Effects of Long-term Fluoride Administration on the Composition and Solubility of Rat Cortical Bone

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Abstract: In the present study, we aimed to investigate changes in the mineral composition and solubility of rat bone under various regimens of fluoride administration in drinking water. Sprague-Dawley rats (male, 4-week-old at the beginning of fluoride administration) were used. We adopted two animal-housing protocols: (1) the age-matched animals were given 0 ppm (control) or 50 ppm fluoride as NaF in deionized water for various periods ranging from 2 to 16 weeks, and (2) the animals were housed for 10 weeks under fluoride regimens of 0 (control), 10, 30, 50, 70 or 90 ppm. At the end of fluoride administration, the animals were sacrificed and then the diaphysial cortical bones of the femora and tibia were harvested. All bone samples were pulverized and deproteinized by low-temperature ashing prior to use in solubility measurements. The solubility of bone crystals was determined through a series of solid/solution equilibration at 25°C under 1.8% CO₂/N₂ gas environment. The results obtained showed that bone mineral composition was highly sensitive to the ingested fluoride, increasing the fluoridation degree of bone crystals up to a plateau around 1 wt%, i.e., one third of the theoretical content for fluorapatite. From the solubility data of bone samples collected according to both animal-housing protocols, it was proved that bone crystal solubility improved most substantially during the initial 6 weeks of fluoride administration and in the concentration range lower than 30-ppm fluoride in the drinking water. Further improvement of the solubility was only modest in magnitude (as indicated by changes in solubility product) even after fluoride ingestion over longer administration periods or at concentrations of 50–90 ppm fluoride.

抄録：本研究では、飲料水へのフッ素添加によるラット骨無機質の組成と溶解度に生じる変化を調べた。SD系4週齢の雄性ラットを使用し、2通りのフッ素投与のプロトコールを設定した。第1のプロトコールでは、飲料水へのフッ素添加濃度を0 ppm（対照群）と50 ppmとして、2週間から16週間の間投与を継続した。第2のプロトコールでは、投与期間を10週間として、フッ素濃度を0、10、30、50、70および90 ppmとした。投与期間を経た動物から大腿骨および頭蓋骨の骨幹部皮質骨を採取した。溶解度の測定に先立ち、すべての骨試料を微粉砕、低温灰化法で有機成分を除いた。骨組織の溶解度は25℃で1.8% CO₂/N₂分圧下で測定した。今回の実験結果から、骨組織はフッ素摂取量に応じて敏感に変化したが、骨組織のフッ素化の最高レベルは重量比で約1%（フルオロアパタイトの化学量論的なフッ素含有量の1/3前後）に止まることが予想した。本実験で採取した骨試料の溶解度の測定結果から、フッ素投与開始から6週間の間、あるいは30 ppm以下のフッ素濃度域で骨溶解度は最も顕著に低下した。ただし、より長期間のあるのは高いフッ素濃度を設定した場合でも、骨溶解度は低下傾向を示したが、溶解度変化量はわずかに停止した。
Introduction

Fluoride may not be essential, but it is ubiquitously involved in mineralization of hard tissues\(^1\,^2\). The use of topical and systemic fluoride for oral health has resulted in a major reduction in dental caries\(^3\). Although the benefits of water fluoridation for dental health are widely accepted, concerns remain about possible adverse effects, particularly effects on bone. Fluoride is an acknowledged bone anabolic agent through an increase in osteoblast activity, and it is believed that a minimum serum fluoride level of 100 ng/ml must be achieved before osteoblasts are stimulated\(^4,^5\). Serum levels associated with drinking water fluoridated to 1 ppm are usually several times lower than this value, but may reach this threshold at concentrations of 4 ppm in the drinking water\(^6,^7\). In accord with this prediction, the majority of animal studies have provided a substantial body of evidence that fluoride at up to 1 ppm does not have an adverse effect on bone strength, bone mineral density or fracture incidence\(^8,^9\). Nevertheless, a narrow therapeutic window and the adverse effects at higher therapeutic doses prevent broad clinical application of fluoride for treatment of diseases of bone loss, such as osteoporosis\(^10,^11\).

