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Fluoride as a Therapeutic Drug in Osteoporosis treatment

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Andrews University
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FLUORIDE AS A THERAPEUTIC DRUG IN OSTEOPOROSIS TREATMENT

An Honors Project
Presented in Partial Fulfillment
of the Requirements for HONS497 Senior Honors
Research in Biochemistry

by
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FLUORIDE AS A THERAPEUTIC DRUG IN OSTEOPOROSIS TREATMENT

"Grandma fell and broke her hip." As the news quickly passes around the family table, the younger members are not concerned. However, as complications like pneumonia, blood clots, and muscle atropy (due to lengthy immobilization) occur, attitudes are changed. Osteoporosis is a disease that affects 20 million people and has been estimated to cost 3.8 billion dollars annually in medical and nursing home care (1). In the United States alone, about 200,000 elderly people experience hip fractures yearly (2).

What is osteoporosis? In a recent issue of Clinical Symposia, osteoporosis was defined as a "generic term referring to a state of decreased mass per unit volume (density) of normally mineralized bone" (3). This decrease in bone density can result from a variety of factors which include: a deficiency in dietary components like calcium, protein, and vitamin D (4); consumption of alcohol (5); genetic factors such as race and sex (6); and skeletal disuse resulting from prolonged bed rest, paralysis, and tumors (3).

In a report issued by the National Institutes of Health in 1984 recommended that calcium and estrogen be used as "mainstays of prevention and management of osteoporosis " (7). Since then the media has exploited calcium and estrogen supplementation in reference to the disease without solid scientific backing. This has had positive effects because media attention has stimulated increased scientific research. Several studies show the beneficial uses of calcium, vitamin D, estrogen, calcitonin, and other drugs. However, conflicting data have been shown with the use of fluoride in osteoporosis treatment.

The use of fluoride as a therapeutic drug is not a novel idea. Studies dealing with the effects of fluoride on the skeletal system date back to the late 1960's (8). Thus, This paper will define osteoporosis, examine methods of detection, and will discuss various methods of treatment with emphasis on the use of fluoride supplements in the form of tablets or fluorinated drinking water. However, to fully understand osteoporosis, a review of basic bone structure, function and histology will make it easier.

BACKGROUND INFORMATION

Bone is "one of the hardest tissues of the human body and is second only to cartilage in its ability to withstand stress" (9). It has many functions, some of which include support of fleshy structures, protection of vital organs, and reservoir for ions--mainly calcium and phosphate which can be released or stored in a controlled manner in order to maintain constant concentrations in body fluids.

Bone is a specialized type of connective tissue which is 70 percent inorganic and 30 percent organic in composition (see Figure 1). Out of the 30 percent which is organic, 98 percent is made up of a cellular matrix consisting of type I collagen and noncollagenous proteins like osteocalcin, osteonecin, bone preoteoglycan, bone proteolipids, and phosphoproteins. The other two percent includes three basic bone cells--osteoblasts, osteocytes, and osteoclasts (3).

Osteoblasts are matrix forming cells whose origin is obscure. However, it is thought that these cells work by activating the production of collagen via somatomedin C, transforming growth factor B, formation factors in the bone matrix, and prostoglandin E2 (10). Osteocytes originate from osteoblasts and are mature bone cells which are found in cavities (lacunae) within the intercellular matrix. Osteoclasts are multinucleated giant cells involved in the resorption and remodeling of bone tissue. This is thought to be done by the secretion of lymphokines and cytokines which in turn stimulate the creation of an acidic cellular environment for the breakdown of bone (11).

Bone remodeling or resorption and formation are an ongoing process in the body. But, when the rate of bone resorption exceeds bone formation, a decrease in bone mass per unit volume, i.e. or density, occurs resulting in osteoporosis. As one gets older, a natural decrease in bone metabolism results in a decrease in bone density. In women, this decrease in density is enhanced by menopause (3). Menopause is a variable period during which the menstrual cycle becomes irregular and eventually disappears. Thus, a decrease in the amount of estrogen produced by the ovarian follicles and corpus leutum (structures associated with the female reproductive system) occurs. Estrogen is a hormone that plays a key role in the process of bone remodelling; however, the basic mechanism is currently obscure. The decrease in bone density due to a lowered estogen levels is

termed Type I osteoporosis or Postmenapausal osteoporosis and only affects females. A decrease in bone density as a result of age is termed Type II osteoporosis or Age-related osteoporosis and affects both males and females of the elderly population (3).

DETECTION

Early detection of osteoporosis has become prevalent in the past four years. An issue of The New Republic describes three methods that are currently used for osteoporosis detection. They are single photon absorptiometry (SPA), dual photon absorptiometry (DPA), and computed tomography (CT) (12). It is interesting to note that one-fourth to one-third of these clinics are owned by doctors, are primarily SPA monitoring facilities, and are not affiliated with hospitals (13).

