FLUORIDE-MEDIATED APOPTOSIS AND DISORDERING OF CELL CYCLE DISTRIBUTIONS DURING *IN VITRO* ORGAN CULTURE OF MOUSE FETAL LONG BONES

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SUMMARY: Effects of fluoride (as NaF) on cell cycle, DNA content, and apoptosis of mouse fetal long bone cultures were examined and analyzed by flow cytometry (FCM). The results showed that NaF at 2.5–5.0 µg/mL (2.5–5.0 ppm) had only slight effects on the DNA content and cell cycle distributions. At 10.0 µg/mL, however, NaF increased the number of cells in S phase but did not change the frequency of the G_0/G_1 and G_2/M phase. At 20.0 µg /mL NaF not only increased the number of cells in S phase but also decreased the frequency of the G_2/M phase. Cell proliferation was also influenced. At 2.5–10.0 µg/mL NaF did not induce increased apoptosis, but the number of apoptotic cells was significantly increased at 20.0 µg NaF/mL. Therefore F damage to bone may involve promoting apoptosis and disordering cell cycle distributions. Although the differences in DNA content, cell cycle distributions, and apoptosis between controls and the two lower NaF concentrations were not statistically significant, there was evidence of hormesis (paradoxical stimulatory) effects. The results indicate that F exerts a dual influence on osteocytes.

Keywords: Apoptosis; Bone cell cycle; Bone DNA content; Fetal bone culture; Flow cytometry; Fluoride and bone; Mouse osteocytes.

INTRODUCTION

Widely distributed in the environment, fluoride (F) is the cause of dental and skeletal fluorosis, two of the most extensive endemic diseases in the world. Although the pathogenesis of endemic fluorosis is clear, the exact biomechanistic features of adverse effects of excess F intake are still not completely understood. In recent years, improvements in the technique of *in vitro* organ culture of bone have opened promising avenues for the study of injury to the skeleton by F. Because the three-dimensional structure of bone tissue can be maintained to some extent in bone culture, bone formation, mineralization, and resorption are observed simultaneously. In previous work, we have found that F can adversely affect the growth, development, and morphological structure of culture long bone.[1]

Compared with *in vivo* experiments, organ culture of bone *in vitro* also has the advantage of rapidity, low cost, ease of operation, controlled dosage, reliable repetition, etc. In contrast to ordinary cell culture experiments, cell proliferation and differentiation in bone organ culture are much more similar to what occurs *in vivo*. On the basis of numerous articles about new methods and techniques relating to F research, the present *in vitro* bone culture study was designed to explore the effects of F on cell cycle distributions and apoptosis in mouse fetal long bone cells.

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Recent investigations have shown that F can cause diploid cell cycle disturbance of rat alveolar macrophages and fibroblasts[2-4] and can also induce apoptosis.[5-6] However, osteocyte cell cycle and apoptosis studies in mouse long bone culture induced by F do not appear to have been reported. As an outgrowth of surveys of skeletal fluorosis due to high F drinking water in Xingjiang province of China, we have developed a method for determining damaging effects of trace elements, including F, on organ culture of mouse fetal long bone.[7] In this investigation, using the method of advanced flow cytometry (FCM), we studied the influence of cell cycle, DNA content, and apoptosis in fetal mouse bone culture at different concentrations of F.

MATERIALS AND METHODS

Experimental animals: Healthy Kunming mice, 40 virgin females (4 months old) weighing 20–30 g, and 20 males (4 to 5 months old) weighing 25–35 g, were chosen for this study. At 10:00 p.m. in the late evening, male and female mice were placed together singly in a ratio of one male to two females. At 8:00 a.m. the next morning, pessus was checked in the female mice. If pessus was found, the mouse was considered to be in the 0th day of pregnancy. After 16 days the pregnant mice were sacrificed by cervical dislocation at 8:00 a.m., and the fetuses were removed. The ulna bones were removed and freed of muscle and soft tissue under a dissecting microscope.

