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Fluoride Treatment Increased Serum IGF-1, Bone Turnover, and Bone Mass, but Not Bone Strength, in Rabbits

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Abstract. We hypothesized that fluoride partly acts by changing the levels of circulating calcium-regulating hormones and skeletal growth factors. The effects of oral fluoride on 24 female, Dutch-Belted, young adult rabbits were studied. The rabbits were divided into two study groups, one control and the other receiving about 16 mg fluoride/rabbit/day in their drinking water. After 6 months of fluoride dosing, all rabbits were euthanized and bone and blood samples were taken for analyses. Fluoride treatment increased serum and bone fluoride levels by over an order of magnitude ($P < 0.001$), but did not affect body weight or the following serum biochemical variables: urea, creatinine, phosphorus, total protein, albumin, bilirubin, SGOT, or total alkaline phosphatase. No skeletal fluorosis or osteomalacia was observed histologically, nor did fluoride affect serum PTH or Vitamin D metabolites ($P > 0.4$). BAP was increased 37% ($P < 0.05$) by fluoride; serum TRAP was increased 42% ($P < 0.05$); serum IGF-1 was increased 40% ($P < 0.05$). Fluoride increased the vertebral BV/TV by 35% ($P < 0.05$) and tibial ash weight by 10% ($P < 0.05$). However, the increases in bone mass and bone formation were not reflected in improved bone strength. Fluoride decreased bone strength by about 19% in the L5 vertebra ($P < 0.01$) and 25% in the femoral neck ($P < 0.05$). X-ray diffraction showed altered mineral crystal thickness in fluoride-treated bones ($P < 0.001$), and there was a negative association between crystal width and fracture stress of the femur ($P < 0.02$). In conclusion, fluoride's effects on bone mass and bone turnover were not mediated by PTH. IGF-1 was increased by fluoride and was associated with increased bone turnover, but was not correlated with bone formation markers. High-dose fluoride treatment did not improve, but decreased, bone strength in rabbits, even in the absence of impaired mineralization.

Key words: Bone density — Bone — Fluoride — Biomechanics — IGF-1—Mineralization.

Fluoride therapy remains a potential, but controversial, treatment for osteoporosis [1]. Moderate to high doses of fluoride increase bone formation and bone mass. Farley et al. [2] suggested that fluoride's anabolic effect on bone was due to a direct effect of fluoride on bone cells. This finding has been supported by some subsequent culture studies [3, 4] and contradicted by others [5, 6]. It has been postulated that the inconsistent results from cell culture are due to the cell populations studied—osteoblast precursors are more responsive to fluoride than osteoblasts [7]—and the fact that fluoride's anabolic effects require the presence of mitogenic growth factors such as insulin-like growth factor-1 (IGF-1) [8]. Fluoride treatment can cause secondary hyperparathyroidism [9] and, therefore, affect levels of some calcium-regulating hormones. It has yet to be determined whether fluoride's anabolic effects on bone are mediated by changes in calcium-regulating hormones or mitogenic growth factors.

Besides increasing bone mass, fluoride treatment has resulted in decreased bone tissue strength in many animal studies [10–15], whereas other animal studies showed no effect of fluoride on bone strength [for example, 16]. Reduced bone strength may be caused by mineralization defects in the bone that can result from high serum fluoride levels [17–21]. This effect of fluoride may have been responsible for increased appendicular fracture incidence in one clinical trial [22].

Fluoride also affects bone strength of well-mineralized bone [23], possibly by altering mineral crystal size and packing [24, 25]. Fluoride tends to increase mineral crystal width [24], and may alter the electrostatic bonding between mineral crystals and the collagen matrix [26]. Both effects may diminish the mechanical properties of the bone [27].

We hypothesized that fluoride partly acts by changing the levels of circulating calcium-regulating hormones and skeletal growth factors. We tested this hypothesis in rabbits by studying the effects of fluoride intake on parathyroid hormone (PTH), Vitamin D metabolites, and two important hormones affecting bone mass: estradiol and IGF-1. Interactions between these serum hormones, bone turnover markers, and tissue-level bone formation are reported. We also studied fluoride effects on bone strength, histomorphometry, and mineral crystal shape and size.

Methods

Animals

We evaluated several animal species as potential models for fluoride effects. Anabolic effects of fluoride in the rat skeleton have not been demonstrated consistently [13, 16]. Furthermore, rat long bones do not undergo osteonal remodeling which is the major

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mechanism for fluoride incorporation in the human skeleton [28]. Chicks have been proposed as a model for fluoride effects on the skeleton [29], although their growing skeleton does not simulate bone remodeling in adult humans well. We therefore rejected these animal models and chose to study rabbits. The skeleton of young adult rabbits undergoes remodeling at a rate of 10–60%/year (unpublished data), and develops osteosclerosis in response to high intakes of fluoride [30, 31].

