DIFFERENTIAL *IN VIVO* GENOTOXIC EFFECTS OF LOWER AND HIGHER CONCENTRATIONS OF FLUORIDE IN MOUSE BONE MARROW CELLS

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SUMMARY: In an *in vivo* genotoxicity investigation of the action of fluoride (F) on bone marrow cells, sodium fluoride (NaF) was administered through the drinking water of 2–3 month old Swiss albino mice for 30 days at lower (7.5, 15, and 30 mg/L) and higher concentrations (100 and 150 mg/L). Mitotic inhibition, chromosomal aberrations, and chromatid breaks were most pronounced in mice that received the relatively low dose of 15 mg NaF/L. The effects became obvious after the first week of treatment and were maximal after 3 months of sustained exposure. Chromosome aberrations induced by one month treatment with 15 mg NaF/L was significantly higher than those found with the 100 and 150 mg NaF/L concentrations. The total number of femur bone marrow cells remained unchanged in all the treatment groups except in the 150 mg NaF/L group, in which it declined significantly. F treatment did not elicit any change in the percentage of viable cells in the bone marrow. Depletion of S-phase fraction of bone marrow cells occurred in the mice receiving 150 mg NaF/ L for 30 days, whereas treatment with 15 mg NaF/L for 90 days elevated the sub-G1 fraction, suggesting inhibition of DNA synthesis and up-regulation of apoptosis, respectively. These results indicate that the action of F *in vivo* is actually more genotoxic at certain lower concentrations than at higher concentrations.

Keywords: Bone marrow cells; Chromosome aberrations; Fluoride genotoxicity; Genotoxicity in mice; Paradoxical concentration effects.

INTRODUCTION

In a recent investigation[1] we found an induction of chromosome aberrations in bone marrow cells in mice following a one-month *in vivo* treatment with 7.5, 15, and 30 mg NaF/L through their drinking water. The lower two levels corresponds to the F concentrations (3.4 and 6.8 mg F ion/L) reported in some potable water supplies in India and other countries. Some reports indicate F can cause lipid peroxidation, chromosome aberrations, induction of sister chromatid exchange (SCE), and DNA damage.[2-16] However, many of the findings regarding the genotoxic potential of F in mammalian cells are seemingly contradictory. Most of these studies have been done either *in vitro* using cell lines or *in vivo* with higher doses of NaF. It was found, for example, that F had no cytotoxic effect at lower concentrations (0–50 mg F/L) in HL-60 cells, but at higher concentrations (100–250 mg F/L) it reduced cell viability, decreased DNA and protein biosynthesis, and induced apoptosis.[17] Thus there is a need for more *in vivo* study on the genotoxic potential of F over a broad range of concentrations and varying time of exposures. The present study of genotoxic effects of F in bone marrow cells of

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mice was therefore undertaken in an effort to resolve some of the earlier contradictions reported on the genotoxicity of F.

MATERIALS AND METHODS

Male Swiss albino mice, aged 2–3 months and weighing 25–30 g were used as in our recent report.[1] The mice were divided into two sets with 4 to 6 mice per group in each set. In one set the animals were exposed to 0, 7.5, 15, 30, 100, and 150 mg NaF/L through their drinking water for 30 days. In the other set, they were exposed to 15 mg NaF/L for 7, 15, and 90 days. All regulations of the institutional Animal Ethics Committee were strictly followed during the experiment. At the end of the treatment the mice were sacrificed by cervical dislocation. For chromosome preparation, bone marrow cells were collected from the femoral shaft, treated with hypotonic KCl solution at 37ºC, fixed in acetic acid-methanol (1:3), placed on clean glass slides, flame dried, and stained with Giemsa by the method previously described.[1] Over 100 well spread metaphase plates were analyzed under a microscope with 100 × 10 magnifications from randomly coded slides. Cell viability was checked by Trypan blue (0.5%) dye exclusion test and counted under microscope using a hemocytometer.