Single photon absorptiometry uses two to five mrad of radiation (where a rad is equal to one unit of absorbed radiation dose and a milirad (mrad) is one-thousandth of a rad (14)), which is applied to the forearm and sensed by a densitometer. A densitometer scans the bone and sends signals back to a detector. The detector then feeds information into a computer which estimates mineral content and produces a print-out to be interpreted by a physician (13). The greater the bone density, the greater the amount of radiation absorbance, and thus the signal generated is decreased.

In a recent issue of FDA Consumer, D. Farley lists three disadvantages of the SPA method. SPA requires uniform soft tissue thickness in bone, exact alignment of area measured, and does not distinguish between compact and spongy bone. Also, "it cannot accurately predict the mineral density at critical fracture sites, such as the proximal femur (thighbone at the hip) and spine." (15). SPA's only advantage is that it is the cheapest method available--it costs about $40 to $120.

Dual photon absorptiometry (DPA) costs from $150 to $300, utilizes 5-15 mrad of radiation, and measure the bone density of the spine and thighbone in a similar manner as a SPA does. DPA produces more accurate results because readings are made at two different energy levels. According to Farley (15), two major disadvantages of this method are that the signals obtained can be distorted by calcium deposits outside the vertebrae, and this method also fails to distinguish between cortical and spongy bone density.

Computed tomography costs from $100 to $300 and uses 200mrad to 2rad of radiation--a significant increase in radiation exposure. This method does distinguish between trabecular and cortical density because x-rays are taken from many different angles, and are fed into a computer which then produces an image using mathematical calculations (16).

These methods of detection determine and estimate bone density; however, several scientists feel that people should not go through routine screening until it is absolutely necessary. In a recent issue of the Annals of Internal Medicine, Susan Ott, M. D., of Harborview Medical Center in Seattle, stated the following:

> "Although the accuracy of these techniques has been shown in the research laboratories that developed them, these findings may not apply to routine clinical laboratories . . . Before these techniques are used in clinical settings, a set of standards should be developed and measured in different geographical locations to enable verification of readings " (17).

TREATMENT

Once osteoporosis has been diagnosed, there are several methods of treatment. Estrogen, calcium, calcitonin, weight-bearing exercises, and fluoride have all been implicated and used in treatment. But in order to fully appreciate the therapeutic effects of fluoride, the other methods of treatment must first be examined.

In the past estrogen via tablets was the most widely used method of treatment. However, this use of estrogen created many side-effects and was found to be a major cause of interuterine cancer (1). Nevertheless, researchers felt that since this type of cancer is "curable" and osteoporosis is "incurable" that the trade off experienced in this mode of treatment is justified (3). Now estrogen is being coupled with progesterone to produce a more balanced effect, but the possibility of interuterine cancer still exists.

Calcium supplementation has gained much popularity in the last four years. This can be attributed to a recommendation made by a panel of specialists convened by the National Institutes of

Health in Bethesda, Maryland, in April, 1984. This panel recommended that an increase in calcium as well as estrogen therapy may be the best way to combat bone resorption (7). However, most researchers agree that calcium supplements and dietary changes have only short-term effects (18). Nevertheless, due to constant bombardment with advertisements for calcium and multi-vitamin supplements, the sale of calcium supplements have increased from $47 million to $125 million dollars just during 1985. In 1986, this figure rose and Marion Laboratories of Kansas City, Missouri, spent a total of 5 million on calcium advertisements alone (2).

Calcium supplementation includes an intake of 1500mg daily in order to be "effective" (19). However, some people can form kidney stones, especially if a history of kidney stones exists (19). The source of calcium is also important to consider. Bone and dolomite (a rock mineral source) are currently the two sources of calcium and may contain lead in amounts that would constitute a risk for infants, children, women of child-bearing age, and possibly the elderly (19). Finally, adequate amounts of vitamin D are required for proper calcium metabolism (20).

Calcitonin is a hormone produced by the thyroid and has been shown to slow bone loss by inhibiting osteoblastic activity (3). Treatment includes subcutaneous injections at critically determined concentrations; however, this method does not prevent the process of bone loss, it just slows it down.

Weight-bearing exercises that work the muscles against gravity have been implicated in osteoporosis treatment and possible prevention. The mechanism in which bone density is increased is unknown, but a significant decrease in bone density can result from a lack of weight-bearing activity (19). Some examples of weight-bearing activities are jogging, walking, biking, and aerobics. Although this method is promising, the extent of bone density increased is not as significant when compared to the other methods, and thus, it should be coupled with other treatment methods.