Twenty-four young adult (3 1/2-month-old) female, Dutch-Belted rabbits were used in this study. The rabbits were divided into two groups of 12; both groups were fed a standard rabbit chow. The experimental group was given drinking water with 100 ppm fluoride for a period of 6 months; the control group received distilled water. The fluoride dose (100 ppm) was chosen because pharmacokinetic studies by Hall et al. [32] showed that it would create serum fluoride levels sufficient to cause mitogenic effects on bone cells, and higher fluoride doses cause osteosclerosis and toxic effects on growth in rabbits [30, 31]. At the end of the study, the animals were anesthetized and 10 cc of blood was drawn by cardiac puncture. The rabbits were then euthanized and tissues were removed for analysis. All tissue and blood specimens removed from the rabbits were identified only by code number. All procedures throughout this experiment conformed with the guidelines of the Animal Care and Use Committee of Indiana University.

Tissue Fluoride Measurements

Fluoride content was measured in the serum, distal half of the left femur, and the L3 vertebra from each rabbit. Bone samples were ashed at 600°C for 6 hours in a muffle furnace and ground to a fine power before fluoride analysis. Fluoride analysis was conducted using the hexamethyldisiloxane (HMDS) (Sigma Chemical Co., St. Louis, MO) microdiffusion method of Taves [33] as modified by Dunipace et al. [34]. After overnight, acid-induced diffusion of the fluoride in each sample into a NaOH trap, the trap was buffered to pH 5.2 with acetic acid and placed under an electrode (Orion #96-909-00). Sample fluoride levels were determined by comparison with a series of fluoride standards.

Clinical Serum Chemistry

A series of serum ''wellness markers'' were measured to evaluate alterations in liver or kidney function, cellular damage, or general modifications in metabolic or physiological status. The analyses included serum creatinine, urea nitrogen, glucose, calcium, phosphorus, uric acid, cholesterol, total protein, albumin, bilirubin, glutamate oxaloacetate transaminase (SGOT), and alkaline phosphatase. These analyses were done using a multichannel auto analyzer (Kodak Ektachem 700).

Bone Turnover Markers

Tartrate-resistant acid phosphatase (TRAP) and the bone-specific isoform of alkaline phosphatase (BAP) were measured as markers of bone turnover. TRAP, a marker of bone resorption, was measured using a modification of the method of Lau et al. [35]. BAP was measured using an immunoradiometric kit (OSTASE, Hybritech, San Diego, CA), a two-site immunoradiometric assay using mouse monoclonal antibodies. Rabbit serum was diluted in parallel with human samples in this assay. All samples were run in the same assay.

Hormones

Serum was assayed for IGF-1, parathyroid hormone (PTH), estradiol, and Vitamin D metabolites. IGF-1 was extracted from serum using a 50:50 mixture of hydrochloric acid and ethanol [36] and measured using a human IGF-1 radioimmunoassay (RIA) kit (Nichols Research Institute, San Juan Capistrano, CA). PTH levels were measured using an immunoradiogrametric assay for the human intact molecule (Nichols Institute Diagnostics, San Juan Capistrano, CA). Both IGF-1 and PTH were shown to cross-react with rabbit serum. Estradiol was measured using the Coat-A-Count RIA kit from Diagnostic Products Corp (Los Angeles, CA). The detection limit of this assay was 10 pg/ml. 25OHD and $1,25(OH)_2D$ were extracted using HPLC and measured by protein binding assays using vitamin D binding protein from human serum for 25OHD and vitamin D receptor protein from calf thymus for $1,25(OH)_2D$. Extraction load was corrected by labeled $1,25(OH)_2D$ and 25OHD recoveries.

Histomorphometry

Approximately 2 weeks prior to the end of the experiment, the rabbits were given a fluorochrome bone label (calcein green at 5 mg/kg I.M.). One week later, this treatment was repeated. The L4 vertebra was taken from each rabbit, fixed in 10% neutral-buffered formalin, and embedded in plastic for histomorphometric analysis. Two thin (5–7 μm) sections were made through the midsagittal plane of each vertebra. One section was stained with Goldner's tetrachrome and the other was left unstained. Static measurements of trabecular bone volume (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), osteoid perimeter (Os.Pm), and osteoid thickness (Os.Th) were made on stained sections using standard methods [37]. Briefly, static histomorphometric measurements were made at ×156 magnification on a Nikon FXA epifluorescent microscope utilizing stereological point-hit (volume-related parameters) and linear intercept (surface-related parameters) methods [38]. The region of bone lying below the primary spongiosa of the caudal portion of the vertebra was analyzed. Dynamic measurements of bone formation rate (BFR), mineral apposition rate (MAR), and mineralizing surface (MS/BS) were made from the fluorochrome bone labels as follows: MS/BS was calculated as half of the single-labeled surface plus the double-labeled surface, MAR was the average distance between the two labels, and BFR was the product of MS/BS and MAR. Measurements were made at ×150 using a Bioquant semiautomatic digitizing system (R & M Biometrics, Nashville, TN) attached to a Nikon Optiphot fluorescence microscope.

Microradiography

Thick sections (120 μm) were cut from each lumbar vertebral specimen in the midsagittal plane and transversely through the femoral midshaft using a Leitz 1600 SawMicrotome. A microradiograph of each section was made using Kodak SOS-343 film at an exposure of 35 kvp, 3 mA for 40 minutes.