Bone culture in vitro: The mouse ulnas were incubated at 37ºC for 48 hr in bone culture medium BGJ obtained from the Grand Island Biological Co, Grand Island, NY, that also contained 10% fetal bone serum (FBS) and 100 units/mL penicillin G and 100 µg/mL streptomycin sulfate in a humidified atmosphere according to the procedure of Ma et al.[7] The culture bottles were set in a continuous rotating device supplemented with 5% CO_2, 50% O_2, and 45% N_2.

Experimental groups: The ulna cultures were divided into five groups. The control group (C) received no sodium fluoride (NaF). The four tests groups were exposed to NaF at 2.5 µg/mL (F1), 5.0 µg/mL (F2), 10.0 µg/mL (F3), and 20.0 µg/mL (F4), respectively.

Flow cytometry (FCM) assay: After 48 hr, the cultured ulnas were washed three times with Hanks solution, sheared into pieces by eye with scissors as small as possible in a Petri dish. The cut fragments were passed through a 200-mesh screen and centrifuged for 5 min at 3000 rpm, followed by discarding of the supernatant. The mono-cell suspension was treated with 1.5 M propidium iodide (PI) dyestuff (100 µg/mL), kept for 30 min in the dark, and used for study by flow cytometry (FCM).

Each mono-cell suspension sample was counted by FCM for 5000 cells. The measured results were analyzed with Elite software (Air Conditioning Contractors of America) to obtain the distribution of cells in G0/G1, S phase, and G2/M phase, as well as the percentage of cells with apoptosis. DNA content was measured by fluorescence intensity.

RESULTS

DNA content and apoptosis: As seen in Table 1, a detectable but statistically nonsignificant increase in DNA content occurred at 2.5 µg NaF/mL (F1). However, the content of DNA decreased significantly in F3 (10.0 µg NaF/mL (P<0.05) and F4 (20.0 µg NaF/mL (P<0.01). Thus higher F concentrations clearly reduced the DNA content of osteocytes. Also recorded in Table 1 was a non-significant inhibition of apoptosis at 2.5 µg NaF/mL (F1). At higher concentrations of NaF apoptosis gradually increased, and in F4 (20 µg NaF/mL) the increase was significant.

Table 1. Effect of NaF on DNA content and apoptosis in *in vitro* mouse fetal bone cultures at different NaF concentrations (No. = number of cell suspensions analyzed)

Group (µg NaF/mL)	No.	DNA content (fluorescence intensity)	Apoptosis (%)
C (0.0)	6	86.20±4.15	10.10±1.16
F1 (2.5)	6	90.50±5.47	8.20±1.65
F2 (5.0)	6	81.00±4.56	12.00±2.66
F3 (10.0)	6	78.00±4.05*	13.50±2.18
F4 (20.0)	6	73.50±3.78†	21.20±3.02*

*P < 0.05; †P< 0.01 compared with the control group C.

Cell cycle distributions: Table 2 reveals there was an opposite effect on cell cycle distributions between 2.5 µg NaF/mL (F1) and 5.0 µg NaF/mL (F2). At 10.0 µg NaF/mL (F3), the number of cells in S phase increased (P<0.05), but those in G0/G1 phase and G2/M phase were unchanged. At 20.0 µ NaF/mL (F4), not only did the number of cells in S phase increase (P<0.01), but the number in G2/M phase was reduced (P<0.05). Thus NaF stagnated the number of cells in S phase and inhibited the change to G2/M.

Table 2. Effect of NaF on cell cycle in *in vitro* mouse fetal bone cultures at different NaF concentrations (No. = number of cell suspensions analyzed)

Group (µg NaF/mL)	No.	Cell cycle distributions (%)		
		G_0/G_1phase	S phase	G_2/M phase
C (0.0)	6	74.95±4.24	16.40±3.08	8.65±2.19
F1 (2.5)	6	75.55±4.30	15.23±3.96	9.05±2.21
F2 (5.0)	6	72.50±5.89	19.28±4.07	8.12±2.30
F3 (10.0)	6	71.08±4.29	22.43±2.84*	6.63±1.58
F4 (20.0)	6	69.13±5.44	25.60±3.92†	5.25±1.68*

*P < 0.05; †P< 0.01 compared with the control group C.