FLUORIDE TREATMENT

Fluoride has been used clinically to increase bone volume in the osteoporotic skeleton (21), but the drug is experimental and controversial (22). The use of fluoride has been shown to increase

the rate of bone formation in humans (23), but its effects are not evenly distributed throughout the skeletal system, and increases as well as decreases in fracture frequency have been reported by patients treated with it (24,25). Some patients do not respond to fluoride, while others seem to develop a tolerance or experience side-effects such as gastrointestinal complaints, joint pain, and increased bone resorption (26). But, in spite of the side effects, fluoride is the most effective agent known to increase bone volume, and the adverse side-effects are not life-threatening. Thus, it may be possible to devise treatment methods in which fluoride's beneficial effects are maximized and undesirable ones minimized.

The mechanism of fluoride action on the skeleton is unknown and there have been few studies on the effects of fluoride on bone turnover in experimental animals--especially a mammalian model. Thus, a study to develop an in vivo rat model to study the mechanism of fluoride action of the bone was devised and conducted by Dr. Russell Turner, Dr. Norman Bell, Kathleen Hannon, Daniel Brown, Joseph Gerand, and I during the summer of 1987 at the Jerry Van Pettis V. A. Hospital in Loma Linda, California. Two experiments were conducted and will be referred to as experiment one and experiment two.

MATERIALS AND METHODS

In experiment one, fourteen male Holtzman rats weighing approximately 190 g were divided into two weight-matched groups of seven animals each. They were housed in group cages and were given standard laboratory chow containing 0.6% calcium and 0.6% phosphorus. All animals were allowed food and water ad libitum. Sodium fluoride was added to deionized water at a concentration of 2.0 mM and given to the experimental group. The controls received the same water without the added fluoride. A twelve hour alternating dark and light cycle was provided. The rats were injected with tetracycline (tetracycline hydrochloride, Sigma Chemical Co., St. Louis, MO) at a dose of 20 mg/kg body weight on the first day of the experiment and again one and two weeks later. Twenty-one days after beginning treatment, serum was obtained, the animals were sacrificed by CO_2 suffocation, and the tibiae were excised.

The second experiment was similar to the first. It differed in the following details: the rats weighed 140 g at the start of the experiment and they were housed individually in hanging wire cages. There were three groups: 1) control rats which received only double distilled water, 2) treated rats which received distilled water with 2.0 mM fluoride added and 3) treated rats which received distilled water with 4.5 mM fluoride added. On the first day of the experiment, animals were anesthetized, subcutaneous pockets were made in the ventral abdominal and thoracic regions with a sterile hemostat and 15 mg of demineralized allogenic bone matrix (DABM) powder was implanted in each of the pockets. Two implants were placed in each animal.

HISTOMORPHOMETERY

Histomorphometric procedures were performed with a SMI-Unicomp semi-automatic image-analysis system (Southern Micro Instruments Inc., Atlanta, GA) consisting of an Apple IIe computer linked to a photomicroscope and an image-analysis system. A high resolution video camera mounted to a microscope was used to display the image of the specimen on a video monitor. The movement of a pen on a graphics tablet superimposed a tracing on the image of the speciman on the video screen. By this method, the region of interest was traced and the line length and area bounded by the tracing were automatically calculated by the computer. The error of this method is less than + 1%.

Histomorphometric analysis of the tibial diaphysis

Ground (transverse) sections were employed for cortical bone anylsis (see Appendix A). Cross sections 150 micrometers (um) thick were cut at the site of the tibia-fibula junction on an Isomet low speed saw with a diamond wafer blade. The sections were ground to a thickness of 15-20 um on a roughened glass plate. The ground sections were examined under reflected ultraviolet light to visualize fluorochrome labeling for the measurement of cortical bone formation. The following measurements were carried out (see Figure 2):

1) Medullary area--the area within the endosteal surface of the specimen.

2) Cross-sectional area--the area within the periosteal surface of the specimen.

3) Total cortical bone area--the area determined by subtracting medullary area from the cross-sectional area.

4) Endosteal bone formation rate--the area between the initial marked endosteal tetracycline label and the endosteal surface multiplied by a standard unit thickness of 1 mm and divided by the labeling period, which was 21 days.

5) Periosteal bone formation rate--the area between the initial periosteal tetracycline label and periosteal surface.

6) Endosteal bone apposition rate--the area between the initial endosteal tetracyclne label and the endosteal surface, divided by the endosteal forming surface length, divided by the 21 day labelling period.

7) Periosteal bone apposition rate--the area betwen the initial periosteal tetracycline label and the periosteal surface, divided by the label length, divided by the 21 day labelling period.

8) Periosteal forming surface length--estimated as the length of the third tetracycline label nearest the periosteal surface.

9) Endosteal forming surface length--estimated as the length of the third tetracycline label which was the label nearest the endosteal surface.

10) Endosteal non-forming surface length--estimated as the length of the endosteal surface not labeled with tetracycline.

Measurements 1-10 are described in greater detail by Baylink et al. (27)

The endosteal bone formation rate determined above is a measure of net bone formation because bone resorption occurs at the sampling site. Resorption of newly synthesized bone matrix and incorporated tetracycline label results in underestimation of bone formation. In the present studies, this error was reduced in magnitude by the administration of tetracycline at weekly intervals. In contrast, measurement of the periosteal bone formation rate is less ambiguous since essentially all of the periosteal surface is forming surface.