Biomechanics

Bone strength was measured in the femur, L5 vertebra, and femoral neck for each rabbit.

Femoral bone strength was measured at the midshaft of the femur using a three-point bending test. Rabbit femora were removed immediately after termination and frozen at −20°C. Before testing, the bones were thawed, and bone strength was measured by applying a load midway between two supports that were 30 mm apart. The femur was positioned so that bending occurred about the medial-lateral axis. Specimens were tested in saline solution at 37°C. Each specimen was submerged in the saline bath for 3 minutes before testing to allow equilibration of temperature. The temperature of the saline bath was constantly monitored throughout the test with a digital thermometer. Temperature in the bath varied by no more than ± 1°C. Load-displacement curves were recorded at a rate of 1 mm/second using a servo-hydraulic materials testing machine (MTS Corp., Minneapolis, MN). Breaking force was calculated as the maximum load sustained by the speci-

men. Bending fracture stress was calculated from the fracture force using the following equation:

$$\sigma_f = \frac{Mc}{I}, \quad (1)$$

where σ_f is bending fracture stress, M is the bending moment at which fracture occurred, c is the distance from the centroid of the cross-section to the periosteal surface, and I is the moment of inertia [39]. The value for moment of inertia used in stress analysis was calculated under the assumption that the femoral cross-sections were elliptically shaped using the following equation:

$$I = (\pi/64)[ab^3 - (a - 2t)(b - 2t)^3] \quad (2)$$

where a is the width of the cross-section in the medial-lateral direction, b is the width of the bone in the anterior-posterior direction, and t is the average cortical thickness. Average cortical thickness was calculated from thickness measurements made in each of four quadrants of the femoral cross-section with a pair of digital calipers accurate to 0.01 mm, with a precision of ± 0.005 mm. Widths a and b were measured at the location of the femur where the top loader contacted the bone. The variable c in (1) was calculated as half of the width of the bone in the anterior-posterior direction. Elastic modulus was calculated using the following equation:

$$E = \frac{F}{d}\frac{L^3}{48I} \quad (3)$$

where F/d is the slope of the force-displacement curve and L is the length between the loading supports [39].

Vertebral bone strength was measured after the posterior processes were removed and the ends of the centrum made parallel using a diamond wafering saw (Buehler Isomet, Evanston, IL). Failure load in each vertebra was measured in compression using the MTS machine. The compressive load was applied at a rate of 50 N/second through a pivoting platen to correct for nonparallel alignment of the faces of the vertebral body [39]. Specimens were tested in saline solution at 37°C. Fracture force was calculated as the maximum load sustained by the specimen. Fracture stress was calculated as the maximum load divided by the gross cross-sectional area πAB, where A and B are the widths in the anterior-posterior and medial lateral directions. Elastic modulus was calculated as the maximum slope of the stress-strain curve.

Femoral neck breaking force was measured by mounting the proximal half of the femur vertically in a chuck and applying downward force at a rate of 1 mm/second on the femoral head until the neck failed. All tests were done at room temperature using the MTS system.

Mineral Size

Samples of bone powder from the tibia were analyzed with a Rigaku diffractometer using Cu Kα radiation and a highly crystalline mineral fluorapatite as a standard. The values $B_{1/2}$ (002) and (130), the widths at one-half the maximum height of the hydroxyapatite reflections, were measured using a step-scanning procedure with 0.4° per step (2θ) and 100 seconds of counting. Because the instrumental broadening was small compared with sample peak breadths, the measured half-widths were corrected for instrumental broadening by subtracting the square of $B_{1/2}$(002) and $B_{1/2}$(130) for the standard (fluorapatite) from the square of the bone value and taking the square root of the difference. ''D'' values, which are related to the crystal size/strain in the long dimension (002) and the cross-section (130) of the apatite crystal, were calculated from the corrected $B_{1/2}$(002) and $B_{1/2}$(130) values using the Sherrer equation [40]:

$$D = \frac{57.3\ K\ \lambda}{B_{1/2}\cos\theta},$$

where 57.3 is a conversion factor from degrees to radians, λ is the X-ray wavelength, $B_{1/2}$ is the breadth at half the height of the 002 and 130 peaks, and θ is the diffraction angle. K is a constant varying with crystal habit and chosen as 0.9 for the elongated crystallites of bone. Each measurement was repeated three times and the results averaged.

Data Analysis

All tissue and blood specimens removed from the rabbits were identified only by code number and all measurements were made without knowledge of the specimen groups. Once all analyses had been completed, samples were decoded; comparisons between fluoride-treated and control groups were made using a *t*-test and relationships between continuous variables were determined using linear regression. All analyses were done on a Macintosh computer using a standard statistics program (Statview, Abacus Concepts, Berkeley, CA). *P*-values less than 0.05 were considered statistically significant.

Results

The initial body weights of the rabbits (mean ± SEM: 1.55 ± 0.04 kg for control and 1.55 ± 0.05 kg for fluoride) and the final body weights (2.24 ± 0.08 kg for control and 2.33 ± 0.08 kg for fluoride) were not significantly different ($P > 0.4$). One rabbit in the control group died before completion of the study. Water consumption averaged 163 ± 5 ml/day and was not significantly different between control and fluoride-treated groups ($P > 0.6$).

