Fluoride Modulates Parathyroid Hormone Secretion in vivo and in vitro

Chaitanya P. Puranik^a Kathleen A. Ryan^b Zhaoyu Yin^c
E. Angeles Martinez-Mier^e John S. Preisser^c Eric T. Everett^d

^aOral Biology PhD Curriculum, ^bOral and Craniofacial Health Sciences, and ^cDepartment of Biostatistics, Gillings School of Global Public Health, and ^dDepartment of Pediatric Dentistry, Carolina Center for Genome Sciences, University of North Carolina at Chapel Hill, Chapel Hill, N.C., and ^eDepartment of Preventive and Community Dentistry, Indiana University School of Dentistry, Indianapolis, Ind., USA

Key Words

Fluoride · Skeletal fluorosis · Parathyroid hormone · Bone · Inbred mouse strains

Abstract

The study objective was to investigate the effects of fluoride on intact parathyroid hormone (iPTH) secretion. Thyro-parathyroid complexes (TPC) from C3H (n = 18) and B6 (n = 18) mice were cultured in Ca²⁺-optimized medium. TPC were treated with 0, 250, or 500 µM NaF for 24 h and secreted iPTH assayed by ELISA. C3H (n = 78) and B6 (n = 78) mice were gavaged once with distilled or fluoride (0.001 mg [F⁻]/g of body weight) water. At serial time points (0.5–96 h) serum iPTH, fluoride, total calcium, phosphorus, and magnesium levels were determined. Expression of genes involved in mineral regulation via the bone-parathyroid-kidney (BPK) axis, such as parathyroid hormone (*Pth*), calcium-sensing receptor (*Casr*), vitamin D receptor (*Vdr*), parathyroid hormone-like hormone (*Pthlh*), fibroblast growth factor 23 (*Fgf23*), α-Klotho (*aKlotho*), fibroblast growth factor receptor 1c (*Fgf1rc*), tumor necrosis factor 11 (*Tnfs11*), parathyroid hormone receptor 1 (*Pth1r*), solute carrier family 34 member 1 (*Slc34a1*), solute carrier 9 member 3 regulator 1 (*Slc9a3r1*), chloride channel 5 (*Clcn5*), and PDZ domain-containing 1

(*Pdzk1*), was determined in TPC, humeri, and kidneys at 24 h. An in vitro decrease in iPTH was seen in C3H and B6 TPC at 500 µM (p < 0.001). In vivo levels of serum fluoride peaked at 0.5 h in both C3H (p = 0.002) and B6 (p = 0.01). In C3H, iPTH decreased at 24 h (p < 0.0001), returning to baseline at 48 h. In B6, iPTH increased at 12 h (p < 0.001), returning to baseline at 24 h. Serum total calcium, phosphorus, and magnesium levels did not change significantly. *Pth*, *Casr*, *aKlotho*, *Fgf1rc*, *Vdr*, and *Pthlh* were significantly upregulated in C3H TPC compared to B6. In conclusion, the effects of fluoride on TPC in vitro were equivalent between the 2 mouse strains. However, fluoride demonstrated an early strain-dependent effect on iPTH secretion in vivo. Both strains demonstrated differences in the expression of genes involved in the BPK axis, suggesting a possible role in the physiologic handling of fluoride.

© 2015 S. Karger AG, Basel

Introduction

Fluoride has recognized actions on bone homeostasis [Everett, 2011]. Yan et al. [2007] investigated in 2 inbred strains of mice, C3H/HeJ (C3H) and C57BL/6J (B6), fluoride actions on bone homeostasis. In C3H mice, short-

Abbreviations used in this paper

<i>Actb</i>	β-actin
B6	C57BL/6J strain
BPK	bone-parathyroid-kidney
C3H	C3H/HeJ strain
<i>Casr</i>	calcium-sensing receptor
<i>Clcn5</i>	chloride channel 5
<i>Fgf1rc</i>	fibroblast growth factor receptor 1c
<i>Fgf23</i>	fibroblast growth factor 23
<i>Gapdh</i>	glyceraldehyde 3-phosphate dehydrogenase
iPTH	intact parathyroid hormone
<i>Pdzk1</i>	PDZ domain-containing 1
<i>Pth</i>	parathyroid hormone
<i>Pth1r</i>	parathyroid hormone receptor 1
<i>Pthlh</i>	parathyroid hormone-like hormone
<i>Slc34a1</i>	solute carrier family 34 member 1
<i>Slc9a3r1</i>	solute carrier 9 member 3 regulator 1
sRANKL	soluble receptor activator of nuclear factor-κB ligand
<i>Tnfs11</i>	tumor necrosis factor 11
TPC	thryo-parathyroid complex
<i>Tpo</i>	thyroperoxidase
TRAP5b	tartrate-resistant acid phosphatase 5b
<i>Vdr</i>	vitamin D receptor
<i>αKlotho</i>	α-Klotho

term systemic exposure to fluoride resulted in increased osteoclastogenesis as evidenced by increases in the following serum osteoclast biomarkers: intact parathyroid hormone (iPTH), soluble receptor activator of nuclear factor-κB ligand (sRANKL), and tartrate-resistant acid phosphatase 5b (TRAP5b), along with a decrease in serum osteoprotegerin levels [Yan et al., 2007]. Osteoclast numbers along bone surfaces and the osteoclast potential of bone marrow cells were increased in C3H mice as well. However, similar fluoride exposure in B6 mice favored anabolic responses, with increases in serum alkaline phosphatase activity and proximal tibia trabecular and vertebral bone mineral density.