For flow cytometry, 1×10^6 bone marrow cells were flushed into 1.0 mL phosphate buffered saline (PBS) in plastic microfuge tubes, centrifuged at 2000 rpm for 7 min, resuspended in RBC lysis buffer (Becton Dickinson [BD], USA), and were kept in ice for 30 min. Following another centrifugation, cells were washed, fixed, and permeated with ice-cold methanol for 1 hr. Then 1 µL RNase (RNase-10 mg, 10 mM Tris, 15 mM NaCl), and 5 µL of propidium iodide (Sigma Chem, USA; PI-0.5mg, PBS -1mL) per sample was added and incubated for 30 min in the dark. The samples were analyzed by a flow cytometer (FACS caliber with Sorter, BD, San Jose, CA, USA). Approximately 10,000 events were acquired from each sample. Data acquisition and processing were performed with Cell Quest software (BD, USA).

The results were analyzed by two-tailed Student's t test by the Origin 6.1 statistical package. A value of $p<0.05$ was considered significant.

RESULTS

Administration of NaF to the mice through their drinking water for 30 days in concentrations ranging from 7.5 to 100 mg/L did not alter the percentage of mitotic indices (MI) of bone marrow cells. A slight increase in the percentage of MI was noticed after treatment with 7.5 mg NaF/L, but the MI decreased significantly ($p<0.05$) at 150 mg NaF/L (Table 1). There was significant rise in the percentage of aberrant metaphases and chromatid breaks (Figure 1) in all the treated groups, the effect being most pronounced in mice that received 15 mg NaF/L. Interestingly, a decline in the severity of these chromosomal changes was noted at higher NaF levels, although the values were still significantly higher than in the controls.

Table 1. Chromosomal aberrations in mouse bone marrow cells following in vivo administration of different concentrations of NaF (mg/L) in the drinking water of the mice for 30 days (Values are mean±SE)

Group (n = 4)	TM[a]	MI(%)[b]	AbM(%)[c]	Ctd.br(%)[d]	Iso.Ctd.br(%)[e]	Exch(%)[f]
Control	612	2.12 ±0.06	1.5±0.29	1.5±0.29	0.00	0.00
NaF-7.5	482	2.35 ±0.26	7.5±0.5*	7.75±0.25*	0.00	0.00
NaF-15	570	1.78±0.39	9.25±0.63*	9.5±0.65*	0.00	1.25±1.25
NaF-30	611	1.85± 0.28	8.25±0.85*	8.5±1.19*	0.00	0.00
NaF-100	1170	1.94±0.22	4.01±0.56*†	4.61±0.73*	0.00	0.00
NaF-150	675	1.55±0.12*	4.09±0.78*†	4.13±0.75*	0.09±0.09	0.00

*$p<0.05$ compared with control group; †$p<0.05$ comared with 15 mg NaF/L treated group; [a]Total metaphases; [b]Mitotic index; [c]Aberrant metaphases; [d]Chromatid breaks; [e]Isochromatid breaks; [f]Exchanges (end-joining of two nonsister chromatids).