Fluoride treatment did not affect the following serum biochemistries: urea, creatinine, phosphorus, total protein, albumin, bilirubin, serum glutamate oxaloacetate transaminase (SGOT), or total alkaline phosphatase ($P > 0.2$). Total serum calcium was decreased 3% ($P < 0.05$), and serum glucose was increased 17% ($P < 0.05$) by fluoride treatment. As expected, fluoride treatment significantly increased fluoride levels in the serum and bones (Table 1). BAP and TRAP were significantly increased by fluoride treatment, indicating that bone turnover was increased. IGF-1 was increased by 40% after fluoride treatment ($P < 0.05$), but no changes occurred in PTH or vitamin D metabolites ($P > 0.4$). Serum levels of estradiol were below the detection limits of the assay in both treated and control groups. BFR was increased 45% by fluoride treatment, though this increase did not reach statistical significance. Increases in BFR were reflected by a significant increase in tibial BV/TV. Fluoride treatment increased the BV/TV by 35%; this change resulted from a 13% increase in the Tb.N and an 18% increase in Tb.Th. However, the increased bone mass resulting from fluoride treatment was not associated with increased bone strength. Fluoride treatment resulted in a 25% decrease in fracture force in the femoral neck, a 19% decrease in fracture stress in the L5 vertebra, and a 21% decrease in femoral elastic modulus. Fluoride treatment increased the average mineral crystal width, as evidenced by changes in the X-ray diffraction patterns of the bone mineral (Table 1).

Serum fluoride was not correlated with IGF-1 levels ($P = 0.4$). However, serum fluoride was positively correlated with BAP (Table 2). Serum IGF-1 was not correlated with BAP ($P > 0.8$), but strongly correlated with TRAP (Table 2).

Bone fluoride levels were strongly correlated with serum fluoride ($r > 0.85$). Fluoride levels were positively correlated with the vertebral BV/TV and tibial ash weight (Table 3). Bone strength parameters were, in general, negatively correlated with bone fluoride content (Table 4). Increases in

Table 1. Results from 6-month fluoride treatment in young adult rabbits (mean ± SEM)

Measurement	Control	Fluoride-treated	Difference (%)
Fluoride levels			
Serum (μM)	2.32 ± 0.10	38.31 ± 4.21	+1551[a]
Vertebral (ppm)	1151.0 ± 41.0	7893.0 ± 244.0	+586[a]
Tibial (ppm)	853.0 ± 38.0	6650.0 ± 279.0	+680[a]
Bone turnover markers			
BAP (ng/ml)	17.4 ± 1.1	23.8 ± 2.1	+37[a]
TRAP (U/Liter)	29.9 ± 1.7	42.5 ± 2.7	+42[a]
Hormones			
IGF-1 (ng/ml)	66.1 ± 5.5	92.4 ± 11.4	+40[a]
PTH (pg/ml)	44.9 ± 5.1	46.2 ± 6.2	+3
1,25 $(OH)_2D$ (pg/ml)	14.4 ± 5.1	15.2 ± 6.2	+6
25 OHD (ng/ml)	33.5 ± 1.2	30.9 ± 3.1	−8
Histomorphometry (L4 vertebra)			
Static			
BV/TV (%)	26.1 ± 1.4	35.2 ± 2.0	+35[a]
Tb.N (mm^{-1})	3.0 ± 0.1	3.4 ± 0.2	+13[a]
Tb.Sp (μm)	396.0 ± 23.0	309.0 ± 27.0	−22[a]
Tb.Th (μm)	87.0 ± 3.0	103.0 ± 6.0	+18[a]
Os.Pm (%)	13.6 ± 3.1	26.9 ± 4.4	+98[a]
Os.Th (μm)	6.6 ± 2.6	11.6 ± 1.7	+76
Dynamic			
BFR ($\mu m^3/\mu m^2$/year)	245.0 ± 44.0	356.0 ± 49.0	+45
MAR (μm/day)	1.14 ± 0.12	1.48 ± 0.14	+30
MS/BS (%)	55.0 ± 6.0	64.6 ± 6.3	+17
Bone Mass			
Tibial ash wt. (g)	2.20 ± 0.06	2.43 ± 0.08	+10[a]
Biomechanics			
Femoral neck			
Fracture force (N)	675.0 ± 41.0	508.0 ± 24.0	−25[a]
L5 Vertebra			
Fracture force (N)	1396.0 ± 97.0	1189.0 ± 63.0	−15
Fracture stress (MPa)	54.9 ± 3.7	44.3 ± 2.9	−19[a]
Modulus (MPa)	1.76 ± 0.20	1.42 ± 0.12	−19
Femur			
Fracture force (N)	368.0 ± 11.0	351.0 ± 8.0	−5
Fracture stress (MPa)	188.0 ± 8.0	169.0 ± 8.0	−10
Modulus (MPa)	7997.0 ± 291.0	6299.0 ± 440.0	−21[a]
Mineral size			
Length (Å)	174.5 ± 2.0	177.8 ± 2.1	+2
Width (Å)	61.2 ± 0.4	66.2 ± 0.9	+8[a]