Histomorphometric analysis of the tibial metaphysis

After fixation in neutral buffered 10% formalin for a minimum of 72 hours, the tibiae were decalcified with 5% formic acid in 10% formalin for four days. The decalcified bone were dissected along their longitudinal axis by means of a flat-edged scapel blade, dehydrated in a graded series of ethanol and infiltrated and embedded in glycol methacrylate (Sorvall). Sections were cut in the midline of the tibial metaphysis at an indicated thickness of 5 um on a Sorvall JB4 microtome with a glass knife, mounted on slides and stained with toluidine blue. A standard sampling site was established in the secondary spongiosa of the metaphyseal (trabecular) bone of the tibiae with an eyepiece graticule square. With an Olympus OM2 microscope, the upper edge of the graticule-square was placed at a constant point one millimeter distal to the middle of the epiphyseal growth plate, perpendicular to and on the long axis of the bone. The trabecular bone was sampled within the graticule square at a constant magnification of times 250 unless otherwise specified. This site is situated distal to the primary spongiosa in the secondary spongiosa. The sampling site was extended bilaterally in each section to exclude the cortical edges. A total metaphyseal area of 1.11 mm^2 was sampled in each animal. Trabecular bone fractional area was expressed as the area of total trabecular bone within one millimeter square of metaphyseal area.

Preparation of implants

Demineralized allogenic bone matrix (DABM) powder was prepared according to a modification of a previously described protocol (28). Diaphyses of femurs and tibiae from normal rats were stripped of soft tissue, crushed with a hammer, rinsed for one hour in five changes of distilled H_2O, then dehydrated for one hour in three changes of 100% ethanol, and extracted for one hour in three changes of a solution of chloroform/methanol (1:1). The fragments were dried overnight at 30^oC, crushed in a mill, and sieved to a particle size which ranged from 90 to 850 um in diameter. The bone powder was decalcified at 25^oC for four hours with 0.5 N HCl (25 meq/g). Following demineralization, the implant material was rinsed for two hours in ten changes of distilled water, dehydrated for one hour in three changes of 100% methanol and extracted for one hour in

three changes of a solution of chloroform/methanol (1:1) and dried overnight at 30°C. The implant powder was stored at -70°C. Aliquots of the powder were analyzed for calcium to ensure that demineralization was complete. The values were less than 0.01mg calcium/10 mg powder. Before implantation, the powder was weighed and 15 mg was used for each implant. The material was rehydrated in phosphate-buffered saline. The powder was implanted subcutaneously in abdominal sites.

Analysis of implants

One implant from each animal was fixed in neutral buffered 10% formalin (pH 6.8-7.0), decalcified, dehydrated, and embedded in JB-4 (Polysciences, Warrington, PA). Sections were cut at an indicated thickness of 5 um on a Sorvall JB4 microtome and stained with toluidine blue. Maximal remodeling implants occurs at three weeks (29). For this reason, histologiccal sections of material implanted for three weeks were used for the histomorphometric determination of residual implanted bone matrix (old bone matrix), newly formed bone matrix, forming surface length and resorbing surface length. Residual or old bone matrix was identified as an acellular collagenous matrix. The new bone matrix formed during the inductive response contained osteocytes and was bordered by cement lines with osteoblasts and resorptive cells (osteoclasts) on free surfaces. The forming surface was identified as a layer of osteoblasts along any free surface of residual and new bone matrix. The cells were identified as having a cuboidal shape and prominent Golgi apparatus situated between the nucleus and the base. Resorbing surfaces were determined by the presence of multinucleated cells along the free matrix surfaces or mononuclear cells within prominent resorption lucanae on the free surfaces of new and residual bone matrix. The calcium content of implants was determined after homogenization of the implants in 2 ml of distilled water with a Polytron probe. The homogenate was centrifuged (500x g). Precipitates were resuspended in 2 ml of 1 N HCl and extracted for 48 hours at 0°C. Following centrifugation, calcium in the supernatants was determined by fluorometric titration assay (Calcette).

The mean values for each section were calculated from measurements of every third field (usually five fields were measure per implant) at a magnification of 250 times. The sample size used to calculate SEM was the number of individual implants measured and not the number of sections measured.

Serum chemistry

Serum calcium was measured by titration (Calcette. Serum phosphate, creatinine, alkaline phosphatase, and serum fluoride were measured by colorimetric automated procedures. Serum 25-(OH)D was measured in duplicate at two concentrations by competitive protein binding with vitamin D-deficient rat serum (30) after extraction with acetonitrile, washing with phosphate buffer, chromatography on C-18 Sep-Pak, and elution with acetonitrile (31). 25-(OH)D was separated from other vitamin D metabolites before the binding assay by chromatography on silica Sep-Pak, and elution with acetonitrile (32). 25-(Oh)D was separated from other vitamin D metabolites before the binding assay by chromatography on silica Sep-pak and elution with hexane-propanol (94:6). Serum $1,25(OH)_2D$ was measured by the method of Reinhardt et al. (32).

