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The Effect of Fluoride and Aluminum on Bone Turnover in Mouse Calvarial Culture

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ABSTRACT *: Fluoride (F), over a narrow concentration range, increases bone formation. Aluminum (Al) too is biphasic in its action on bone, being mitogenic at very low levels and inhibitory at higher levels. Both F and Al are present in finished drinking water where the chemical interaction of these two agents is well characterized. F and Al, given individually, accumulate preferentially in bone. In addition, in vivo studies have shown that F causes the co-accumulation of Al in bone. Thus, it was necessary to determine the interactive effect of these two agents on bone mitogenesis. Calvaria were obtained from neonatal CD-1 mice and cultured with various concentrations of F (0.05~19 ppm) as NaF, Al (2 ppb~2 ppm) as $AlCl_3$, or F and Al for 3 days at 37°C on a rotating roller drum. Alkaline phosphatase activity in calvaria and β-glucuronidase activity in culture medium were determined as a measures of bone turnover. Alkaline phosphatase activity in calvaria was significantly increased by F (0.05~2 ppm) treatment and β-glucuronidase activity was slightly increased in the culture medium of calvaria treated with 0.3 ppm Al. The combination of 19 ppm F and 0.3 ppm Al increased alkaline phosphatase activity in calvaria, but did not affect β-glucuronidase activity, suggesting the interactive effect of fluoride and aluminum on bone turnover.*

Key Words *: Fluoride, Aluminum, Bone turnover, Mouse calvarial culture, Alkaline phosphatase activity, β-glucuronidase activity*

I. INTRODUCTION

Fluoride (F; approximately 0.8 ppm F) is added to municipal water supplies of residents of Jinhae, chong-ju, Gwa-chun and so on in Korea for the prevention of dental caries. Now, growing numbers of communities in Korea tend to favor fluoridation. However, as the level of F increases, an increasing number of toxic side effects are seen (Krishnamachari, 1987). Among them, fluorosis is the clearest and least controversial toxicity of F. Early reports that fluorosis produced osteosclerosis (Chuttani *et al.*, 1962) stimulated studies on the potential utility of F for the treatment of osteoporosis. The effect of F is characterized by an increase in the rate of bone formation, the number of osteoblasts in bone, and the serum activity of skeletal alkaline phosphatase (Farley *et al.*, 1990). However, F is known to have a biphasic effect of bone formation. F induces a stimulation of the production of osteoblasts, but decreases their activity at high doses (Chavassieux, 1990). Clinical trials have proven controversial. Farley and coworkers (1992) reported a decrease in the spinal fracture rate in osteoporotic patients treated with F (30 mg/day), while Riggs and coworkers (1990) reported no change in the vertebral fracture rate and even an increase in nonvertebral fracture rate in patients treated with NaF (75 mg NaF/day).

Aluminum (Al) is ubiquitous in the environment. Al is found in food and pharmaceutical preparations. The accumulation and toxicity of Al is mostly seen following parenteral administration of Al or fluids contaminated with Al, because this route of administration bypasses the very effective gastrointestinal barrier to Al absorption (Klein, 1990). Generally, more Al has been found to accumulate in bone than in soft tissues and Al has been reported to be a bone toxin. For examples, Al disrupts skeletal mineralization and reduces the number and activity of osteoblasts (Posner *et al.*, 1986; Cournot-Witmer *et al.*, 1986). Also, Al in-

duces cell-mediated calcium efflux from bone and increases β-glucuronidase release into the culture medium, which suggests that Al stimulates osteoclasts in mouse calvarial culture (Sprage and Bushinsky, 1990). The accumulation of Al in bone has been implicated in the pathogenesis of osteomalacia and adynamic lesions of bone. Although Al toxicity to bone and bone cells is widely accepted, some studies have reported that at low doses Al stimulates neo-osteogenesis in the trabecular bone of normal beagles (Quarles *et al.*, 1990). Using osteoblast cell lines from chicken and human, Lau and coworkers (1991) found that micromolar concentrations of aluminum sulfate (10~25 μM) consistently stimulated [^{3}H] thymidine incorporation into DNA and increased cellular alkaline phosphatase activity.