Fluoride is known to exert physicochemical effects on bone crystals at much lower levels than the concentrations required for provoking effects on bone cells\(^12,^13\). Regarding the fluoride effect on bone mineral, there is general consensus that fluoride increases the stability of the apatite lattice, reduces specific surface area, and decreases the solubility of the apatite crystals\(^14\). The degree and type of progressive changes of bone structure due to fluoridation in human and animal models are known to be influenced by various factors, such as age, level and timing/duration of fluoride ingested, and environmental factors such as temperature\(^15,^16\). At present, however, there is still a paucity of quantitative information showing how the fluoride dosage affects the crystal structure and properties of bone mineral. It is now becoming clear that biominerals including bone crystals are most adequately characterized as carbonatoapatites containing various impurities\(^17,^18\), but the solubility of bone mineral content under controlled partial pressures of CO\(_2\) remains to be determined.

In the present study, we aimed at filling the gap in our knowledge of the solubility properties of rat bone mineral obtained under various fluoride regimens. The solubility properties of rat bone mineral were determined under constant CO\(_2\)/N\(_2\) atmosphere. We also attempted to address an unsolved issue, whether the long-term administration of fluoride steadily alters bone mineral chemistry, reaching to the stoichiometry of fluorapatite or maximizing the thermodynamic stability of bone crystals.

Materials and Methods

1. Animals

Sprague-Dawley rats (male, 4 weeks old) were purchased and, after acclimation for one week in the animal facility of the Nippon Dental University, weight-matched animals without any signs of illness and abnormal behaviour were used in this study according to the following animal-housing protocols. In the first protocol, the animals were divided into two groups that were given 0 ppm (control) or 50 ppm fluoride (as NaF) in deionized drinking water for various periods ranging from 2 to 16 weeks. Animals in each group were sacrificed at timed intervals of 2, 4, 6, 8, 10, 12, 14 and 16 weeks after commencement of fluoride administration. In the second, the animals were housed for 10 weeks under fluoride regimens of 0 (control), 10, 30, 50, 70 or 90 ppm. The animal groups were named as follows: N and F stand for non-fluoridated (control) and fluoride-ingested groups, respectively. The animal groups were further identified in terms of fluoride dose used (in parentheses) and experimental period; thus, N-16 indicates the control animal groups sacrificed after 16 weeks housing, while F (50)-16 means the animals given drinking water containing 50 ppm fluoride for 16 weeks. We did not prevent access to natural fluoride-including laboratory diets (F content: less than 2.5 ppm) and other sources. The animals from each group were sacrificed by inhalation of CO\(_2\) gas at the end of the predetermined schedule. Long bones (femora and...
tibia from bilateral sides) were harvested and pooled for use in the designed analyses and solubility measurements. All animal care and treatment procedures were conducted in compliance with guidelines of the Institutional Animal Care and Use Committee.

2. Preparation of bone powder and plasma-ashing for removal of the organic matter in bone

The individual bone samples were lyophilized and then stored at −20°C until used. The diaphysis of the long bones was dissected using a hard-tissue cutting machine (Exact) under cooling by running water. The periosteum and bone marrow tissues were removed. Prior to analyses of the mineral phase, the pooled solid was pulverized by hand grinding with an agate mortar and pestle. Since it was of concern that grinding hard mineral substances may give rise to heating effects, by which acid phosphate in bone mineral could be converted into pyrophosphate, the bone pieces were frozen in liquid N2 prior to grinding. The resulting powder was passed through a 270-mesh sieve. Prior to determination of chemical composition and equilibration experiments, all powdered samples were plasma-ashed with oxygen activated by a radio frequency at approx. 60°C. In practice, each of the bone powders was placed inside a Plasmod reaction chamber (Tegal Co.). The oxidation of the organic matter was conducted at a power level of 25 kW. Weight losses of bone samples were monitored periodically and the treatment was continued until a constant weight was obtained, which usually occurred within 48 h.