Statistical analysis

Student's unpaired t-test and Pearson's R coeffficient were performed with a personal computer (Apple IIe) using the Steinmetz program for statisfical analysis.

RESULTS

The effect of fluoride in drinking water on growth and fluoride intake are shown on Table 1. In both experiments, the initial body weights were the same, whereas the final mean body weight and growth rate did not differ between the controls and rats administered 2.0 mM of fluoride. However, the final weight and growth rate was significantly decreased in the rats given 4.5 mM of fluoride compared to control groups. The water intake did not differ between the cage with control rats and the cage with rats administered 2.0mM fluoride (data not shown). In the second experiment, the

water intake did not differ between the control rats and rats administered 2.0 mM fluoride, but the water intake was significantly increased in rats given 4.5 mM of fluoride.

The effects of fluoride on serum chemistry are shown in Table 2. There were significant increases in serum fluoride and alkaline phosphatase in rats given 2.0 mM and 4.5 mM fluoride. Serum fluoride correlated with fluoride uptake (R=0.95; p 0.001). Serum phosphorous and calcium were decreased compared to the controls in rats administered 2.0 mM fluoride, but there were no changes in rats given the 4.5 mM fluoride. Fluoride had no significant effect on serum 25(OH)D, $1,25(OH)_2D$ or creatinine.

The effects of fluoride on static bone measurements is seen in Table 3. 2.0 mM fluoride resulted in a significant increase in trabecular bone fractional area as compared to the controls while 4.5 mM fluoride resulted in a significant decrease in this measurement. In both experiments, however, there were increases in medullary area in fluoride treated rats. Also, endosteal nonforming surface increased and there was a corresponding decrease in forming surface length. There was no significant change in periosteal forming surface length. Cross-sectional area was increased in rats fed 2.0 mM fluoride but was unchanged in rats fed 4.5 mM.

The effect of fluoride on dynamic bone histomorphometry in the tibial diaphysis is shown on Table 4. The periosteal bone formation rate and apposition rate increased significantly at 2.0 mM fluoride, but did not differ from the controls at 4.5 mM. The endosteal bone formation rate and apposition rate decreased compared to the controls in rats fed 4.5 mM fluoride. The endosteal bone formation rate decreased compared to the controls in rats fed 2.0 mM fluoride in one experiment was was unchanged in the second experiment. The corresponding endosteal bone apposition rates did not change compared to the controls and was increased in the two experiments respectively. Fluoride resulted in increased neutral and total surface length and decreases in forming surface length and calcium content. There was no change in resorbing surface length.

The effect of fluoride on DABM implants are shown in Table 5. There were increases in new bone matrix and decreases in implant calcium in rats treated with 2.0 mM and 4.5 mM fluoride.

There was no change in residual matrix. Fluoride treatment resulted in an increase in neutral surface, no changes in resorbing surface and a decrease in forming surface.

DISCUSSION

One of the more interesting results of the present study was the remarkable difference in the skeletal response of rats fed 2.0 mM fluoride when compared to the animals fed 4.5 mM. At the lower concentration, the drug stimulated periosteal bone formation and increased endosteal bone turnover at the tibial diaphysis. It also increased trabecular bone volume at the metaphysis. It is significant that the growth rate of these rats was normal. Further, the changes in serum chemistry and cortical bone histomorphometry were very similar in two separate experiments. In contrast, 4.5 mm fluoride did not alter periosteal bone formation, inhibited endosteal bone formation, increased endosteal bone resorption, and reduced trabecular bone volume. The growth rate of these rats was reduced compared to the controls. Thus, there was a narrow concentration range separating the beneficial effect of fluoride to increase bone volume from toxic effects.

We were able to demonstrate that 2.0 mM serum fluoride increases trabecular bone volume in the rat while 4.5 mM reduces trabecular bone volume. This finding is of interest because trabecular bone is especially susceptible to loss in osteoporotic patients. Although these studies were conducted in growing rats, the trabecular bone measurements were made at the secondary spongiosa and reflect turnover and not growth (33). Previous attempts to observe an effect of fluoride on trabecular bone in the rat were unsuccessful (34, 35). In these studies, high doses of 4.5 mM and 5.4 mM fluoride were administered and had no effect and reduced trabecular bone volume respectively.

