P	X^2 P	X^2 P	X^2 P	X^2 P	X^2 P
Control						
N Ab (448-84)	4.76**	5.36**	17.33**	56.50**	79.91**	88.76**
1 ppm		.26*	4.04**	22.25**	35.91**	40.05**
5			2.16*	16.91**	28.60**	31.98**
10				7.37**	16.22**	18.58**
50					2.01*	2.70*
100						.04*

N = Number of scored cells with normal chromosomes; Ab = Number of scored cells with chromosomal aberrations; * = Not significant at 5% level of significance; ** = Significant difference at 5%; X^2 value of 3.841 has a P value of .05; X^2 value of 6.635 has a P value of .01.

of .10-.05); 10 ppm did not differ significantly from either 50 ppm (chi-square value .23 with a P value of .70-.50) or 100 ppm (chi-square value 3.37 with a P value of .10-.05) and the latter did not significantly differ from 200 ppm (chi-square value 1.37 with a P value of .30-.20).

In the six weeks duration for spermatocytes, the total frequency of chromosomal aberrations observed after treatment with 5 ppm did not differ significantly from that observed after 10 ppm (chi-square value 2.16 with a P value of .2-.10). Furthermore, the frequency of observed aberrations after 50, 100, or 200 ppm did not differ significantly from each other. This may be explained on the basis that concentrations above 10 ppm cause cell death.

Table 5

Frequency Distribution of Chromosomal Changes in Spermatocytes in Anaphase I (A_I + Telophase I (T_I) and Anaphase II (A_{II}) + Telophase II (T_{II})

Treatment	No. Cells Studied		$A_I + T_I$ B*	$A_I + T_I$ B+F**	$A_I + T_I$ F***	A_{II} T_{II} B	A_{II} T_{II} B+F	A_{II} T_{II} F	Total Aberrations
Initial Control	449	No.	20	13	5	28	2	5	73
		%	4.5	2.9	1.1	6.3	0.4	1.0	16.2
Three Weeks									
Control	440	No.	17	17	4	27	12	2	79
		%	3.5	3.5	0.7	5.5	2.4	0.4	16.0
1 ppm	239	No.	15	8	4	15	7	2	51
		%	6.2	3.3	1.7	6.2	3.0	1.0	21.4
5 ppm	225	No.	15	8	3	15	6	5	52
		%	6.7	3.6	1.3	6.7	2.7	2.2	23.2
10 ppm	200	No.	19	5	4	19	7	7	61
		%	9.5	2.5	2.0	9.5	3.5	3.5	30.5
50 ppm	221	No.	24	7	5	25	9	6	76
		%	10.9	3.2	2.2	11.3	4	2.7	34.3
100 ppm	201	No.	31	7	6	23	8	6	81
		%	15.4	3.5	3.0	11.4	4.0	3.0	40.3
200 ppm	193	No.	32	8	4	25	13	6	88
		%	16.6	4.1	2.1	12.9	6.7	3.1	45.5
Six Weeks									
Control	532	No.	31	12	5	28	5	3	84
		%	5.8	2.2	1.0	5.2	1.0	0.6	15.8
1 ppm	245	No.	17	4	4	16	7	4	52
		%	6.9	1.6	1.6	6.5	2.9	1.6	21.1
5 ppm	216	No.	15	4	3	17	8	2	49
		%	6.9	2.0	1.4	7.9	3.7	0.9	22.8
10 ppm	222	No.	26	6	4	20	6	4	66
		%	11.7	2.7	1.8	9.0	2.7	1.8	29.7
50 ppm	211	No.	33	7	8	27	8	4	87
		%	15.6	3.3	3.8	12.8	3.8	2.0	41.3
100 ppm	180	No.	26	8	8	29	10	6	87
		%	14.4	4.4	4.4	16.1	5.6	3.3	48.2
200 ppm	201	No.	31	10	9	33	11	7	101
		%	15.4	5.0	4.5	16.4	5.5	3.5	50.3

B* = bridges alone; B+F** = bridges with fragments; F*** = fragments alone

The types of chromosomal aberrations observed in the bone marrow were acentric fragments, ring chromosomes, translocations, dicentrics, and anaphase or telophase bridges with or without fragments. Multipolar anaphases, aneuploidy, euploidy and ball-mataphase were also observed by other investigators studying the cytological effects of fluoride on somatic mammalian chromosomes (6,10-11). In the spermatocytes, the observed abnormalities were mainly bridges, bridges plus fragments and fragments alone.

Discussion

It has been found (18) that fluoride affects enzymatic activities,and this effect could delay mitotic and meiotic cycles causing chromosomal breakages. Such chromosomal aberrations may eventually lead to the formation of structural changes and fragmentations as observed in the present studies and by other investigators(2-6,11. 19-20). Since it has

been shown that fluoride can inhibit nucleic acid synthesis (21) it is suggested that chromosomal damage induced by fluoride could be the result of an attack on the enzymes responsible for DNA synthesis or repair,rather than a direct attack on the DNA molecule (5,11).