F and Al which are both present in finished drinking water are known to form a strong complex. Given individually, both elements accumulate preferentially in bone. Further, high doses of F (50 ppm F) administered in the drinking water to rabbits for 10 weeks increased bone Al measured as accumulation in tibia (Ahn *et al.*, 1995). Even so, very few studies have evaluated the effect of these two agents on bone metabolism. Kawase *et al.* (1991) showed that a combination of F and Al stimulates DNA synthesis of MOB 3-4-F2 cells which is an osteoblast-like cell line derived from neonatal mouse calvaria. Therefore, it was demanded to compare the effect of a combination of F and Al with F or Al alone on bone turnover, using mouse calvarial culture. Alkaline phosphatase activity, both in calvaria and released into the culture medium, was determined as an index of osteoblast, or bone-forming cell activity (Farley *et al.*, 1983). Release of β-glucuronidase is known to correlate with osteoclastic-mediated calcium efflux (Eilon and Raisz, 1978). Therefore β-glucuronidase activity in the culture medium was measured as an index of osteoclast, or bone-resorbing cell activity.

II. MATERIALS AND METHODS

1. Materials

All the reagents for calvarial culture and enzyme measurement were purchased from Sigma chemical company (St. Louis, MO, USA). CD-1 mice, weighing 20~25 g, were provided from Charles River, Portage, MI, USA. After 1 week acclimatization period, three female and one male mice were housed in one cage in a temperature-controlled room with a 12 hr light : dark cycle. Pregnant mice were housed individually and 4~6 day neonates removed for use.

2. Calvarial culture

This method was originally described by Stern and Krieger (1983). Neonatal (4-to 6-day old) CD-1 mice of either sex were killed by cervical dislocation. Calvaria (frontal and parietal bones) was removed aseptically and trimmed of any excess connective tissue and the occipital bone. The calvaria was cultured in 16×150 mm tubes with 2 m*l* Dulbecco's Modified Eagle's Medium supplemented with 15% heat-inactivated (56°C, 1 hr) horse serum, 1.4% L-glutamine, 10 U/m*l* heparin, 10 U/m*l* penicillin and 10 μg/m*l* streptomycin. Each tube was gassed with 50% oxygen, 45% nitrogen, 5% carbon dioxide, and stoppered tightly with a screw cap. The tubes were placed on a rotating roller drum (1 rpm, PGC scientific) in an incubator and incubated for 72 hr at 37°C. Addition of F, Al, a combination of these two agents, or hormones to the medium was made at the start of the culture. The medium was changed and re-gassed every 24 hr. To exclude the possibility of Al contamination, deionized distilled water was used in all solutions. Alkaline phosphatase and β-glucuronidase activities were measured in calvaria after 72 hr incubation or in culture medium after 48 hr incubation.

3. Experimental conditions

Calvaria were cultured in the presence of F (0.05~19 ppm) as NaF, Al (2 ppb~2 ppm) as $AlCl_3$, or a combination of these two agents. F concentrations were chosen to cover the range of plasma levels found in individuals drinking fluoridated water, in F-treated osteoporotic women, and in F-intoxicated rats (0.05, 0.23 and 1.23 ppm respectively, Krishnamachari, 1987). Al concentrations

were selected to cover the range of Al levels found to increase cellular alkaline phosphatase specific activity in cultured embryonic chicken calvarial cells (0.05~2 ppm, Lau *et al.*, 1991). Calvaria were also cultured with bovine parathyroid hormone (PTH; 10 nM) or salmon thyrocalcitonin (36 nM) as positive controls. PTH is known to increase and calcitonin to decrease β-glucuronidase activity, in culture medium (Sprague and Bushinsky, 1990). A consistent increase or decrease of β-glucuronidase activity in culture medium of calvaria treated with these hormones suggests that the calvaria are viable under these experimental conditions.