Total serum alkaline phosphatase activity was used as an index of osteoblast activity. Although bone formation in the tibia and serum alkaline phosphatase activity were both significantly increased in rats fed 2.0 mM fluoride, alkaline phosphatase activity was elevated without a corresponding increase in bone formation in rats fed 4.5 mM fluoride. The cause of the discrepancy is not known. Fluoride has a direct effect <u>in vitro</u> to stimulate alkaline phosphatase activity in primary cultures of cells derived form embryonic bone (36). However, in the present experiments,

total serum alkaline phosphatase activity was not significantly correlated with serum fluoride (R=0.30; p 0.05). Thus, it is possible that the drug increases the activity of the enzyme independently of its effect to increase bone formation. Alternatively, the tibia may not have been representative of the skeleton. If this was the case, there may have been increased bone formation at other skeletal sites in rats treated with 4.5 mM fluoride. Finally, the elevated serum alkaline phosphatase may have been due to increases in the lives and/or intestinal isoenzymes (see Appendix B). Whatever the cause, it is clear that total serum alkaline phosphatase in fluoride treated rats did not reflect the rate of bone formation in the tibia.

There was an excellent correlation between fluoride intake and serum fluoride. A similar relationship was previously observed (37). Also, it was shown that serum fluoride reaches a steady state level in the plasma as early as 14 days after the start of treatment.

We observed an increase in corical bone turnover with fluoride treatment similar to that previously described in younger rats (38). Periosteal bone formation was increased in rats fed 2.0 mM Fluoride. Medullary area and endosteal non-forming surface length was increased in fluoride treated rats, indicating that bone resorption was increased (38-40). The endosteal bone formation rate was decreased slightly in one experiment using rats fed 2.0 mM fluoride and was unchanged in the second experiment. The corresponding bone apposition rate were unchanged and increased respectively. These findings suggest that although the forming surface length was decreased in fluoride treated rats because of increased bone resorption, the activity of osteoblasts lining the forming surface was not inhibited. The mechanism mediating the increase in bone turnover in fluoride treated rats is unknown. Our data suggests that the optimal concentration of fluoride for increased bone resorption is higher than that for increased formation. If this is correct, the effects of fluoride in bone formation and resorption may be mediated through two different mechanisms and may be dissociable.

The response of DABM implants to fluoride appears to be similar to changes observed in bone in the present and previous studies; there was an increase in bone matrix and a decrease in mineralization. Fluoride resulted in a decrease in forming surface in DABM implants with no change

in resorbing surface and increased bone matrix suggesting that osteoblast activity was increased. Osteoinduction by DABM implants should provide a useful model for studying the effects of fluoride on the organic and mineral phases of bone as well as on differentiation of bone cells. This in vivo model has advantages over whole animal studies. Experiments can be designed using the implant model such that the entire process of bone cell differentiation, bone matrix synthesis and mineralization occur in the presence of fluoride. Previous studies have demonstrated that osteoinduction by DABM implants is sensitive to systemic factors known to regulate bone metabolism (26, 40) including vitamin D, serum calcium, and ovarian hormones.

The present in vivo studies demonstrate that rats respond to fluoride with altered growth rate, water intake, serum chemistry, radial growth, and endosteal modeling in cortical bone, trabecular bone volume, and osteoinduction by DABM impants. Importantly, these changes occurred at serum concentrations of fluoride similar to that in patients treated with fluoride for osteoporosis (41). The beneficial effects of fluoride on trabecular bone volume, as well as potentially detrimental effects on forming surface length and decreased mineralization that have been observed in patients undergoing treatment for osteoporosis (41) were seen in the rat.

These findings suggest that the rat may be a relevant model for fluoride treatment of osteoporotic humans, and that the use of fluoride at a key dosage has many advantages and few, if any, disadvantages. Fluoride supplementation may be accomplished through tablet form or through fluorinated drinking water. However, much more research on possible combinations of fluoride with calcium, estrogen, tamoxifen, and other agents is necessary to obtain an optimum treatment method.

Appendix A

In the body there are two basic types of bone. One is called cortical or compact bone, is dense, and does not possess cavities in cross-sections. The other type, trabecular or spongy bone, is found in areas with interconnecting cavities, and is more flexible than companct bone. The metaphysis contains primarily spongy bone while the diaphysis has compact bone.

APPENDIX B

An enzyme is a substance that catalyzes a chemical reaction in the body. Isoenzymes (lysozymes) are enzymes which occur in different molecular forms in the same species, same tissue, or even the same cell. The different forms of the enzyme catalyze the same reaction, but each lysozyme has different kinetic properties and different amino acid composition and/or sequence (42). Alkaline phosphatase is an isoengyme that is found in the liver, intestine, and bone. Thus changes in any of these locations could cause an increase in alkaline phosphatase levels in the body.