Many investigators have suggested that fluoride affects the rate of cellular protein synthesis (22 - 24) and the amino acids sequence of newly synthesized protein (25). Since the spindle apparatus is composed primarily of protein, then fluoride could be responsible for the disruption of the microtubules of the spindle, causing multipolar anaphases and/or unequal chromosomal distribution leading to aneuploidy and euploidy.

The high frequency of ball-metaphases in the treatments could be due to the physiological effects of fluoride (19), to a breakdown in the mitotic process due to the toxicity of fluoride, or to both (10, 19). The chromosome stickiness observed in these studies may also be due to enzymatic changes and/or changes in the composition of chromosomal proteins induced by fluoride treatment affecting chromatin condensation. Also when the chromosomes are fully contracted, the submicroscopic chromatin strands may persist causing the chromosomes to stick to each other (26). The degree of failure of the chromosome to move in anaphase will depend upon the amount of stickiness. Due to this movement, chromatin bridges will be formed when the sticky chromosomes fail to separate and fragmentation would result. In the extreme case, complete failure of chromosomes to separate may occur due to ball-metaphase formation causing lethal or sublethal conditions to the cell.

In comparing the concentrations of fluoride in body ash with frequencies of chromosomal changes observed in mitosis or spermatocytes, it is evident from these data that both follow the same pattern. The correlation coefficient value for bone marrow cell chromosomal abnormalities and the amount of body ash fluoride for three and six week series were .91 and ..85, respectively. The values for spermatocytes and body ash fluoride were .97 for three weeks and .30 for six weeks. This indicates a high correlation between the amount of fluoride in the body ash and the frequency of chromosomal aberrations, except for the six-week series in spermatocytes where the correlation was not as high. This relatively low correlation could be due to the toxic effect of fluoride preventing the damaged cells from continuing the meiotic cycle.

Whereas some investigators (7-9) stated that fluoride is unable to induce chromosomal changes significantly higher than the controls, the present studies show clearly that fluoride is able to induce chromosomal damage in bone marrow cells as well as in spermatocytes. Findings similar to those observed in this study have been reported by others (6, 10-11). It should be realized, however, that some of these observed abnormalities will be eliminated during sperm maturation as well as during bone marrow cell divisions. Therefore to arrive at a conclusive result cytological tests should be carried out on the progeny of treated individuals.

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LEUKOCYTE RESPONSE IN YOUNG MICE CHRONICALLY EXPOSED TO FLUORIDE

by

S.R. Greenberg
N. Chicago, Illinois

SUMMARY: The study, by light and fluorescent microscopy, of sternal and femoral bone marrow taken from young Swiss mice exposed for periods up to 280 days to elevated levels of sodium fluoride in drinking water, has revealed morphologic abnormalities in cell structure and mitotic figure formation in immature leukocytes. Alterations in the content and distribution of RNA and DNA also appear after several weeks of exposure. These findings, interpreted in relation to other reported data, bear compatability with a possible shift of these cells toward anaplasia.

Introduction

The widespread utilization of fluoride as a dental protective measure has raised serious questions concerning its potential hazzard to humans. It has been established that repeated exposure to fluoride induces inhibition in the mineralization of bone (osteomalacia), often accompanied by increased osteoclastic activity and osteoid formation (1). Belanger and coworkers (2) have reported the suppression of calcium absorption through the intestinal wall by fluoride-treated suckling pigs. Other investigators (3, 4) have described experimental settings in which fluoride stimulated the formation of new bone.

The chief cells in the parathyroid glands of rats, chronically exposed to fluoride, undergo an increase in metabolic activity (5). Similar observations have been made in the human parathyroid glands (6). The effects of fluoride upon parenchymous organs have proved to be something less than dramatic. The only positive change found in the liver and kidneys of rats after prolonged fluoride exposure, is a rise in lipid and glycogen deposition (7,8).* Recently alterations in white blood cells in relation to fluoride treatment have been reported (9-11).

The hematopoetic system is constantly being exposed to the slightest presence of fluoride (12). Changes in mature fluoride-exposed murine leukocytes have been previously documented (13). Therefore it is deemed of special significance to investigate the results of fluoride upon rapidly dividing immature cells.

Materials and Methods

Young Swiss mice of both sexes were divided into three groups. Twenty animals (group I) received only distilled drinking water containing sodium

* Editor: See Ramseyer et al., J. of Gerontology, 12:14-19, 1957.

From Dept. of Path. U.of Health Sciences,Chicago Med. School, N. Chicago, Ill.