To differentiate between enzymatic and non-enzymatic effects, a single calvaria was cut in half and two halves obtained from 2 mice were cultured as described. The other 2 halves were devitalized by heating the bones to 75°C in 1 m*l* culture medium for 5 min (Miyahara *et al.*, 1980) prior to culture. Alkaline phosphatase and β-glucuronidase activities in dead calvaria were markedly low and did not respond to bovine parathyroid hormone.

4. Measurement of alkaline phosphatase activity

After incubation, individual calvaria were homogenized in saline (9 g/liter NaCl) containing 0.1% Triton X-100 (Canalis, 1983). One hundred μ*l* culture medium or bone homogenate was incubated with 30 mM p-nitrophenyl phosphate (substrate) and 400 mM alkaline buffer (2-amino-2 methyl-1-propanol buffer, pH 10.5) at 37°C for 30 minutes, for the measurement of alkaline phosphatase activity (Lowry, 1954). Also, a series of known concentrations of p-nitrophenol (0~200 nM) was incubated to make a standard curve. The reaction was terminated by adding 2 m*l* ice cold 0.1 N NaOH. The amount of p-nitrophenol released was measured as absorbance at 410 nm, based upon the standard curve. Alkaline phosphatase activity was expressed as nanomoles of p-nitrophenol released per calvaria per 30 min.

5. Measurement of β-glucuronidase activity

Immediately after culturing, 100 μ*l* medium was incubated with 1.5 mM phenolphthalein glucuronidate in 75 mM phosphate buffer (pH 6.8) for 72 hr at 37°C. The reaction was terminated by adding 2.5m*l* glycine buffer (200 mM, pH 10.4), and the amount of phenolphthalein released was measured spectrophotometrically at 540 nm. Phenolphthalein (1.6 μg) was used to make a standard curve. β-Glucuronidase activity was calculated as micrograms of phenolphthalein released per calvaria per hour.

6. Statistics

Results are expressed as the mean±S.E. The differences in alkaline phosphatase or β-glucuronidase activities between treatment groups were determined by student's t-test or paired t-test, respectively. A significant difference was reported when $P \leq 0.05$.

III. RESULTS

1. The effect of F on bone turnover

Alkaline phosphatase activity in calvaria treated with various concentrations of F (0.05~4 ppm) as NaF is illustrated in Fig. 1. Fluoride, ranging from 0.05 ppm to 0.2 ppm, significantly increased alkaline phosphatase activity in mouse calvaria. However, this increase was not seen in mouse calvaria

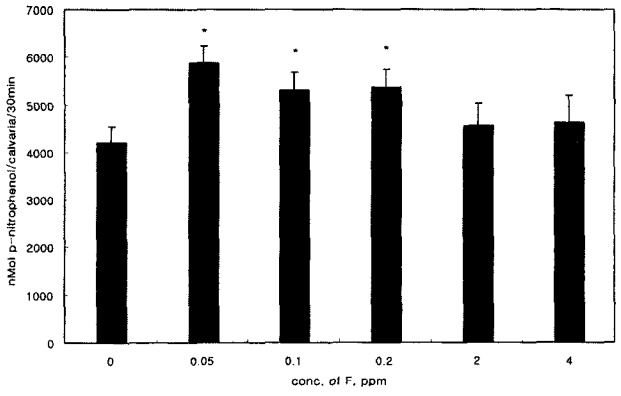

Fig. 1. Alkaline phosphatase activity in calvaria treated with F (0~4 ppm) as NaF for 3 days, expressed as nanomoles of p-nitrophenol released per calvaria per 30 min. incubation. Results are shown as mean and S.E. (n=8). Alkaline phosphatase activity in calvaria treated with F 0.05, 0.1 and 0.2 ppm was significantly increased compared to control, $P \leq 0.05$ (assessed by student's t-test).