FIGURE 1--Composition of Bone

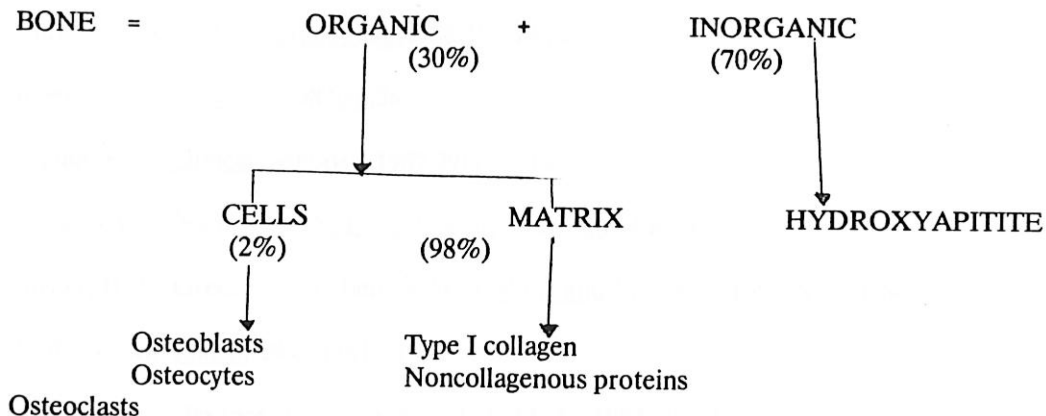

FIGURE 2--Anatomy of a Long Bone

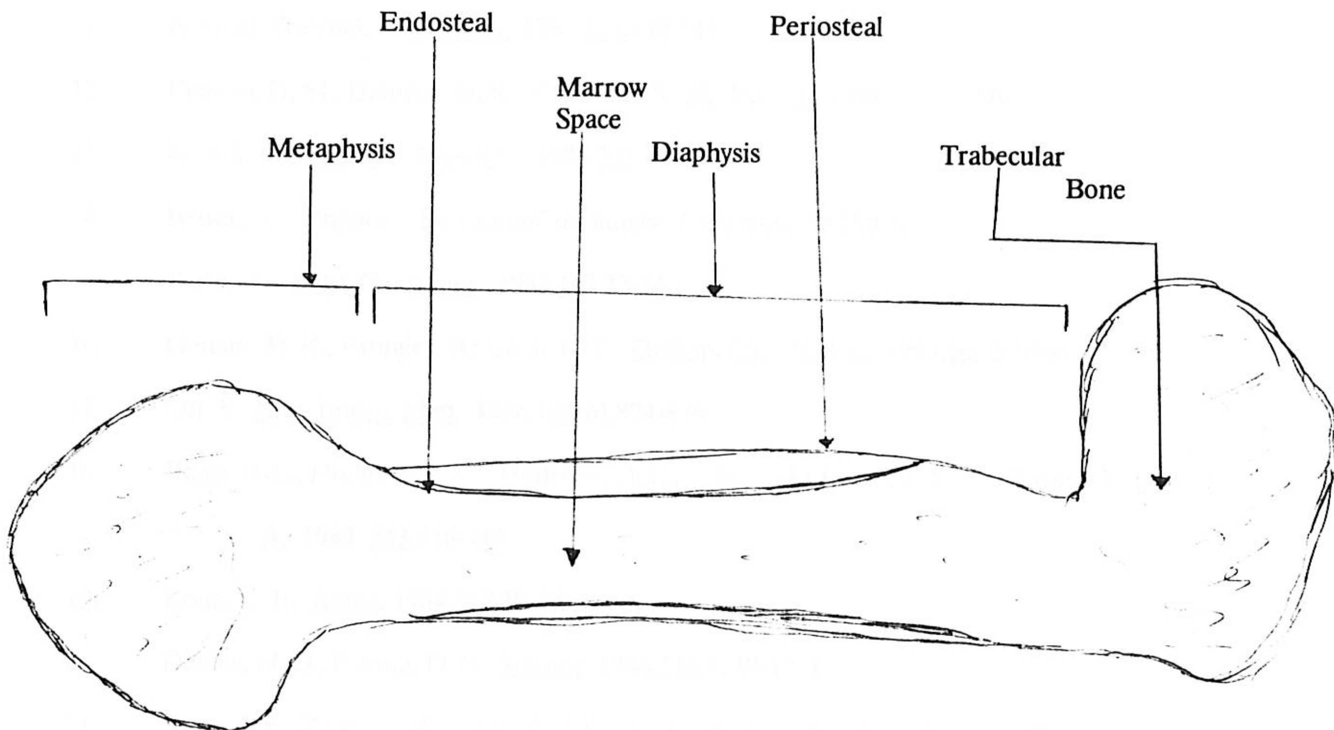

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Table 1: The effect of fluoride in drinking water on growth and fluoride intake.