PTH is responsible for calcium homeostasis and is released in response to hypocalcemia to normalize serum calcium levels [Potts, 2005]. PTH is an important component of the bone-parathyroid-kidney (BPK) feedback loop responsible for mineral homeostasis [Bergwitz and Juppner, 2010; Torres and De Brauwere, 2011]. Skeletal fluorosis results from systemic exposure to fluoride leading to calcification of ligaments, bone deformities, fractures, functional limitations, a disturbed mineral balance, and occasional pseudo-hyperparathyroidism [Teotia and Teotia, 1973; Teotia et al., 1998]. The mechanisms underlying altered PTH levels due to fluoride exposure are not understood. We hypothesized that PTH secretion is in-

fluenced by direct effects of fluoride on the parathyroid gland. Our in vitro and in vivo experiments were directed to investigating early events following fluoride exposure on PTH secretion.

Materials and Methods

Animals

Ninety-six 5- to 6-week-old male mice, C3H/HeJ (C3H) and C57BL/6J (B6) (The Jackson Laboratory, Bar Harbor, Maine, USA), were placed on a constant-nutrition no-fluoride diet [No. 5861; Test Diet®, Richmond, Ind., USA; fluoride: 0 ppm, calcium: 0.60%, phosphorus: 0.57%, vitamin D₃: 2.2 IU/g, and energy: 4.09 (kcal/g)²] and were provided with distilled drinking water ad libitum for 1 week for acclimatization. The mice were housed in the Division of Lab Animal Medicine facility at The University of North Carolina at Chapel Hill, an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International)-accredited unit. Mice were placed in 12:12-hour light-dark cycles at a 21°C ambient temperature. All procedures involving vertebrate animals were approved by the Institutional Animal Care and Use Committee of The University of North Carolina at Chapel Hill.

Parathyroid Gland Dispersed Cell Culture

Mice were euthanized using CO₂, and ventral neck dissections were performed to isolate and harvest thryo-parathyroid complexes (TPC) after microdissections. TPC were finely minced and transferred to 2-ml centrifuge tubes with digestion buffer containing 1 mg/ml collagenase (Stem Cell Technologies, Vancouver, B.C., Canada) in dispase solution (Stem Cell Technologies). TPC were incubated at 37°C for 3 h with intermittent pipetting for homogeneous dispersion of cells. Dispersed cells were plated onto 6-well plates and incubated at 37°C at 5% CO₂ in 2 ml prewarmed α-MEM (Sigma-Aldrich, St. Louis, Mo., USA) media with 10% FBS (Clontech Laboratories, Mountain View, Calif., USA) and 1% antibiotic-antimycotic (Sigma-Aldrich). Total calcium, magnesium, and phosphorus levels in media were adjusted to that of the serum in each strain of mice as follows: C3H (calcium: 10.6 mg/dl, phosphorus: 8.7 mg/dl, and magnesium: 2.1 mg/dl) and B6 (calcium: 10.4 mg/dl, phosphorus: 10.3 mg/dl, and magnesium: 2.9 mg/dl). TPC from C3H and B6 mice were prepared on 2 separate occasions using a total of 12 mice per strain. An initial study used 2 F concentrations (0 and 500 μM). This was repeated using additional mice, and 3 F concentrations were examined (0, 250, and 500 μM). Media supernatants were collected at 24 h, centrifuged, and stored at -80°C until analysis.

Gavage and Sample Collections

Following acclimatization, 72 mice from both strains were randomly divided in 12 groups of 6 mice (0.5, 1, 3, 6, 12, or 24 h) based on the time of sacrifice after the gavage dose, with control and test groups based on fluoride levels (0 or 100 ppm) in gavage. Additionally, 6 mice from both strains were assigned to the no-gavage-at-baseline group. At the time of gavage, the mice were weighed and the gavage dose was calculated for each mouse (0.001 mg [F⁻]/g of body weight). Mice were anesthetized with ketamine-HCl (90 mg/kg) and xylazine (14 mg/kg) intraperitoneally. Sera were

collected from anesthetized mice and stored at -80°C until use. TPC, kidneys, and humeri were harvested, snap-frozen in liquid nitrogen, and stored at -80°C .

ELISA and Biochemistry Assays

Media supernatants and sera were analyzed for iPTH using 1-84 PTH ELISA Kits (Immutopics, San Clemente, Calif., USA). The intra- and interassay precision (coefficient of variation) for the iPTH ELISA was 3.2 and 8.4, respectively. Each ELISA plate had 6 standard and 2 control solutions for the determination of R^2 values. The threshold R^2 value was set at 0.95. All ELISA plates demonstrated R^2 values >0.98 . Each ELISA plate was read with 6 samples each from the control and test group in triplicates.