[a] Significantly different from the control group ($P < 0.05$ by a *t*-test)

Table 2. Relationships between mitogenic factors and bone turnover markers measured in serum

	TRAP	BAP
Serum F	r = 0.38, $P = 0.07$	r = 0.53, $P < 0.01$
IGF-1	r = 0.52, $P < 0.05$	NS
PTH	NS	NS

NS = not significant ($P > 0.1$)

Table 3. Relationships between fluoride levels and bone mass

	BV/TV	Tibial ash wt
Serum F	r = 0.54, $P < 0.01$	r = 0.41, $P < 0.05$
Vertebral F	r = 0.63, $P < 0.001$	r = 0.48, $P < 0.05$
Tibial F	r = 0.68, $P < 0.0005$	r = 0.57, $P < 0.005$

F = fluoride; BV/TV = trabecular bone volume in the L4 vertebra

bone mass resulting from fluoride treatment did not lead to increased bone strength. In fact, many biomechanical parameters, namely, vertebral and femoral fracture stress, and femoral elastic modulus, were *negatively* correlated with bone mass (Table 4). This seems counterintuitive, however, the bone samples with the highest bone mass also had the highest fluoride content, and the negative effect of increased fluoride content on bone strength far overwhelmed the positive effect of increased bone mass.

Fluoride treatment did not cause mineralization defects in femoral or vertebral bone as viewed by microradiography. The osteoid surface and osteoid thickness on the vertebral bone were increased by fluoride treatment (Table 1). However, these parameters were not significantly correlated

Table 4. Relationships among bone fluoride content, bone mass, and bone strength

	Vert F	Tibia F	BV/TV	Tibial ash wt.
Fem. neck force	$r = -0.75$ $P < 0.005$	$r = -0.72$ $P < 0.005$	$r = -0.51$ $P = 0.06$	NS
Vert. force	$r = -0.39$ $P = 0.07$	$r = -0.36$ $P = 0.09$	NS	NS
Vert. modulus	NS	NS	NS	NS
Vert. σ	$r = -0.44$ $P < 0.05$	$r = -0.43$ $P < 0.05$	$r = -0.42$ $P < 0.05$	$r = -0.36$ $P = 0.09$
Fem. force	NS	NS	NS	NS
Fem. modulus	$r = -0.61$ $P < 0.005$	$r = -0.60$ $P < 0.005$	$r = -0.54$ $P < 0.01$	$r = -0.65$ $P < 0.001$
Fem. σ	$r = -0.39$ $P = 0.07$	$r = -0.41$ $P < 0.05$	$r = -0.45$ $P < 0.05$	$r = -0.67$ $P < 0.0005$

with any bone strength measurement ($P > 0.1$). The bone crystal width in the tibia was significantly increased with fluoride treatment (Table 1). Bone crystal width was negatively correlated with fracture stress in the femur (Fig. 1), but did not correlate with any other bone strength parameter.

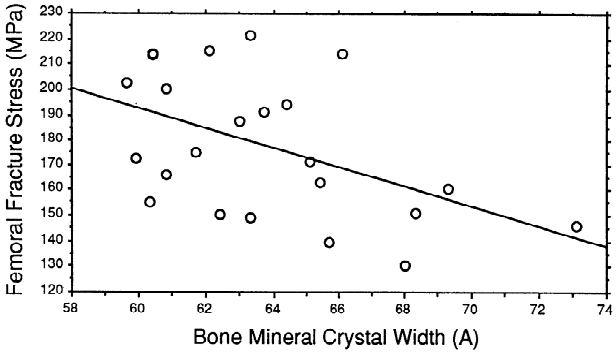

Fig. 1. Fracture stress of the femur was negatively correlated with mineral crystal width measured in the tibia ($r = 0.49$, $P < 0.02$). Fluoride treatment increased crystal width, which may have been the cause of the fluoride-induced reduction in bone strength.

Discussion

We found that fluoride treatment increased bone turnover and bone mass in rabbits. Fluoride also increased serum levels of IGF-1. However, serum fluoride, but not IGF-1, was closely associated with skeletal alkaline phosphatase, suggesting that increased bone mass was a direct result of serum fluoride levels rather than indirectly mediated through IGF-1. The increased bone mass did not lead to increased bone strength. In fact, bone strength was decreased by fluoride treatment and there was a negative association between the fluoride-induced new bone mass and bone strength. This suggests that the quality of the new bone was impaired by fluoride. We found that fluoride changed the average width of mineral crystals and there was a negative association between crystal width and fracture stress of the femur. However, fluoride treatment did not cause mineralization defects and the mineral structure, viewed by microradiography, appeared normal.