3. Equilibrations of bone powder in dilute H3PO4 solutions under controlled Pco2

The experimental set-up for studies on the solubility of the plasma-ashed bone sample was the same as that used in the previous studies on the solubility of enamel and dentin samples19,20. A weighed amount of each of the bone powder specimens (60 mg) was equilibrated with a dilute phosphoric acid solution (100 ml in volume) at 25 ± 0.1°C. Three acid solutions with concentrations of 0.06, 0.1, and 0.6 mM were prepared using reagent grade H3PO4 in 0.1 M KNO3 solutions. The presence of background electrolyte facilitates the potentiometric measurements, i.e., determinations of pH values and Ca ionic activities. In all equilibrations, an ultrapure N2–CO2 gas mixture, which corresponded to 1.8% partial pressure of CO2 (hereafter Pco2) were used. The gas mixture used was presaturated with water vapor from a 100 mM KNO3 solution containing 0.5% (v/v) chloroform to minimize bacterial growth. Equilibrium conditions were assumed when the solution composition did not change significantly for a period of 3–4 days. It usually took from 14 to 21 days, so that each solid/solution equilibration was continued for a maximum of 26 days. The solubility of bone crystals was evaluated by averaging the values of the ionic activity product, IP = (Ca2+)3(PO43−)3(OH−), which was calculated from the individual saturated solutions. A concern commonly raised in the solubility measurement of biomineral is whether the original mineral could show a congruent equilibration behaviour or repeat dissolution/precipitation during the solution/solid equilibration19. In order to critically test such equilibration processes, the initial series equilibration using two batches of bone samples, namely N-16 and F (50)-16, were conducted in the absence and presence of 0.9 mM Mg ion in the solution. The rationale for using the concentration of Mg ion is that the ion is a potent inhibitor of apatite precipitation and the concentration used is close to that found in serum12. Our previous studies also indicated that the corresponding Mg exerted sufficient inhibition of precipitation reaction during the equilibration of dentin mineral under 1.8% Pco220. As described below, the addition of Mg ion to the equilibration solution was verified to prevent or retard kinetically recrystallization of the original bone mineral during the equilibration, so that the rest of solid/solution equilibrations were executed in the presence of 0.9 mM Mg ion.

4. Chemical analyses and x-ray diffraction

Calcium and magnesium were measured using atomic absorption; sodium was determined with the same instrument in the emission mode. Total phosphorus was analyzed colorimetrically. The carbonate content was determined by Conway’s microdiffusion method21 with CaCO3 as a standard. The HPO4 content was measured according to the pyrolysis method.
Fig. 1  X-ray diffraction patterns of rat cortical bone samples which were pulverized, plasma-ashed and washed with deionized water, and finally lyophilized prior to use for the solubility measurement. (A) Control group: bone samples were collected at timed intervals according to the time schedule. Numbers 2 through 16 indicate animal housing periods in weeks. N-0 corresponds to bone sample harvested from animals of 5-week-old at the beginning of animal-housing. (B) The animals ingested the drinking water containing 50 ppm fluoride for 2 through 16 weeks. (C) The animals were kept for 10 weeks under various fluoride regimens. Fluoride concentrations in the drinking water are shown in parentheses. Note the sharpening of apatite reflections in the 2θ range of 31 and 35 degrees for fluoride-ingested animals.

reported in detail elsewhere. The estimated uncertainties of these chemical determinations were: Ca, 3%; total P, 1.5%; Mg, 3%; Na, 4%; CO3, 8%; and HPO4, 6%. For the determination of the calcium ionic activity in solution, a specific calcium-ion electrode (Orion) and a reference electrode were used. The Ca²⁺ activity in each of the experimental solutions was also calculated using a generalized program that takes into account the formation of calcium ion pairs with phosphate and bicarbonate ions. The pH value of the experimental solutions was determined using a glass-reference combination electrode.

X-ray diffraction pattern of the bone powder was recorded using a D/Max x-ray powder diffractometer (Rigaku) equipped with a rotating anode and a graphite monochromator. In all cases, a non-reflection sample holder made out of quartz was used to minimize the sample size (about 20 mg) and the diffuse background scattering.