Serum levels of fluoride were determined using the direct fluoride microanalysis method [Vogel et al., 1990]. In this method, micropipettes pulled from glass capillary tubes are combined with a custom-made micropipette holder and used to dispense nanoliter-sized standards and serum samples (diluted 9:1 with TISAB III). Serum samples are extracted over a light table using the micropipette/micropipette holder and dispensed onto the surface of an inverted fluoride electrode (F-ISE) under a layer of mineral oil to prevent loss of fluoride. Using a micromanipulator and a microscope for viewing, the reference electrode is touched to the standard or sample drop, resulting in an electrometer millivolt reading. Both the F-ISE and the hand-pulled reference electrode are connected to an electrometer and a computer with plot program monitoring/recording software. Samples are run in triplicate, with periodic electrode conditioning and standard checks.

Calcium, phosphorus, and magnesium levels were determined by the Animal Clinical Laboratory, University of North Carolina at Chapel Hill, and the Clinical Pathology Laboratory, College of Veterinary Medicine at North Carolina State University.

RNA Extractions and cDNA Preparation

RNA was extracted from TPC, kidney, and humerus samples using an RNeasy Midi Kit (Qiagen Inc., Valencia, Calif., USA). Quantitative analysis of the RNA sample was performed using an Agilent Bioanalyzer with Nano LabChip at the Genomic Core Facility, University of North Carolina at Chapel Hill. All RNA samples had RIN values in the range of 7.5–10. A High-Capacity Reverse Transcription Kit (Applied Biosciences, Carlsbad, Calif., USA) was used to prepare cDNA. After preparation, the cDNA was stored at -80°C .

Quantitative Polymerase Chain Reaction

cDNA samples free of genomic DNA contamination were subjected to quantitative polymerase chain reaction for a suite of genes including parathyroid hormone (*Pth*), calcium-sensing receptor (*Casr*), α -Klotho (α Klotho), fibroblast growth factor receptor 1c (*Fgf1rc*), vitamin D receptor (*Vdr*), parathyroid hormone-like hormone (*Pthlh*), tumor necrosis factor 11 (*Tnfs11*), fibroblast growth factor 23 (*Fgf23*), parathyroid hormone receptor 1 (*Pth1r*), chloride channel 5 (*Clcn5*), solute carrier 9 member 3 regulator 1 (*Slc9a3r1*), PDZ domain-containing 1 (*Pdzk1*), and solute carrier family 34 member 1 (*Slc34a1*). Expression of these genes was normalized using 2 housekeeping genes comprising β -actin (*Actb*) and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). Thyroperoxidase (*Tpo*) was used as a surrogate marker for the quality of the TPC dispersed cell culture. All of the primers were from SA Biosciences (Qiagen). Data were analyzed using the $2^{-\Delta\Delta C_t}$ method [Livak and Schmittgen, 2001; Yuan et al., 2006].

Fig. 1. TPC histology and iPTH levels in supernatant media. **a** Histological section of TPC; the arrow indicates parathyroid tissue. **b** iPTH $\pm$ SE in C3H TPC at 3 calcium concentrations (1.1, 10.5, and 25 mg/dl; $n = 3$ in each group). ** $p < 0.001$.

Statistical Analysis

Data are reported as means $\pm$ SE. One-way ANOVA was used to compare the effect of calcium concentration on iPTH secretion in the TPC dispersed cell culture model. For in vitro experiments, 6 groups (each consisting of 6 mice) were compared using a 2-way ANOVA based on strain (C3H or B6) and the level of fluoride exposure (0, 250, or 500 μM). For in vivo experiments, 24 groups were compared using a 3-way ANOVA based on strain (C3H or B6), the level of fluoride exposure (0 or 100 ppm), and time points (0.5, 1, 3, 6, 12, and 24 h). A preliminary 2-way ANOVA was first used to confirm the absence of a time effect on water gavage when baseline

gavage (0 h) was included for both strains of mice (total $n = 84$), which would justify the omission of baseline gavage data at 0 h in order to maintain balance in the 3-way ANOVA. Three-way ANOVA were also evaluated for serum total calcium, phosphorus, and magnesium levels for each strain followed by treatment with 0 or 100 μM fluoride concentrations at 0.5, 1, 3, 6, 12, and 24 h. A natural log transformation was used for iPTH and serum fluoride (but not for calcium, magnesium, or phosphorus) to address skewness. ANOVA main effect and interaction p values ≤ 0.05 were considered statistically significant, followed by Bonferroni adjustments for post hoc comparisons of levels of factors. SAS version 9.3 (SAS Inc., Cary, N.C., USA) was used for statistical analysis.