Measurement	Experiment #1 Control	Experiment #1 Fluoride 2.0 mM	Experiment #2 Control	Experiment #2 Fluoride 2.0 mM	Experiment #2 Fluoride 4.5 mM
Starting Weight (g)	190±3	192±3	145±2	140±3	140±3
Ending Weight (g)	305±5	296±12	298±8	295±7	268±15*
Growth Rate (g)	5.29±0.21	4.94±0.44	6.39±0.27	6.46±0.21	5.11±0.55**
Water Intake (ml/d)	ND	ND	29.5±1.0	28.2±1.4	40.6±4.2*
Fluoride Intake (mgNaF/d)	ND	ND	0	2.8±0.1***	9.0±0.9***

Values are expressed as mean ± 1 SEM (n = 7-8), significance is compared to control group.
ND is not determined.
* $p<0.05$
** $p<0.025$
*** $p<0.001$

Table 2: The effect of fluoride on serum chemistry.

Measurement	Control	Fluoride 2.0 mM	Fluoride 4.5 mM
Fluoride (mg/dl)	0.19±0.01(8)	0.41±0.02***(8)	1.10±0.21****(8)
Alkaline Phosphatase (u/l)	96±8(8)[1]	124±9*(8)	140±11**(8)
Creatinine (mg/dl)	0.50±0.03(15)	0.53±0.03(15)	0.57±0.03(8)
Calcium (mg/dl)	10.5±0.1(15)	10.1±0.1**(15)	10.5±0.1(8)
Phosphorus (mg/dl)	9.1±0.2(15)	8.2±0.2**(15)	9.2±0.05(8)
25(OH)D (Ng/ml)	17.2±1.6(6)	20.4±2.2(7)	ND
$1,25(OH)_2D$ (pg/ml)	39.8±3.4(6)	35.3±3.2(7)	ND

Values are expressed as mean ± 1 SEM (N), significance is compared to control group.
ND is not determined.

* $p<0.05$
** $p<0.025$
*** $p<0.01$
**** $P<0.001$

[1]Experiment #2 (In experiment #1, alkaline phosphatase was 120±9 (6) for the control and 158±4 (6) for rats treated with 44 ppm fluoride.

Table 3: The effect of fluoride on static bone measurements.

Measurement	Experiment #1		Experiment #2		
	Control	Fluoride 2.0 mM	Control	Fluoride 2.0 mM	Fluoride 4.5 mM
Tibial Metaphysis					
Trabecular Bone (fractional area)	ND	ND	0.18±0.02	ND	0.18±0.01
Tibial Diaphysis					
Medullary Area (mm^2)	1.00±0.04	1.11±0.06	0.99±0.09	1.15±0.10	1.22±0.06*
Cross Sectional Area (mm^2)	3.73±0.14	3.87±0.10	3.83±0.13	4.20±0.16*	3.97±0.12
Periosteal Forming Surface (mm)	7.19±0.08	7.25±0.13	7.20±0.13	7.52±0.18	7.28±0.11
Endosteal Forming Surface (mm)	3.53±0.12	2.83±0.32*	3.12±0.13	2.50±0.42	1.83±0.28***
Endosteal Non-forming Surface (mm)	0.16±0.08	1.06±0.02**	0.61±0.27	1.46±0.51*	2.31±0.40***

Values are expressed as mean ± 1 SEM (n = 7-8), significance is compared to control group.
ND is not determined.
* $p<0.05$
** $p<0.025$
*** $p<0.001$

Table 4: The effect of fluoride on dynamic bone histomorphometry in the tibial diaphysis.

Measurement	Experiment #1		Experiment #2		
	Control	Fluoride 2.0 mM	Control	Fluoride 2.0 mM	Fluoride 4.5 mM
Periosteal Bone Formation Rate ($mm^3 \times 10^{-3}$/day)	38.0±1.0	44.1±3.0*	46.0±2.1	54.6±3.6*	47.2±5.0
Periosteal Bone Apposition Rate (um/day)	5.32±0.19	6.02±0.34*	6.39±0.27	7.27±0.37*	6.43±0.59
Endosteal Bone Formation Rate ($mm^3 \times 10^{-3}$/day)	7.6±0.2	5.4±0.4**	6.4±0.4	5.7±1.2	2.6±0.4
Endosteal Apposition Rate (um/day)	2.10±0.004	1.99±0.15	1.65±0.27	2.19±0.24*	1.25±0.17

Values are expressed as mean ± 1SEM; n=5-7, significance is compared to control groups.
* $p<0.05$
** $P<0.025$

Table 5: The effect of fluoride on demineralized bone matrix implants.

Measurement	Control	Fluoride 2.0 mM	Fluoride 4.5 mM
Old Bone Matrix (fractional area)	0.442±0.029	0.484±0.037	0.426±0.043
New Bone Matrix (fractional area)	0.132±0.010	0.156±0.012	0.207±0.042*
Implant Calcium (mg/implant)	0.277±0.022	0.161±0.022**	0.123±0.016***

Values are expressed as mean ± 1 SEM; N=7, significance is compared to control group.

* $p<0.05$

** $p<0.01$

***$p<0.001$