Table 1. β-glucuronidase activity in culture medium (mean±SE, n=8)

Treatment (ppm)	β-glucuronidase activity (μg phenolphthalein/calvaria/hour)
control	0.45±0.077
F(0.05)	0.46±0.074
F(0.1)	0.47±0.071
F(0.2)	0.42±0.048
F(2.0)	0.44±0.053
F(4.0)	0.48±0.062

treated with higher F level (2~4 ppm). Although alkaline phosphatase activity in the culture medium was increased with F treatment, this increase was not statistically significant due to large variability. β-Glucuronidase activity in culture medium was not influenced by F treatment (0.05~4 ppm) (Table 1).

2. The effect of Al on bone turnover

Alkaline phosphatase activities in both calvaria and culture medium were not changed due to the treatment of Al (0.002~2 ppm) as $AlCl_3$. However, β-glucuronidase activity in medium, which is shown in Fig. 2, was slightly increased with the treatment of 0.3 ppm Al, although this increase (P= 0.08) was not statistically significant at $P \leq 0.05$.

3. The effect of a combination of F and Al on bone turnover

In vitro studies evaluating the effect of F and Al

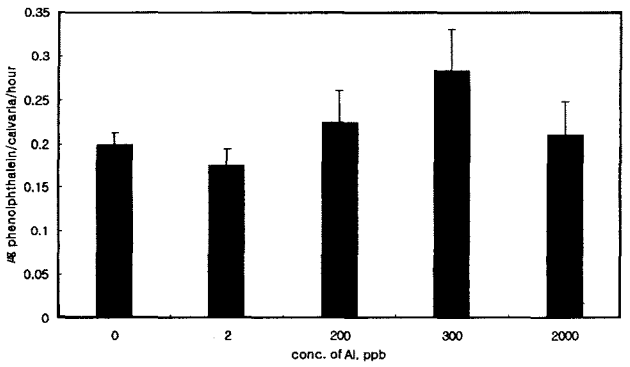

Fig. 2. β-glucuronidase activity in culture medium following 2 day culture of calvaria treated with Al (0~2 ppm) as $AlCl_3$, expressed as micrograms of phenolphthalein released per calvaria per hour. Results are shown as mean and S.E. (n=8). β-glucuronidase activity in culture medium resulting from incubation with 0.3 ppm Al was slightly increased compared to control (P= 0.084), assessed by paired t-test.

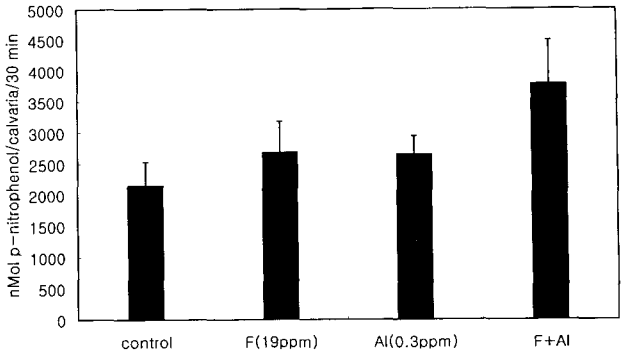

Fig. 3. Alkaline phosphatase activity in calvaria treated with F, Al or a combination of F and Al for 3 days, expressed as nanomoles of p-nitrophenol released per calvaria per 30 min. incubation. Results are shown as mean and S.E. (n=6). Alkaline phosphatase activity in calvaria treated with a combination of F and Al was slightly increased compared to control (P=0.088), assessed by student's t-test.

on adenylate cyclase showed that the guanine nucleotide-binding regulatory component was maximally activated at the ratio of F : Al (62.5 : 1 w/w, which is 88.8 : 1 on a molar basis; Sternweis and Gilman, 1982). Since 0.3 ppm Al increased β-glucuronidase release into culture medium, the combination of 19 ppm F and 0.3 ppm Al (F : Al=62.5 : 1) was used to determine the effect of F and Al combined on bone turnover. Neither 19 ppm F alone, nor combined with 0.3 ppm Al had any effect on β-glucuronidase activity. However, the combination of 19 ppm F and 0.3 ppm Al increased alkaline phosphatase activity in calvaria as shown in Fig. 3, although this increase was not statistically significant (P=0.09).