IGF-1 and serum glucose were increased substantially by fluoride treatment. It is possible that IGF-1 changes resulted from changes in serum glucose, but this cannot be proven since other regulators of serum glucose, e.g., insulin, were not measured. Furthermore, it is not clear why fluoride affected serum glucose in this study, although it has been suggested that fluoride might inhibit the activity of the insulin receptor [41]. Our previous studies in rats have shown no effects of high fluoride intakes on serum glucose [20, 34, 41]. IGF-1 levels were significantly correlated with a marker of bone resorption, TRAP, but were not correlated with any measure of bone formation. This suggests that the changes in IGF-1 contributed to the increased bone turnover observed in the fluoride-treated animals. IGF-1 was not associated with increased bone formation, but serum fluoride was positively correlated with a serum marker of bone formation, BAP. It appears, therefore, that fluoride directly increased bone formation with no indirect effects of IGF-1. Levels of PTH and vitamin D metabolites were not changed by fluoride treatment. Thus, fluoride did not cause secondary hyperparathyroidism, as is sometimes observed in patients treated with fluoride [9]. A possible explanation for this discrepancy is the fact that rabbit chow contains high levels (1.2%) of calcium, which are 4–5 times the average calcium intake of human (on a per calorie basis, assuming 800 mg/day for human Ca intake). Rabbit chow also is well fortified with Vitamin D_3 (0.7 IU/calorie). Calcium and vitamin D_3 supplementation has been shown to reverse the fluoride-induced hyperparathyroidism in patients [9]. The serum calcium level was reduced slightly, but significantly, by fluoride treatment but this change was not reflected in altered phosphate, PTH, or 1,25 $(OH)_2D$ levels, suggesting that the ionized fraction of calcium was not affected. Thus, fluoride treatment may have affected the protein-bound fraction of serum calcium.

There was a positive association between bone fluoride levels and bone mass, but a negative association between bone fluoride and bone strength. Fluoride impaired the new bone quality so much that no strength was added with the new bone mass. In fact, fracture stress, which is normalized for bone size, was negatively associated with bone mass, suggesting that the newly formed bone had considerably less strength. This finding is consistent with the findings of Lafage et al. [12]; they showed no correlation between vertebral failure load and bone volume in fluoride-treated minipigs. Furthermore, Lafage's group showed a significant, negative correlation between trabecular elastic modulus in the spine and bone fluoride content. We observed negative correlations between bone fluoride content and femoral

neck strength, vertebral fracture stress, femoral fracture stress, and femoral elastic modulus. The bone fluoride levels measured in the current study (6500–8000 ppm) were higher than those reported by Lafage et al., but in the same range as those reported by Sogaard et al. [43] for osteoporotic patients treated with fluoride for 5 years. Sogaard observed over a 50% decrease in the strength of bone biopsies taken before and after 5 years of therapy. These studies indicate that high doses of fluoride impair bone quality resulting in reduced tissue-level strength. Our data further indicate that bone quality defects may be manifested more in the femoral neck than in the spine or long bones.

Mineral crystals in the tibia became thicker after fluoride treatment and mineral thickness was negatively correlated with bone strength in the femur. Thus, the changes in mineral crystal size and packing caused by fluoride may impair bone strength. Fluoride treatment caused increases in osteoid surface and thickness in the vertebrae. Increased osteoid surface, as was expected, is a result of the fluoride-induced increase in bone formation rate. The increase in osteoid thickness suggests a slight mineralization defect in the bone, however, this increase was not large or statistically significant. Microradiography did not show any differences in bone mineralization between fluoride-treated and control animals. This is in contrast to histological observations in fluoride-treated humans. Light microscopy studies of bone biopsies by Lundy et al. [19] and Boivin et al. [17], and backscattered electron imaging studies by Grynpas et al. [18] showed focal and linear mineralization defects within the bone tissue from fluoride-treated subjects. The probable reason why mineralization defects were not observed in the present study is the relatively high calcium and vitamin D levels in the normal rabbit diet, which may have improved mineralization.

The fluoride treatment used in the present study did not perfectly mimic treatment regimens used for osteoporosis therapy. Although fluoride pharmacokinetics were not measured in the present study, they were probably different in rabbits drinking fluoridated water compared with patients taking two to three fluoride tablets per day. The average serum fluoride in the rabbits was 38 μM, which was equivalent to the peak fluoride level attained in humans after ingesting 20 mg F [44]; it is six times the serum levels achieved by slow-release fluoride tablets (11.3 mg F) [45]. Perhaps the negative effects of fluoride on bone quality could have been prevented if a lower dose of fluoride was used. Bone fluoride levels measured in the present study were similar to those achieved after 5–6 years of fluoride therapy (18–30 mg F/day) [43, 46], but greater than those from slow-release therapy of 23 mg F/day [47]. This distinction is important because the patients studied by Sogaard et al. [43] had greatly reduced bone strength in biopsy specimens, and many of the patients studied by Boivin et al. [46] had mineralization defects. Conversely, the slow-release treatment regimen used by Pak et al. [47] resulted in well-mineralized new bone and significantly reduced vertebral fracture rate.