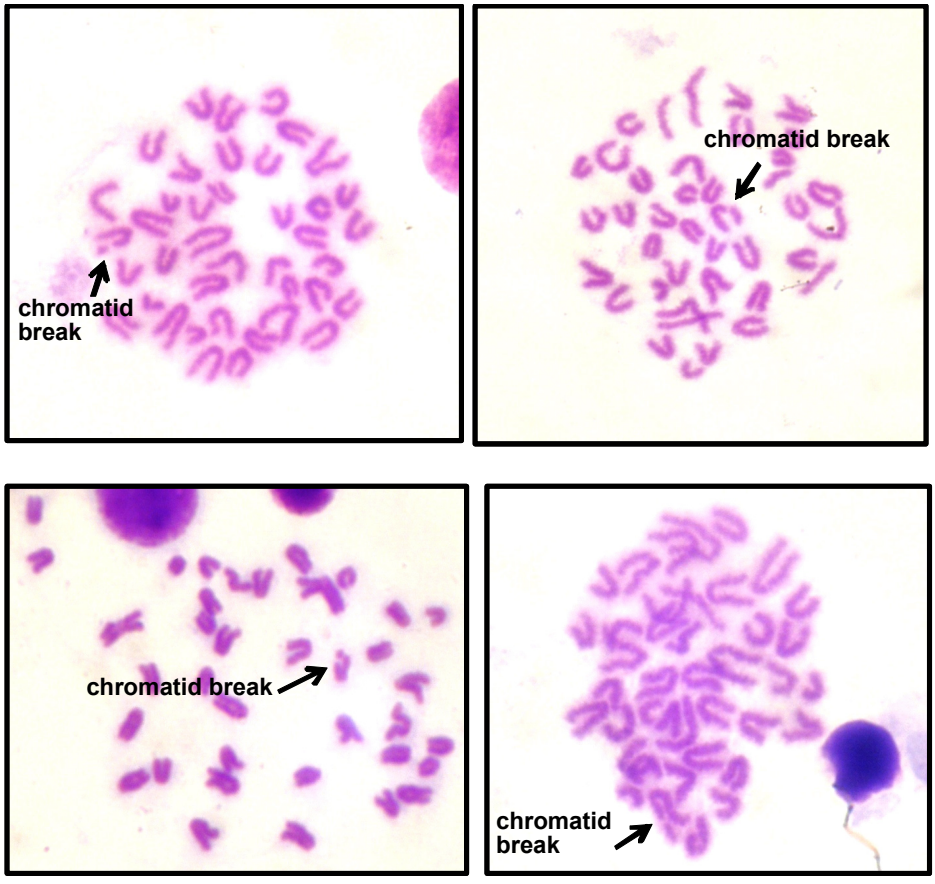

Figure 1. Chromatid breaks induced in bone marrow cells of mice after *in vivo* exposure to NaF in the drinking water.

At 15 mg NaF/L, suppression of MI was observed by day 7 post-treatment, but no discernible change was recorded in MI with prolonged exposure to F. A significant increase ($p<0.05$) in the frequency of aberrant metaphases and chromatid breaks was found after day 15, reaching a peak on day 90 post-treatment (Table 2).

Table 2. Chromosomal aberrations in mouse bone marrow cells following *in vivo* administration of 15 mg NaF/L in the drinking water of the mice for 7-90 days (Values are mean±SE)

Group (n = 4)	TM[a]	MI (%)[b]	AbM (%)[c]	Ctd.br (%)[d]	Iso.Ctd.br (%)[e]	Exch (%)[f]
Control	612	2.12±0.06	1.50 ±0.29	1.50±0.29	0.00	0.00
NaF-15 mg/L (7 days)	510	1.31±0.15*	1.80±0.49	1.83±0.48	0.00	0.00
NaF-15 mg/L (15 days)	900	2.12±0.15	5.84±1.19*	6.28±1.05*	0.00	0.00
NaF-15 mg/L (30 days)	570	1.78±0.39	9.25±0.63*	9.5±0.65*	0.00	1.25±1.25
NaF-15 mg/L (90 days)	753	2.52±0.46	9.51±1.48*	9.90±1.27*	0.25±0.25	0.25±0.25

*p< 0.05 compared with control. [a]Total metaphases; [b]Mitotic index; [c]Aberrant metaphases; [d]Chromatid breaks; [e]Isochromatid breaks; [f]Exchanges (end-joining of two nonsister chromatids).

The total number of nucleated cells per femur remained relatively unaltered in all treated groups except in 150 mg NaF/L group where it was drastically reduced (Table 3). Compared to the controls, the percentage of nonviable cells in bone marrow remained higher in all the treatment groups, although the change was not significant.