DISCUSSION

Although excess F intake causes skeletal fluorosis, information about the exact mechanism of toxic effects of F on bone is still very limited. In the present study the effects of different concentrations of F on cell cycle and apoptosis in mouse fetal long bone organ cultures were investigated.

Our previous investigations revealed a toxic dose concentration-response relationship of F to bone, liver, and kidney.[8-9] We found that the molecular mechanism of this toxicity might involve F-mediated apoptosis and disordering of cell cycle distributions. As is well known, cell cycle refers to the period in which cells pass from one division phase to another, including the G_0, G1, G2, S, and M period. Although various studies have reported effects of F on cell cycle and apoptosis,[10-16] the present investigation accurately demonstrated the relationship

of F concentration to apoptosis and cell cycle distribution using an organ culture method. We found that 2.5 μg NaF/mL did not adversely affect the DNA content and cell cycle distribution in organ culture of mouse fetal long bone. However, at 10.0 μg/mL, NaF increased the number of cells in S phase, and at 20.0 μg/mL, NaF not only increased the number of cells in S phase, but it also decreased those in G2/M phase, thereby suggesting F damage to DNA synthesis and increased cell cycle disorder. As a stimulative factor, F evidently interrupted normal cellular signal transduction by inhibiting the cells in the S phase to G2/M phase transition and causing the cell cycle to stagnate in S phase. So far, the study of F effects on bone cell cycle is not well developed, and it is not known exactly how F influences cell cAMP, how it affects cyclin dependence on the activity of Cyclin cdks, or how it interacts with the regulator genes (P53, Rb, P16, C-fos, C-myc, etc.) that participate in cell cycle. Clearly, additional studies are needed.

Apoptosis is programmed cell death in which a host gene controls cell death to maintain internal milieu stability. Apoptosis has been observed by Machalinska et al.[17] in both human bone marrow and cord blood hematopoietic progenitor cells exposed to different concentrations of NaF. In the range of 50 μg NaF/L, a larger number of cells entered the early phase of apoptosis. In addition, Machalinski et al.[18] also found that apoptosis was induced by NaF and Na_2SiF_6 in four different human leukemic cell lines (HL-60, HEL, TF-1, and K562). In those studies, elevated (but not relatively low) F concentrations induced and accelerated apoptosis. In the present work, NaF at 2.5–10.0 μg/mL did not significantly induce apoptosis *in vitro*, but the number of apoptotic cells showed a significant increase at 20.0 μg/mL. It appears possible, therefore, that F damage to bone may be related to promotion of apoptosis. This apoptosis could be induced by activation of G-protein-dependent signaling pathways. Conceivably, a sustained rise in intracellular cAMP might form part of the effective system controlling apoptosis and disordering cell cycle distributions.

It has also been suggested that oxidative stress plays a major role in various forms of cell death, including apoptosis. Anuradha et al.[19] reported that perhaps NaF induced apoptosis by oxidative stress-induced lipid peroxidation, causing loss of mitochondrial membrane potential, thereby releasing cytochrome c into the cytosol and further triggering the caspase cascade leading to apoptotic cell death in HL-60 cells. Such effects of F on osteocytes need further study.

Finally, it is worth noting that although the differences in DNA content and apoptosis between the 2.5 and 5.0 μg NaF/mL and control cultures were not statistically significant, there was definite evidence of increased DNA content and inhibition of apoptosis at 2.5 μg NaF/mL. Moreover, an opposite effect was seen in the cell cycle distributions between 2.5 and 5.0 μg NaF/mL. This dual type effect on osteocytes is an example of a stimulatory hormesis or paradoxical dose-response effect of F.[20]

ACKNOWLEDGEMENT

This research was sponsored by the National Natural Science Foundation of China (Grant No. 30160079).

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