Results

Responsiveness of the TPC Dispersed Cell Culture Model

The TPC (fig. 1a) dispersed cell culture model was utilized in order to determine the direct effects of fluoride on the parathyroid gland. All TPC dispersed cell cultures demonstrated *Tpo* gene expression (a surrogate marker for TPC dispersed cell culture quality) with a Ct value of 24.8 ± 2.1 . The responsiveness of the dispersed cell culture model to changes in extracellular calcium, a known PTH mediator, was used to validate the culture model. TPC cultured in varying calcium concentrations (hypocalcemic: 1.1 mg/dl, normocalcemic: 10.5 mg/dl, or hypercalcemic: 25 mg/dl) demonstrated changes in iPTH secretion into the media ($p < 0.001$). Based on a Bonferroni-corrected significance level of $0.05/3 = 0.017$ and after 24 h of incubation in the hypocalcemic condition, iPTH secretion (129.4 ± 27.1 pg/ml) was significantly increased ($p = 0.007$) compared to the normocalcemic condition (49.2 ± 6.2 pg/ml) (fig. 1b). The hypercalcemic condition led to a further statistically significant decline in iPTH relative to the normocalcemic condition ($p = 0.012$). Though not shown, TPC in culture remained responsive to changes in calcium for 3 days.

Effect of Fluoride on TPC

The fluoride exposure experiments using TPC from B6 and C3H mice were performed at 2 different times. In an initial experiment, TPC from B6 ($n = 6$) and C3H ($n = 6$) mice were cultured in media normalized to calcium, phosphorus, and magnesium levels present in the sera for each strain. Calcium, phosphorus, and magnesium are known modulators of iPTH and can serve as confounding variables and hence were kept at the levels commonly seen in the sera of each strain. Hence, constant levels of calcium, phosphorus, and magnesium were present in the test and control groups. This was followed by treatment

Fig. 2. iPTH levels in supernatant media (in vitro study). Mean iPTH $\pm$ SE in C3H and B6 TPC after 24 h of incubation at 3 fluoride concentrations (0, 250, and 500 μM ; $n = 6$ in each group; ** $p < 0.005$).

with 2 fluoride concentrations (0 and 500 μM). Media supernatants were collected at 24 h and iPTH levels were determined. TPC from C3H mice demonstrated a significant ($p < 0.001$) decrease in iPTH with increasing fluoride concentrations from 0 μM (145.7 ± 13.1 pg/ml) to 500 μM (72.2 ± 5.9 pg/ml). TPC from B6 mice demonstrated a similar decrease ($p < 0.001$) in iPTH with increasing fluoride concentrations from 0 μM (88.3 ± 6.1 pg/ml) to 500 μM (47.1 ± 5.3 pg/ml). A replicate experiment was performed using TPC from B6 ($n = 6$) and C3H ($n = 6$) mice cultured in media normalized with calcium, phosphorus, and magnesium as described above and then followed by treatment with varying fluoride concentrations (0, 250, and 500 μM). Media supernatants were collected at 24 h and iPTH levels were determined. In the 2-way ANOVA, main effects (both $p < 0.001$) and interaction ($p = 0.042$) were statistically significant, indicating that iPTH differs significantly between the 2 strains and the 3 fluoride levels, with the effect of fluoride exposure on iPTH being different for C3H versus B6. Based on a Bonferroni-corrected significance level of $0.05/6 = 0.008$, for

Fig. 3. Serum iPTH levels at 0, 6, 12, and 24 h. Mean serum iPTH $\pm$ SE after 0 or 100 ppm fluoride gavage in C3H (a) and B6 (b) mice sacrificed at serial time points (0, 6, 12, and 24 h) compared to untreated mice ($n = 6$ in each group; Bonferroni-corrected significance level; * $p < 0.008$, ** $p < 0.001$).

C3H, all 3 levels of fluoride exposure were significantly different from one another, with a higher fluoride level leading to a lower value of iPTH as follows: 0 μM (128.5 ± 10.6 pg/ml) to 250 μM (86.2 ± 12.4 pg/ml, $p < 0.001$) or 500 μM (52.3 ± 8.9 pg/ml, $p < 0.001$). TPC from B6 mice demonstrated less of a decrease in iPTH with increasing fluoride concentrations from 0 μM (64.3 ± 14.7 pg/ml) to 250 μM (50.9 ± 12.2 pg/ml) or 500 μM (38.1 ± 5.1 pg/ml), with the only significant pairwise comparison relating to fluoride concentrations at 0 versus 500 μM ($p < 0.001$) (fig. 2). In the initial and replicate studies, C3H had higher initial iPTH levels in the media compared to B6 TPC ($p < 0.01$). TPC from both strains showed F dose-dependent decreases in iPTH secretion at 24 h.

Serum iPTH after a Single Fluoride Dose via Orogastic Gavage

As in the *in vitro* experiments, *in vivo* studies were done in two different instances. The initial experiment involved 42 mice per strain. Serum iPTH in C3H and B6 mice was measured at 4 time points (baseline and 6, 12, and 24 h) after fluoride gavage (0.001 mg $[\text{F}^-]$ /g of body weight) or gavage with an equal volume of 0 ppm fluoride

water. At baseline with 0 ppm fluoride water gavage, the hour effect was not significant for main effect and interaction, and therefore the baseline observations were dropped from the analysis. The 3-way interaction between strain, hour, and gavage was not significant, but all 2-way interactions were significant (strain $\times$ gavage: $p = 0.001$, strain $\times$ hour: $p < 0.001$, gavage $\times$ hour: $p = 0.009$). Although significance was not reached, at 6 h after 100 ppm fluoride gavage, serum iPTH levels increased modestly for C3H (243.9 ± 13.4 pg/ml) and B6 (102.1 ± 7.3 pg/ml) compared to baseline untreated levels (C3H: 210.5 ± 16.2 pg/ml and B6: 86.6 ± 8.9 pg/ml) and control levels (0 ppm fluoride gavage; C3H: 189.1 ± 19.8 pg/ml and B6: 77.1 ± 4.8 pg/ml). In the C3H strain, iPTH dropped below baseline at 24 h (126.7 ± 10.2 pg/ml, $p < 0.001$) (fig. 3a), whereas in B6 iPTH increased at 12 h (234.8 ± 34.1 pg/ml, $p < 0.001$) after 100 ppm fluoride gavage (fig. 3b).