Table 3. Total number of nucleated cells in each femur and the percentages of dead cells in bone marrow of mice administered various concentrations of NaF (mg/L) in the drinking water (Values are mean±standard error)

Group (n = 4)	Duration of treatment (days)	TNC x 10^6/femur	% of dead cells
Untreated	30	24.9±2.12	2.64±0.64
NaF- 7.5	30	21.27±2.37	4.84±1.19
NaF- 15	30	21.18±1.30	5.05±0.79
NaF- 30	30	24.52±0.73	4.73±0.74
NaF- 100	30	26.51±4.43	4.68±0.57
NaF- 150	30	17.62±1.76*	3.93±0.19
NaF- 15	90	30.08±1.23	4.36±0.80

TNC, Total nucleated cells; *p< 0.05, significantly different from control group.

There was no appreciable change in the percentage of bone marrow cells at different phases of the cell cycle in mice receiving 15 mg NaF/L for 30 days (Table 4, Figure 2). After 90 days of treatment, however, sub-G_1 peak was significantly elevated ($p<0.05$), suggesting an up-regulation of apoptosis. There was a decline in G_0/G_1 and a rise in S phase fraction at this time point, but the changes were not significant ($p>0.05$). However, a significant decline in S phase fraction was recorded with 150 mg NaF/L, indicating inhibition of DNA synthesis by the high F concentration.

Table 4. Percentages of bone marrow cells in different stages of cell cycle in mice treated with NaF (mg/L) (Values are mean±standard error).

Group (n = 6)	Days	Sub G_1	G_0/G_1	S	M
Untreated	30	0.37±0.11	62.85±0.87	5.98±0.61	30.83±1.02
NaF- 15	30	0.34±0.07	63.08±0.87	5.33±0.38	31.24±0.88
NaF- 150	30	0.35±0.06	64.62±0.68	3.48±0.28*	30.55±0.50
NaF- 15	90	3.85±0.43*	60.02±0.81	8.02±0.40	28.08±0.82

*p< 0.05, significantly different from control group.

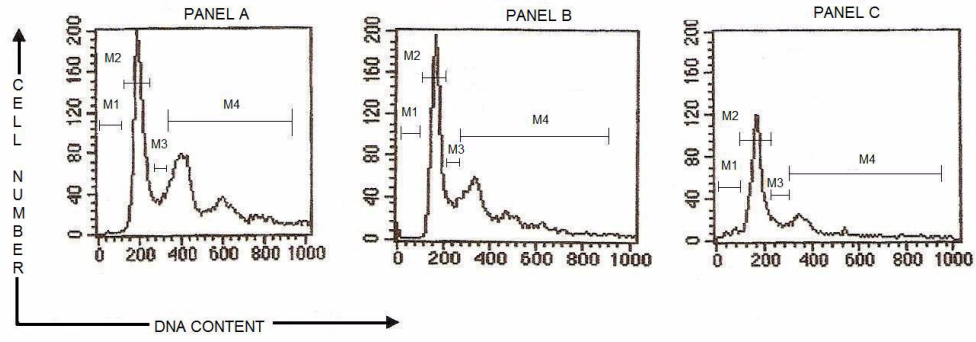

Figure 2. Flow-cytometry analysis of mouse bone marrow cells in control (panel A) and following exposure of the mice with 15 mg in the drinking water for 30 (panel B) and 90 days (panel C). The DNA content (x axis) and cell numbers (y axis) have been determined. Gates were set to assess the percentage of cells in different cycles. M1 = <2n DNA (Sub G1 cells); M2 = 2n DNA (G0/G1 cells); M3 = 2n - 4n DNA (cells in S phase); M4 = 4n DNA (G2/M cells). Bars denote the boundaries of cell cycle phases.

DISCUSSION

Based on *in vivo* assays in human populations, *in vitro* assays with human cell lines, and *in vivo* experiments with rodents, the 2006 US NAS-NRC report on F in drinking water,[20] noted that, the genotoxic effects of F at environmental concentrations is contradictory. Gaps in the information on F prevented the review panel from making final judgments about the safety or the risks of F at concentrations of 2 to 4 mg/L. Spittle,[21] in his recent book on F fatigue, observed that "The numerous fluoridation studies in the past failed to rigorously test for changes in GI symptoms and there are no studies on drinking water containing fluoride at 4 mg/L in which GI symptoms were carefully documented."