Results

There were no differences among animal groups in initial body weight, which averaged 63.8 ± 0.2 g. Body weights of the animals after housing for 16 weeks were about 400–420 g in both control and 50 ppm fluoride-given groups. No animals showed signs of intoxication or fracture of long bones during the entire experimental period. Exostoses or other gross anatomical alterations were not seen in any bones collected after 16 week administration periods at 50 ppm. The animals which ingested the highest fluoride concentration (90 ppm) also exhibited no marked changes in body weight or bone morphology, but their cortical bone was white and opaque visually, and hard and brittle by hand grinding in the agate mortar.

Figure 1 shows x-ray diffraction patterns of the plasma-ashed bone samples used in the solubility measurements. All reflections were broadened, due to the low crystallinity (i.e., small particle sizes and
Fig. 2 Results of chemical analyses of bone mineral. The date are presented in terms of molar ratios Ca/P, Mg/Ca, Na/Ca, CO₃/Ca and F/Ca, except for the acid phosphate content in terms of % of the total P. Solid and open circles represent control (non-F) and experimental (50 ppm F) groups, respectively. Note the difference in unit for F/Ca ratio between control and fluoride-ingested groups.

Figure 2 shows the results of chemical analyses of bone powders collected according to the first protocol. Fluoride intake through drinking water induced an increasing trend in the fluoride content of the cortical bone and, moreover, the fluoride content increased steadily during the first 6—8 weeks of fluoride administration, followed by a plateau around 1% wt of the bone mineral. Bone mineral of the control group also showed a marginal increment in the F/Ca ratio as a function of age although the external source of fluoride remained limited. At the end of the longest housing period (16 weeks), the fluoride content of bone in the fluoride-ingested group was approximately 50 times higher than that in the control animals. Other remarkable features of the fluoridated bone mineral were the Ca-deficiencies, i.e. lower than the theoretical value of hydroxyapatite or fluorapatite (1.67), and the enrichment of magnesium and sodium in comparison with the control group. The carbonate decreased inversely with increasing fluoride content of bone mineral, but appreciable amounts of carbonate still remained stable. With respect to the acid phosphate, there were no marked differences between the control and fluoride-ingested bone samples over the experimental periods.

Figure 3 illustrates representative time courses of equilibration condition with respect to the control and fluoridated bone samples in the form of a potential plot in terms of log \([\text{Ca}^{2+} \cdot \text{OH}^{-2}] \) vs log \([\text{H}^{+}]^{3} \cdot \text{PO}_{4}^{3-}\)]. The two bone samples showed marked
Fig. 3 Potential plots of the solution composition during equilibration with bone solid in terms of log \([\text{Ca}^{2+}(\text{OH}^-)^2]\) versus log \([\text{H}^+]^3(\text{PO}_4^{3-})]\). Triangles and circles represent bone samples of control and experimental (50 ppm F in water) groups, respectively. Equilibration was conducted either in the absence or presence of 0.9 mM Mg in media. Solid and open symbols represent the solubility data obtained with and without the addition of Mg ion, respectively. Note the minute changes in the solution condition at the last period of equilibration in each system.

Fig. 4 X-ray diffraction patterns of rat bone samples recovered at the end of equilibration. Note the marked improvement of apatite crystallinity of F(50)-10 sample after equilibration in the absence of Mg ions, supporting the inhibitory effect of Mg ion in solution on recrystallization of bone apatite during equilibration.

The above-mentioned dissolution/precipitation processes taking place during equilibration was confirmed by a comparison of x-ray diffraction patterns of solid samples before and after equilibration (Fig. 4). The solids equilibrated in the absence of Mg ion accompanied improvement of their crystallinity without any evidence of precipitation of non-apatitic salts. As one can anticipate due to the accelerating effect of fluoride on apatite precipitation\(^{18}\), such improvement of apatite crystallinity during equilibration was much more prominent for the fluoridated F(50)-16 sample in comparison with the control N(0)-16.