The *in vivo* studies were replicated using an additional 78 mice per strain. As before, serum iPTH was measured at various time points (0, 0.5, 1, 3, 6, 12, and 24 h) in C3H and B6 mice after fluoride gavage (0.001 mg $[\text{F}^-]$ /g body weight) or gavage with an equal volume of 0 ppm fluoride water. A preliminary 2-way ANOVA re-

Fig. 4. Serum iPTH levels at 0.5, 1, 3, 6, 12, and 24 h. Mean serum iPTH $\pm$ SE after 0 or 100 ppm fluoride gavage in C3H (a) and B6 (b) mice sacrificed at serial time points (0.5, 1, 3, 6, 12, and 24 h) compared to untreated mice ($n = 6$ in each group; * $p < 0.05$, ** $p < 0.005$). B = Baseline.

stricted to baseline gavage 0 μM fluoride at 6 time points and strain as the second factor gave a nonsignificant interaction and a nonsignificant time effect consistent with the assumption of no differences between the mean serum iPTH for baseline gavage (0 h) and 0 μM fluoride at any time point. In the 3-way ANOVA, the 3-way interaction was not statistically significant, but all 2-way interactions were significant (strain $\times$ gavage: $p = 0.017$, strain $\times$ hour: $p < 0.001$, gavage $\times$ hour: $p < 0.001$). This means that each factor has effects on iPTH that vary across the levels of a second factor when averaged over the third factor. Based on a Bonferroni-corrected significance level of $0.05/6 = 0.008$, fluoride levels of 0 and 100 ppm have a statistically different mean iPTH only at 0.5 h ($p = 0.004$) and 24 h ($p < 0.0001$) for either strain. Specifically, at 0.5 h, gavage with a fluoride level of 100 ppm yielded C3H (358.4 ± 19.4 pg/ml) and B6 [149.9 ± 21.2 pg/ml compared to control (0 ppm fluoride gavage) levels (C3H: 244.4 ± 35.5 pg/ml and B6: 98.6 ± 6.4 pg/ml)]. However, gavage with a fluoride level of 0 ppm yielded higher mean iPTH levels at 24 h ($p \leq 0.001$). In the C3H strain, iPTH dropped below baseline at 24 h (82.0 ± 10.7 pg/ml, $p =$

0.002) (fig. 4a), whereas in B6, iPTH increased at 12 h (176.9 ± 20.8 pg/ml, $p < 0.001$) and then returned to baseline at 24 h (95.6 ± 19.9 pg/ml) (fig. 4b). The peak iPTH value in C3H (358.4 ± 19.4 pg/ml) was twice the magnitude of the peak iPTH value in B6 (176.9 ± 20.8 pg/ml).

Since the iPTH levels in C3H mice dropped below baseline at 24 h, additional time points up to 96 h were examined. The serum iPTH in C3H mice after a single 100-ppm fluoride gavage returned to baseline at 48 h (fig. 5). These data demonstrated a difference in the effects of fluoride on iPTH secretion in 2 mice strains: C3H and B6.

Serum Fluoride Levels after Gavage

A calculated dose of fluoride was delivered by orogastric gavage. Serum fluoride concentrations were measured at serial time points (0.5, 1, 3, 6, 12, and 24 h) after a single gavage dose. The fluoride levels in the serum peaked in both strains, i.e. C3H (61.0 ± 5.6 μM) and B6 (26.7 ± 3.1 μM), 0.5 h ($p < 0.001$) after gavage. Fluoride levels reached baseline (0 ppm) in C3H (8.7 ± 1.7 μM) and B6 (3.5 ± 0.4 μM) after 6–9 h (fig. 6). The interaction be-

Fig. 5. Serum iPTH levels at 48–96 h. Mean serum iPTH $\pm$ SE (pg/ml) after 0 or 100 ppm fluoride gavage in C3H mice harvested at extended time points (48, 72, and 96 h) compared to untreated mice ($n = 6$ in each group; ** $p < 0.005$). B = Baseline.

Fig. 6. Serum fluoride levels. Mean serum fluoride $\pm$ SE after 100 ppm fluoride gavage in C3H and B6 mice sacrificed at serial time points (0.5, 1, 3, 6, 12, and 24 h) compared to baseline (no gavage) ($n = 6$ in each group; ** $p < 0.005$). B = Baseline.

tween hour and strain was significant ($p < 0.0001$), indicating that the pattern of fluoride kinetics is different across the strains. In particular, fluoride levels in B6 were significantly lower than in C3H at 0.5, 1, 3, and 12 h ($p < 0.001$). The peak fluoride concentration in C3H ($61.0 \pm 5.6 \mu\text{M}$) was nearly twice that in B6 ($26.7 \pm 3.1 \mu\text{M}$).