From the present study, it is apparent that drinking of water contaminated with F could be injurious to bone marrow by causing chromosomal changes, reducing the rate of cell division, and inducing apoptosis, depending on the dosage and duration of exposure. As little as 7.5 mg NaF/L (3.4 mg F ion/L) in the drinking water appeared to be genotoxic, whereas higher concentrations seemed to be less toxic. The reason for this is currently unknown, as is the mechanism of F action at the subcellular level. Such paradoxical effects of F were also noticed on MI, where the MI% showed slight increase after 7.5 mg NaF/L treatment, but decreased significantly after the treatment with 150 mg NaF/L (Table 1). Similarly, the MI% decreased significantly within one week following exposure to 15 mg NaF/L, although it rose above the control level after prolonged treatment for 90 days. These findings appear to be another addition to the number of reports on paradoxical dose-response effects of F as cited by Burgstahler.[22]

The chromosomal breaks and apoptosis observed in the present study could be mediated by reactive oxygen species generated during F-induced oxidative stress. Our hypothesis thus corroborates an earlier report[23] on a remarkable rise in MDA and consequent elevation in the activity of antioxidant enzymes in the liver of F-

treated rats. Interestingly, F was found to stimulate lipid peroxidation at lower concentrations and inhibit it at higher concentrations.[24]

The findings of the present *in vivo* study are at variance with earlier reports that showed lack of genotoxic potential of F.[2-7] Our results also contradict the observations of Song et al.[17] in HL-60 cells *in vitro*. However, the disparity may be due to the inherent differences between *in vivo* and *in vitro* test systems for the assessment of the genotoxicity. In addition, factors like variability in age, animal species, kind of tissue examined, dosage and mode of F exposure could play an important role in modulating genotoxicity of fluoride.[25] Moreover, variability in diet and daily water consumption was also considered to influence F toxicity.[26]

Stimulation of apoptosis, as evident from the rise in sub-G_1 fraction in NaF-treated mice in the present investigation, was consistent with apoptosis-inducing activity of F *in vitro*.[14-19] However, sub-G_1 fraction was present only in mice treated for 90 days with 15 mg NaF/L, whereas chromosomal changes were observed within 2 weeks of exposure at this level. This clearly indicated the potency of F to induce chromosome aberrations in the bone marrow cells of mice within two weeks of their exposure to a relatively low level of F, while a longer duration of exposure is essential for induction of apoptosis. The pattern of induction of apoptosis and alteration of cell cycle corroborate an earlier *in vitro* organ culture study with mouse fetal long bones.[18] Our observations also suggest that not only at higher amount but also prolonged duration of exposure could be a potential factor for the induction of apoptosis.

A fall in MI of bone marrow cells within a week of exposure of the mice to 15 mg NaF/L without having any effect at the chromosomal level is difficult to explain. But the decline in the percentage of cells in S phase at the higher exposure level of 150 mg NaF/L as detected by flow cytometry correlates well with the reduced percentage of mitotic cells recorded by us. Further study is necessary to demonstrate whether a differential rate of activation of a cellular detoxification pathway and/or DNA repair mechanisms are responsible for the pattern of genotoxicity exhibited at the different levels of *in vivo* F exposure.

Since NaF in dilute aqueous solution dissociates completely to generate Na and F ions, the observations in this study strongly support the view of the 2006 NRC report that the current Maximum Contaminant Level Goal (MCLG) of F promulgated by the US Environmental Protection Agency (EPA) is not safe enough. This agency's MCLG of 4 mg F/L in drinking water can induce significant chromosome aberrations in mouse bone marrow cells. Tice et al. using the genetic activity profile database concluded that humans are more than an order of magnitude more sensitive than rodents to most genotoxic agents.[27] In view of the prolong exposure of F to human population, it is justified to argue that the MCLG of F in drinking water should be zero.

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