Figure 5 shows plots of the solubility data in the form of mean activity product, \((\text{IP})^{1/3}\), which were obtained for control and 50 ppm fluoride-ingested animals. The solubility of the control group remained essentially constant throughout the experimental period up to 16 weeks, regardless of the chronological age of the animals. In sharp contrast, the solubility of
Fig. 5 Changes in the solubility of bone mineral as a function of the experimental periods. Open circle, bone samples collected for the animals given the drinking water containing 50 ppm F; solid circles, bone samples of control (non-fluoride administrated). All equilibrations were conducted in the presence of 0.9 mM Mg ion in the initial solution. The solubility of bone mineral was expressed in terms of mean ionic activity product (IP)$^{1/t}$, where $IP = (Ca^{2+})^{5}(PO_4^{3-})^3(OH^-)$ at equilibrium and $t$ (a sum of stoichiometric coefficients) = 5 + 3 + 1. The results are presented as the average (SD) of three independent equilibrations using the different phosphoric acid solutions. Note the rapid decrease in the IP values of bone apatite at the early stage of fluoride ingestion. See text for the details of solid-solution equilibration.

Fig. 6 Changes in the F/Ca molar ratios (open hexagons) and solubility (solid circles) of bone samples as a function of fluoride concentration in the drinking water. Error bars are shown for the values of (IP)$^{1/9}$ obtained as explained in Fig. 3, but not for F/Ca ratios because of the very close values obtained.

the fluoride-ingested bone mineral decreased substantially in the initial 6 weeks, followed by a plateau over the all of the experimental periods. This decrease in bone solubility was generally consistent with the improvement of bone apatite crystallinity as shown in Fig. 1 B. It is notable that the difference in the solubility between the control and fluoridated bone mineral was in the third order in magnitude in terms of (Ca$^{2+}$)$^5$ (PO$_4^{3-}$)$^3$(OH$^-$), i.e., $2 \times 10^{-53}$ for N-16 and $1 \times 10^{-56}$ for F(50)-16. It is pertinent to add that the values of $2 \times 10^{-53}$ and $1 \times 10^{-56}$ correspond to 1.39 and 0.60, respectively, in terms of mean ionic activity product (IP)$^{1/t}$, where $IP = (Ca^{2+})^{5}(PO_4^{3-})^3(OH^-)$ at equilibrium and $t$ (a sum of stoichiometric coefficients) = 5 + 3 + 1.

Based on the solubility behaviour of bone apatite mentioned above, we harvested bone samples at 10 weeks in the second experimental protocol in order to compare the effects of fluoride ingestion at various concentrations on bone solubility around the supposed plateau levels. Figure 6 plots the solubility data of these bone samples. The changes in bone solubility appeared to occur in a two-steps manner as a function of the fluoride concentration given in drinking water, i.e., the first steady decrease in the range of 0 through 30 ppm and the following marginal decrease up to 90 ppm. For comparison, the values of F/Ca molar ratio for the bone samples are also plotted. Comparison of the two sets of plots suggested that the enrichment of fluoride into bone mineral progressed consistently with the level of fluoride concentration in the drinking water, whereas the solubility of bone crystals did not exactly follow the fluoride intake.

**Discussion**

It is well known that fluoride participates in many aspects of calcium phosphate formation *in vivo* and has enormous effects on this process and on the nature and properties of the formed biomineral. The most well-documented effect of fluoride is that this ion substitutes for a column hydroxyl in the apatite structure, giving rise to a reduction of crystal volume and a concomitant increase in structural stability. The present results of chemical and solubility measure-
ments prove that bone mineral composition of growing animals is highly sensitive to the ingested amount of fluoride from an external source. An important conclusion derived from the present results is that fluoridation of bone apatite is not necessary to remedy the state of Ca-deficiency and substitution of impurities in bone apatite. Concerning the content and state of Mg and Na in bone samples, it may be considered that some Mg and Na ions are incorporated into the lattice positions, whereas substantial portions of the Mg and Na content are located on crystal surfaces or in matrices, as reported for dentin mineral. It is of note that increasing fluoridation of bone apatite is still accompanied by incorporation of impurities available in the media, rather than exclusion of impurities, because fluoride can accelerate the kinetics of crystallization even in the presence of various impurities in media. An unexpected finding was that the HPO₄ remained at high levels, regardless of the fluoride content. Acid phosphate has generally been recognized as a marker of immature mineral phase including acidic precursors of bioapatite formation. Crystal growth studies in vitro also verified that the presence of fluoride at concentrations higher than 0.4 ppm promote de novo precipitation of apatite without OCP. Taken together, the enrichment of acid phosphate in both control and fluoride-ingested bone samples may reflect accumulation of the ions on the surface of small bone crystals, rather than occupying the lattice position, because HPO₄ is the dominant phosphate species in neutral conditions as expected for the bone mineralizing milieu.