Serum Total Calcium, Phosphorus, and Magnesium Levels

Serum total calcium levels were relatively consistent between 0 and 100 ppm fluoride gavage in both C3H (fig. 7a) and B6 mice (fig. 7d). We observed a significant reduction ($p < 0.05$) in serum total calcium only at 0.5 h in the 100 ppm fluoride gavage group (C3H: $8.7 \pm 0.2 \text{ mg/dl}$ and B6: $8.5 \pm 0.2 \text{ mg/dl}$) compared to the 0 ppm control (C3H: $8.9 \pm 0.1 \text{ mg/dl}$ and B6: $9.5 \pm 0.2 \text{ mg/dl}$) and untreated baseline (C3H: $9.3 \pm 0.1 \text{ mg/dl}$ and B6: $9.2 \pm 0.2 \text{ mg/dl}$) groups.

The strain $\times$ gavage interaction in the 2-way ANOVA for serum phosphorus levels was significant ($p = 0.034$). Hour was removed from the model as it was not statistically significant. Therefore, when time was not consid-

ered, there was an overall difference between 0 and 100 ppm fluoride gavage for C3H ($p = 0.009$) (fig. 7b) but not for B6 (fig. 7e) mice. No statistically significant differences in magnesium levels were observed between 0 and 100 ppm fluoride gavage at any time point for either C3H (fig. 7c) or B6 (fig. 7f) mice.

Quantitative Polymerase Chain Reaction: Expression of Genes Involved in the BPK Feedback Loop

We investigated the expression of various genes involved in the BPK feedback loop using TPC, humeri, and kidneys. Expression of the genes in tissues obtained from 100 ppm fluoride gavage C3H and B6 mice at 24 h were normalized with an average of 2 housekeeping genes (*Actb* and *Gapdh*). Gene expression was further normalized to the control 0 ppm fluoride gavage group, and fold differences of genes in TPC (fig. 8a), humeri (fig. 8b), and kidneys (fig. 8c) were calculated for C3H and B6 mice using the $2^{-\Delta\Delta\text{Ct}}$ method. In TPC, *Pth* ($p = 0.001$), *Casr* ($p = 0.012$), *α Klotho* ($p = 0.003$), *Fgf1rc* ($p = 0.035$), *Vdr* ($p = 0.001$), and *Pthlh* ($p = 0.03$) were significantly upregulated in C3H compared to B6. In humeri, only *Pthlh* ($p =$

(For legend see next page.)

0.001) was significantly upregulated in C3H compared to B6 mice. In kidneys, *Pth1r* ($p = 0.005$), *Clcn5* ($p = 0.003$), *α Klotho* ($p = 0.05$), *Fgf1rc* ($p = 0.004$), and *Slc9a3r1* ($p = 0.04$) were significantly downregulated in C3H compared to B6.

Discussion

Skeletal fluorosis is characterized by a spectrum of radiographic bone changes ranging from osteoporosis to osteosclerosis [Wang et al., 1994]. Although clinical cases of skeletal fluorosis are rare in the USA [Kurland et al., 2007; Whyte et al., 2008], in other parts of the world where skeletal fluorosis is more common, patients often demonstrate pseudo-hyperparathyroidism [Teotia and Teotia, 1973; Teotia et al., 1998; Gupta et al., 2001; Xu et al., 2010; Koroglu et al., 2011]. Epidemiologic studies have shown that individuals of a population exposed to similar fluoride levels demonstrate variable skeletal features ranging from excessive bone formation to bone resorption [Teotia et al., 1998; Harinarayan et al., 2006]. Therefore, the mechanisms that underlie the actions of fluoride on the bone and PTH are not clearly understood. Yan et al. [2007] demonstrated a link between fluoride exposure and iPTH levels using the C3H and B6 inbred strains of mice. Fluoride exposure in C3H led to enhanced osteoclastogenesis including elevated serum iPTH, whereas in B6 an anabolic action of fluoride was favored. Additionally, these strains are widely studied for their differences in bone biology. C3H mice have a high bone mass, peak bone density, and alkaline phosphatase activity and a low bone resorption capacity compared to B6 mice [Chen and Kalu, 1999; Linkhart et al., 1999; Turner et al., 2000, 2001].

Direct or indirect effects of fluoride on the parathyroid gland have not been investigated. We developed a murine TPC dispersed cell culture model using inbred

Fig. 7. Mean ($\pm$ SE) serum calcium (Ca), phosphorus (P), and magnesium (Mg) levels after 0 or 100 ppm fluoride gavage in C3H (a–c, respectively) and B6 (d–f, respectively) mice harvested at serial time points (0.5, 1, 3, 6, 12, and 24 h; $n = 6$ in each group; * $p < 0.05$, ** $p < 0.005$). B = Baseline.