IV. DISCUSSION

In vitro Studies have an advantage over whole animal studies since all external influences can be removed and controlled by scientists. However, this altered environment might cause inconsistent results between *in vitro* and *in vivo* studies, since the vascular proliferation, muscular stress, and endogenous substances including hormone are not present in culture systems (Bingham and Raisz, 1974). Nevertheless, bone organ culture and bone cell line culture, especially using osteoblast or osteoprogenitor cells have been widely used. Not only it is very difficult to obtain a homogeneous bone

cell culture, but osteoblasts do not function in isolation. Bone is constantly undergoing formation and resorption, so that bone organ culture containing coupled osteoblast and osteoclast activities might be more realistic than isolated bone cell culture. The most widely used bone organ culture systems are fetal rat long bones and neonatal mouse calvaria. Stern and Krieger (1983) showed that neonatal mouse calvarial culture in roller drum exhibits greater sensitivity to stimuli such as parathyroid hormone and 1, 25-dihydroxy vitamin D_3 than does limb bone culture. Furthermore, calvarial culture involves a simpler dissection than the limb bones. Therefore, mouse calvarial culture, as described by Stern and Krieger (1983) was used in this study.

F accumulates in bone by substituting for OH^- ions of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), resulting in mixed fluoro- and hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_{2-x}F_x$) (Grynpas, 1990). Since the ionic radius of F^- (1.29 Å) is similar to that of OH^- (1.33 Å), and both ions share the same charge and primary hydration number, F^- ion readily substitutes for OH^- (Messer, 1984). F may be directly incorporated into apatite during initial crystal formation or may displace OH^- ions from previously deposited mineral. Besides the replacement of hydroxyl ion by F, a considerable amount of F is adsorbed onto the crystal surface. Denbesten and coworkers (1992) also reported that F can bind to matrix proteins of mineralizing tissues *in vivo*, not by covalent organic F binding, but an ionic exchangeable fluoride bond.

F has previously been shown to increase osteoblast activity in cultures of chick calvaria (Farley, 1983). In this study, the F concentration causing a maximal increase of alkaline phosphatase activity was not exactly the same as that reported by Farley (0.05 ppm F in this study vs. 0.19 ppm F in Farley's study), but the range of F concentrations (0.05 ppm to 0.2 ppm), where alkaline phosphatase activity was increased, was comparable to that reported by Farley (1983). He reported that exposure of embryonic chick calvaria to 0.19 ppm F as NaF for 3 days increased alkaline phosphatase activity maximally and half of this maximal effect was seen with 0.05 ppm F. Bellows and coworkers (1990) demonstrated that F (0.19 ppm) increased the expression of osteoprogenitor cells, obtained from fetal rat calvaria. However, at higher concentrations of F, the F effect on bone was lost due to F cytotoxicity in both rat and chick. Therefore, F effect on bone is similar in both rat and chick.

While we saw increased osteoblast activity with F, we did not see any F effect on osteoclast activity. F has been variously reported to stimulate (Brearley and Storey, 1970), or have no effect on osteoclast activity (Bellows *et al.*, 1990). At higher F levels, a preponderance of studies report inhibition (Bellows *et al.*, 1990). F concentration (10~19 ppm) has been reported to decrease the number of resorption lacunae and the resorbed area per osteoclast in isolated rabbit osteoclasts cultured on thin slices of devitalized compact bovine bone (Okuda *et al.*, 1990). Therefore, it had been expected to see both an increase in bone formation and decrease in bone resorption with the treatment of F.