In light of the plasticity or accommodation of bone composition to water-borne fluoride, the finding of interest was that the steady uptake of fluoride into the cortical bone of the growing rat continued for the first 4 through 6 weeks after commencement of fluoride administration. This progressive fluoridation of bone crystals supports the theory that the metabolically active pool of bone mineral, originally carbonatoapatite substituting less than 0.01%wt of fluoride, is readily replaced by a new solid-solution of fluoride-enriched carbonatoapatite. From a theoretical point of view, the maximum fluoride content of apatite structure corresponds to 3.3%wt in terms of fluoroapatite Ca₅(PO₄)₃F. The present analytical results regarding the fluoride content showed that the bone mineral formed under high-fluoride regimes approaches a plateau around 1%wt, one third of the theoretical content for fluoroapatite. In relation to the limited fluoridation even after the long-term ingestion of 50—90 ppm fluoride in the drinking water, the exact mechanism remains elusive but several possible explanations are available. One plausible explanation is that substantial fractions of bone crystals, perhaps two-thirds of the total mass, remain less accessible to the ingested fluoride. Another possibility is that the observed F/Ca ratios might reflect the composition of medium surrounding bone crystals, because previous in vitro studies proved that, if sufficient amounts of fluoride ions are available during precipitation of carbonatoapatite, the resulting composition of fluoridated apatite, at least with respect to Ca and fluoride, reflects the ratios of the corresponding ions (more strictly speaking, the ratios of free ion concentrations or activities) in solution. If this is the case, the plateau level of fluoridation of bone apatite obtained leads to an estimated value of F/Ca around 0.3/5 in the mineralization milieu under high-fluoride regimes. At present, no solid information is available about the concentration of Ca and fluoride ions at sites of bone mineralization, but it is an interesting simulation that a free concentration of Ca ion at bone mineralization site might be around 0.5 mM as supposed on the basis of Ca concentration in serum, so that fluoride concentration at the mineralizing sites could be estimated around 3×10⁻⁵ mol/l under the fluoride regimes according to the analytically determined F/Ca ratios of bone solid. Previous studies on seeded crystal growth demonstrated that this level of free fluoride ion is sufficient to promote the kinetics of apatite precipitation.

The present solubility work provides evidence that the equilibrium composition is achievable in a reproducible manner under the constant Pco₂, but the equilibration of bone mineral likely includes two processes, i.e., dissolution of the original bone mineral and precipitation of a new solid-solution phase(s). X-ray diffraction patterns of the fluoridated bone powder disclosed a marked improvement in the crystallinity
after equilibration. At present, we do not have data regarding the stoichiometry of a new phase(s) distinct from the original bone mineral, but it is reasonable to assume that a new phase accumulated more fluoride available after dissolution of the bone mineral, thereby decreasing the solubility properties and improving crystallinity. Similar recrystallization in the presence of excess of fluoride occurs continuously in bone tissue as indicated by the progressive promotion of fluoridation of bone apatite. Interestingly, magnesium, which is one of the major ionic constituents in serum and mineralization milieu, is likely involved as a regulator in bone recrystallization during normal metabolism and under fluoride regimen.

In conclusion, the overall findings indicate that fluoridation evokes substantial differences in the composition and solubility properties of bone mineral in growing rats. Remarkable features of the stoichiometry of the fluoridated bone mineral include Ca deficiencies and the enrichment of magnesium, carbonate and acid phosphate in the crystal lattice. The current approach to interpreting the solubility data of fluoridated bone apatite still contains uncertainties regarding the stoichiometry and solubility in association with involvement of the second precipitating crystalline phase. Further work is required to refine the stoichiometry model of bone mineral and to define the specific gas and solution environments under which congruent dissolution of bone mineral is realized.

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References