In conclusion, fluoride appeared to directly stimulate bone formation in rabbits. IGF-1 levels were increased by fluoride treatment, but there was no evidence that IGF-1 stimulated bone formation. Fluoride significantly increased bone mass but decreased bone strength, so no beneficial effect of the greater bone mass was achieved. Decreased bone strength with fluoride treatment was not associated with abnormal mineralization, but mineral crystal width was significantly increased and this increase was negatively correlated with bone strength. Therefore, fluoride's tendency to increase bone crystal size may contribute to its negative effects on bone quality.

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References

1. Heaney RP, Baylink DJ, Johnston CC Jr, Melton LJ III, Meunier PJ, Murray TM, Nagant de Deuxchaisnes C (1989) Fluoride therapy for vertebral crush fracture syndrome. A status report. Ann Intern Med 111:678–680
2. Farley JR, Wergedal JE, Baylink DJ (1983) Fluoride directly stimulates proliferation and alkaline phosphatase activity of bone-forming cells. Science 227:330–332
3. Hall BK (1987) Sodium fluoride as an initiator of osteogenesis from embryonic mesenchyme in vitro. Bone 8:111–116
4. Khokher MA, Dandona P (1990) Fluoride stimulates (^{3}H)thymidine incorporation and alkaline phosphatase production by human osteoblasts. Metabolism 39:1118–1121
5. Kopp JB, Robey PG (1990) Sodium fluoride does not increase human bone cell proliferation or protein synthesis in vitro. Calcif Tissue Int 47:221–226
6. Chavassieux P, Chenu C, Valentin-Opran A, Delmas PD, Boivin G, Chapuy MC, Meunier PJ (1993) In vitro exposure to sodium fluoride does not modify activity or proliferation of human osteoblastic cells in primary cultures. J Bone Miner Res 8:37–44
7. Kassem M, Mosekilde L, Eriksen EF (1994) Effects of fluoride on human bone cells in vitro: differences in responsiveness between stromal osteoblast precursors and mature osteoblasts. Eur J Endocrinol 130:381–386
8. Lau KHW, Farley JR, Freeman TK, Baylink DJ (1989) A proposed mechanism of the mitogenic action of fluoride on bone cells: inhibition of the activity of an osteoblastic acid phosphatase. Metabolism 38:858–868
9. Dure-Smith BA, Farley SM, Linkhart SG, Farley JR, Baylink DJ (1996) Calcium deficiency in fluoride-treated osteoporotic patients despite calcium supplementation. J Clin Endocrinol Metab 81:269–275
10. Beary DF (1969) The effects of fluoride and low calcium on the physical properties of the rat femur. Anat Rec 164:305–316
11. Faccini JM (1969) Fluoride and bone. Calcif Tissue Res 3:1–16
12. Lafage MH, Balena R, Battle MA, Shea M, Seedor JG, Klein H, Hayes WC, Rodan GA (1995) Comparison of alendronate and sodium fluoride effects on cancellous and cortical bone in minipigs. A one-year study. J Clin Invest 95:2127–2133
13. Sogaard CH, Mosekilde L, Schwartz W, Leidig G, Minne HW, Ziegler R (1995) Effects of fluoride on rat vertebral body biomechanical competence and bone mass. Bone 16:163–169
14. Turner CH, Hasegawa K, Zhang W, Wilson M, Li Y, Dunipace AJ (1995) Fluoride reduces bone strength in older rats. J Dent Res 74:1475–1481
15. Wolinsky I, Simkin A, Guggenheim K (1972) Effects of fluoride on metabolism and mechanical properties of rat bone. Am J Physiol 223:46–50
16. Einhorn TA, Wakley GK, Linkhart S, Rush EB, Maloney S, Faierman E, Baylink DJ (1992) Incorporation of sodium fluoride into cortical bone does not impair the mechanical properties of the appendicular skeleton in rats. Calcif Tissue Int 51:127–131
17. Boivin G, Duriez J, Chapuy M-C, Flautre B, Hardouin P,