Quarles and coworkers (1991) reported that addition of Al (0.03~1.3 ppm) as $AlCl_3$ to quiescent osteoblasts (MC3T3-E1 cells) increased [^{3}H] thymidine incorporation into DNA in concentration-dependent manner. Also, aluminum sulfate (15~50 μM), aluminum nitrate and aluminum chloride were reported to significantly increase the specific activity of cellular alkaline phosphatase in a monolayer chicken calvarial cell culture, which was enriched in osteoblasts and osteoblast precursors (Lau *et al.*, 1991). No studies have evaluated the effect of Al on osteoblast activity in bone culture. In the study reported here, we failed to observe a significant increase in alkaline phosphatase activity in either culture medium or calvaria treated with various concentrations of Al (2 ppb~2 ppm). The difference between our results and others may be explained in part by the fact that preosteoblast and osteoblast cells were used by Quarles and Lau, while whole calvaria was used in our study.

Although some studies report an uncoupled increase in bone formation by Al, numerous studies have shown that an increased load of bone Al is associated with a decrease in the activity and number of osteoblasts (Cournot-Witmer, 1986; Dunstan, 1984). Al is also known to accumulate at the min-

eralization front, the interface between the osteoid and the calcified matrix. This is characteristic in patients with Al-induced osteomalacia. Posner and coworkers (1986) have proposed that Al ions may adsorb onto growth sites on the surface of previously formed hydroxyapatite nuclei, which prevent further hydroxyapatite development. He suggested that small amount of aluminum phosphate may adsorb as hydroxyapatite crystal growth poisons. On the other hand, Meyer and Thomas, Jr. (1986) suggested that citrate complexes of Al act as effective inhibitors of calcium phosphate crystal growth. Alternatively, later work has shown that toxicity of Al may act on the osteoblast which was proposed to inhibit the mineralization process (Cournot-Witmer *et al.*, 1986). Any specific mechanism of inhibition of mineralization is not clear yet.

In our study, 0.05 ppm F alone and 0.3 ppm Al alone caused maximum increase of alkaline phosphatase activity and β-glucuronidase acitivity, respectively. However, the combination of 0.05 ppm F and 0.3 ppm Al didn't change alkaline phosphatase activity and β-glucuronidase acitivity in culture medium. In most studies evaluating the interactive effect of F and Al, the ratio of F : Al (62.5 : 1 w/w) was used since the maximal activation of G-protein is observed at this ratio. Kawase and coworkers (1989) have shown that in cultures of MOB 3~4 cells, which are osteoblast-like cells obtained from neonatal mouse calvaria, 0.3 ppm Al maximizes the stimulatory effect of 19 ppm F on prostaglandin synthesis. Kawase and coworkers suggested that this may be via G-protein stimulation. Two years later, they showed that the combination of 19 ppm F and 0.3 ppm Al significantly increase [^{3}H] thymidine incorporation into MOB 3~4 cell line (Kawase *et al.* 1991). This finding is in direct agreement with our study where the tendency of increase in alkaline phosphatase activity was found in calvaria treated with 19 ppm F and 0.3 ppm Al. Our previous study showed Al levels of tibia and sterna were significantly increased by the addition of high concentration of F (50 ppm and 79 ppm) to the drinking water, even in animals receiving no Al in their drinking water (Ahn *et al.*, 1995). Therefore, the exposure to high level of F results in the presence of both F and Al in bone, where the interactive effect of these two elements is suggested in this study. However, F and Al levels used in our study are very high concentration since the maximum contaminant levels of F and Al in finished drinking water are 1.5 ppm and 0.2 ppm, respectively. Additional study is required to determine the interactive effect of F and Al at such low levels.

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