Fig. 8. Expression of genes involved in the BPK feedback loop. Fold difference $\pm$ SE in the C3H and B6 100-ppm fluoride gavage groups normalized to the control, the 0-ppm fluoride group, and the housekeeping gene pool (*Actb* and *Gapdh*) in TPC (a), humeri (b), and kidneys (c) ($n = 6$ in each group; * $p < 0.05$, ** $p < 0.001$).

mouse strains with a predictable iPTH secretion response to changes in calcium. Similar TPC dispersed cell culture models have been employed using bovine, human, and rat parathyroid tissues to study parathyroid hormone regulation [Wongsurawat and Armbrecht, 1987; Nielsen et al., 1996; Nakajima et al., 2010]. Calcium, phosphorus, and magnesium are known to mediate changes in iPTH secretion and therefore the levels of these in the media were kept equivalent with strain-specific serum levels to prevent unwanted effects on iPTH secretion. In both C3H and B6, fluoride exposure resulted in dose-dependent reductions in iPTH secretion into the media measured at 24 h. This reduction was more evident for C3H, which at baseline secretes almost twice the iPTH that B6 does.

The fluoride kinetics observed for B6 and C3H strains were consistent with previous studies where serum fluoride reached a peak within 20–30 min [Ekstrand et al., 1977; Whitford, 1994]. It is generally accepted that after absorption fluoride is incorporated in newly mineralized bone or is excreted in urine, resulting in a decline in serum levels, reaching baseline by 6–8 h [Ekstrand et al., 1980]. In order for fluoride to elicit a rapid iPTH response, it is hypothesized that fluoride interacts with the parathyroid gland directly through modulation of iPTH release from vesicles in the chief cells. The exact molecular mechanism of this response is not understood. However, our in vitro findings suggest the presence of such a direct interaction of fluoride on the parathyroid glands.

In the current study, C3H mice had higher baseline serum iPTH levels compared to B6 mice. This is consistent with our TPC baseline values. C3H mice were more responsive to the single fluoride exposure, perhaps because they reached a higher peak serum fluoride level. This enhanced response was shown after the gavage when the levels of iPTH were higher in C3H compared to B6. At 24 h, iPTH levels were lower than baseline values for both strains. Both strains also showed bimodal responses to fluoride. For C3H, iPTH peaked at 0.5 h and declined below baseline at 12 h, reaching a nadir at 24 h, and then over the next 24 h it returned to baseline and remained at baseline at 96 h. B6 mice showed a similar but lower-magnitude response.

Modulation of iPTH secretion as a response to fluoride exposure may also be mediated by changes in calcium levels. Decreased serum calcium levels after acute hydrofluoric acid inhalation have been documented in humans [Zierold and Chauviere, 2012]. Acute exposure to fluoride through injection of hydrofluoric acid (1.6 mg/kg) in

rats led to a decrease in serum ionized calcium and total calcium after 30 and 300 min, respectively [Santoyo-Sanchez et al., 2013]. A similar decrease in plasma calcium has been observed in rats due to acute fluoride exposure [Imanishi et al., 2009]. The reduced serum calcium is hypothesized to be due to the formation of CaF_2 complexes resulting in a net reduction in calcium levels leading to a hypocalcemic response. In our study, we observed a transient decrease in calcium in both strains of mice only at 0.5 h, with a return to baseline within 1 h. There were no significant changes in phosphorus or magnesium at any time point.

The BPK feedback loop has been extensively studied to understand the interaction of various genes involved in mineral homeostasis. The cross talk between bone, the parathyroid gland, and the kidney is responsible for effective regulation of minerals. The effect of fluoride on these individual tissues after a single fluoride dose in the 2 strains of mice will help us to understand the effect of fluoride on regulatory aspects of mineral homeostasis. Changes in gene expression patterns due to fluoride exposure have been studied in the past [Li and DenBesten, 1993; Nair et al., 2011; Pei et al., 2012], but our study focused only on the expression of the genes that are involved in calcium and phosphorus homeostasis and are part of the BPK feedback loop. Fold differences in the expression of *Pth*, *Casr*, *α Klotho*, *Fgf1rc*, *Vdr*, and *Pthlh* in TPC suggest that at the molecular level fluoride has a differential impact on parathyroid tissues in C3H and B6. The expression of these genes reflects a compensatory mechanism to counter changes in iPTH secretion. It is clear that fluoride effects are not limited to *Pth* but extend to *Pth* homologues such as *Pthlh*. Expression of *Fgf1rc- α Klotho* (receptor couple for *Fgf23*) and *Pth1r* is suggestive of an effort to intercept conservation of calcium and excretion of phosphorus in the kidneys to achieve homeostasis after the initial iPTH secretion. Overall, there exists a difference in expression of BPK genes in the 2 strains of mice. This suggests a difference in physiologic handling of fluoride by the 2 strains. It will be interesting to study the effect of a repeated fluoride dose on expression of the key genes in C3H and B6.