Meunier PJ (1993) Relationship between bone fluoride content and histological evidence of calcification defects in osteoporotic women treated long term with sodium fluoride. Osteoporosis Int 3:204–208
18. Grynpas MD, Holmyard DP, Pritzker KPH (1994) Bone mineralization and histomorphometry in biopsies of osteoporotic patients treated with fluoride. Cells Mat 4:287–297
19. Lundy MW, Stauffer M, Wergedal JE, Baylink DJ, Featherstone JD, Hodgson SF, Riggs BL (1995) Histomorphometric analysis of iliac crest bone biopsies in placebo-treated versus fluoride-treated subjects. Osteoporosis Int 5:115–129
20. Turner CH, Owan I, Brizendine EJ, Zhang W, Wilson ME, Dunipace AJ (1996) High fluoride intake causes osteomalacia and diminished bone strength in rats with renal deficiency. Bone 19:595–601
21. Wiers BH, Francis MD, Hovancik K, Ritchie CK, Baylink DJ (1990) Theoretical physical chemical studies of the cause of fluoride-induced osteomalacia. J Bone Miner Res 5:S63–S70
22. Riggs BL, Hodgson SF, O'Fallon WM, Chao EYS, Wahner HW, Muhs JM, Cedel SL, Melton LJ III (1990) Effect of fluoride treatment on the fracture rate in postmenopausal women with osteoporosis. N Engl J Med 322:802–809
23. Turner CH, Dunipace AJ (1993) On fluoride and bone strength (letter). Calcif Tissue Int 53:289–290
24. Fratzl P, Roschger P, Eschberger J, Abendroth, Klaushofer K (1994) Abnormal bone mineralization after fluoride treatment in osteoporosis: a small-angle X-ray-scattering study. J Bone Miner Res 9:1541–1549
25. Grynpas MD, Rey C (1992) The effect of fluoride treatment on bone mineral crystals in the rat. Bone 13:423–429
26. Walsh WR, Labrador DP, Kim HD, Guzelsu N (1994) The effect of in vitro fluoride ion treatment on the ultrasonic properties of cortical bone. Ann Biomed Eng 22:404–415
27. Catanese J, Keaveny TM (1996) Role of collagen and hydroxyapatite in the mechanical behavior of bone tissue. J Bone Miner Res 11:S295
28. Turner CH, Boivin G, Meunier PJ (1993) A mathematical model for fluoride uptake by bone. Calcif Tissue Int 52:130–138
29. Lundy MW, Farley JR, Baylink BJ (1986) Characterization of a rapidly responding animal model for fluoride-stimulated bone formation. Bone 7:289–293
30. Brearley LJ, Storey E (1970) Osteofluorosis in the rabbit: macroscopic and radiographic changes. Pathology 2:231–247
31. Malcolm AS, Storey E (1971) Osteofluorosis in the rabbit: microradiographic studies. Pathology 3:39–51
32. Hall LL, Kilpper RW, Smith FA, Morken DA, Hodge HC (1977) Kinetic model of fluoride metabolism in the rabbit. Environ Res 13:285–302
33. Taves DR (1968) Separation of fluoride by rapid diffusion using hexamethyldisiloxane. Talanta 15:969–974
34. Dunipace AJ, Brizendine EJ, Zhang W, Wilson ME, Miller LL, Katz BP, et al. (1995) Effect of aging on animal response to chronic fluoride exposure. J Dent Res 74:358–368
35. Lau KHW, Onishi T, Wergedal JE, Singer FR, Baylink DJ (1987) Characterization and assay of tartrate-resistant acid phosphatase activity in serum: potential use to assess bone resorption. Clin Chem 33:458–462
36. Crawford BA, Martin JL, Howe CJ, Handelsman DJ, Baxter RC (1992) Comparison of extraction methods for insulin-like growth factor-I in rat serum. J Endocrinol 134:169–176
37. Parfitt AM, Mathews CHE, Villanueva AR, Kleerekoper M, Frame B, Rao DS (1983) Relationships between surface, volume, and thickness of iliac trabecular bone in aging and in osteoporosis. J Clin Invest 72:1396–1409
38. Garetto LP, Chen J, Parr JA, Roberts WE (1995) Remodeling dynamics of bone supporting rigidly fixed titanium implants: a histomorphometric comparison in four species including humans. Implant Dent 4:235–243
39. Turner CH, Burr DB (1993) Basic biochemical measurements of bone: a tutorial. Bone 14:595–608
40. Klug H, Alexander L (1974) X-ray diffraction procedures for polycrystalline and amorphous materials. John Wiley & Sons, New York, NY, pp 687–692
41. Viñals F, Testar X, Palacín M, Zorzano A (1993) Inhibitory effect of fluoride on insulin receptor autophosphorylation and tyrosine kinase activity. Biochem J 291:615–622
42. Dunipace AJ, Wilson CA, Wilson ME, Zhang W, Kafrawy AH, Brizendine EJ, Miller LL, Katz BP, Warrick JM, Stookey GK (1996) Absence of detrimental effects of fluoride exposure in diabetic rats. Arch Oral Biol 41:191–203
43. Sogaard CH, Mosekilde LI, Richards A, Mosekilde LE (1994) Marked decrease in trabecular bone quality after five years of sodium fluoride therapy—assessed by biomechanical testing of iliac crest bone biopsies in osteoporotic patients. Bone 15: 393–399
44. Ekstrand J, Spak C-J (1990) Fluoride pharmacokinetics: its implications in the fluoride treatment of osteoporosis. J Bone Miner Res 5(suppl 1):S53–S61
45. Pak CYC, Sakhaee K, Piziak V, Peterson RD, Breslau NA, Boyd P, Poindexter JR, Herzog J, Heard-Sakhall A, Haynes S, Adams-Huet B, Reish JS (1994) Slow-release sodium fluoride in the management of postmenopausal osteoporosis. Ann Inter Med 120:625–632
46. Boivin G, Chapuy M-C, Baud CA, Meunier PJ (1988) Fluoride content in human iliac bone: results in controls, patients with fluorosis, and osteoporotic patients treated with fluoride. J Bone Miner Res 3:497–502
47. Pak CYC, Zerwekh JE, Antich PP, Bell NH, Singer FR (1996) Slow-release sodium fluoride in osteoporosis. J Bone Miner Res 11:561–564