Our study investigated the early events involved in fluoride interaction on the parathyroid gland in vitro and in vivo. Fluoride can act on the parathyroid gland directly to modulate iPTH secretion in vitro and in vivo. It is still not clear if the decrease in total serum calcium levels is responsible for fluoride-mediated iPTH modulation. The effect of fluoride on iPTH secretion is rapid

and observable even after a single fluoride dose. The overall effects of fluoride are not limited to the parathyroid gland, but changes in the expression of the genes involved in the BPK feedback loop suggest a broader impact of fluoride on multiple aspects of bone metabolism.

Acknowledgements

Ms. Staci Love is gratefully acknowledged for her help with in vitro studies. The research reported in this publication was supported by the National Institute of Dental and Craniofacial Research (NIDCR) of the National Institutes of Health under award No. R01DE018104 to E.T.E.

References

- Bergwitz, C., H. Juppner (2010) Regulation of phosphate homeostasis by PTH, vitamin D, and FGF23. *Annu Rev Med* 61: 91–104.
- Chen, C., D.N. Kalu (1999) Strain differences in bone density and calcium metabolism between C3H/HeJ and C57BL/6J mice. *Bone* 25: 413–420.
- Ekstrand, J., G. Alvan, L.O. Boreus, A. Norlin (1977) Pharmacokinetics of fluoride in man after single and multiple oral doses. *Eur J Clin Pharmacol* 12: 311–317.
- Ekstrand, J., M. Ehrnebo, G.M. Whitford, P.O. Jarnberg (1980) Fluoride pharmacokinetics during acid-base balance changes in man. *Eur J Clin Pharmacol* 18: 189–194.
- Everett, E.T. (2011) Fluoride's effects on the formation of teeth and bones, and the influence of genetics. *J Dent Res* 90: 552–560.
- Gupta, S.K., T.I. Khan, R.C. Gupta, A.B. Gupta, K.C. Gupta, P. Jain, A. Gupta (2001) Compensatory hyperparathyroidism following high fluoride ingestion – a clinico-biochemical correlation. *Indian Pediatr* 38: 139–146.
- Harinarayan, C.V., N. Kochupillai, S.V. Madhu, N. Gupta, P.J. Meunier (2006) Fluorotoxic metabolic bone disease: an osteo-renal syndrome caused by excess fluoride ingestion in the tropics. *Bone* 39: 907–914.
- Imanishi, M., T. Dote, H. Tsuji, E. Tanida, E. Yamadori, K. Kono (2009) Time-dependent changes of blood parameters and fluoride kinetics in rats after acute exposure to subtoxic hydrofluoric acid. *J Occup Health* 51: 287–293.
- Koroglu, B.K., I.H. Ersoy, M. Koroglu, A. Balkarli, S. Ersoy, S. Varol, M.N. Tamer (2011) Serum parathyroid hormone levels in chronic endemic fluorosis. *Biol Trace Elem Res* 143: 79–86.
- Kurland, E.S., R.C. Schulman, J.E. Zerwekh, W.R. Reinus, D.W. Dempster, M.P. Whyte (2007) Recovery from skeletal fluorosis (an enigmatic, American case). *J Bone Miner Res* 22: 163–170.
- Li, R., P.K. DenBesten (1993) Expression of bone protein mRNA at physiological fluoride concentrations in rat osteoblast culture. *Bone Miner* 22: 187–196.
- Linkhart, T.A., S.G. Linkhart, Y. Kodama, J.R. Farley, H.P. Dimai, K.R. Wright, J.E. Wergedal, M. Sheng, W.G. Beamer, L.R. Donahue, C.J. Rosen, D.J. Baylink (1999) Osteoclast formation in bone marrow cultures from two inbred strains of mice with different bone densities. *J Bone Miner Res* 14: 39–46.
- Livak, K.J., T.D. Schmittgen (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-delta delta C(T))} method. *Methods* 25: 402–408.
- Nair, M., Z.R. Belak, N. Ovsenek (2011) Effects of fluoride on expression of bone-specific genes in developing *Xenopus laevis* larvae. *Biochem Cell Biol* 89: 377–386.
- Nakajima, K., T. Okazaki, T. Okamoto, H. Kimura, K. Takano, K. Sato (2010) Genes up- or down-regulated by high calcium medium in parathyroid tissue explants from patients with primary hyperparathyroidism. *Endocr J* 57: 153–159.
- Nielsen, P.K., U. Feldt-Rasmussen, K. Olgaard (1996) A direct effect in vitro of phosphate on PTH release from bovine parathyroid tissue slices but not from dispersed parathyroid cells. *Nephrol Dial Transplant* 11: 1762–1768.
- Pei, J., B. Li, Y. Gao, Y. Wei, L. Zhou, H. Yao, J. Wang, D. Sun (2012) Fluoride decreased osteoclastic bone resorption through the inhibition of NFATc1 gene expression. *Environ Toxicol* 29: 588–595.
- Potts, J.T. (2005) Parathyroid hormone: past and present. *J Endocrinol* 187: 311–325.
- Santoyo-Sanchez, M.P., M. del Carmen Silva-Lucero, L. Arreola-Mendoza, O.C. Barbier (2013) Effects of acute sodium fluoride exposure on kidney function, water homeostasis, and renal handling of calcium and inorganic phosphate. *Biol Trace Elem Res* 152: 367–372.
- Teotia, S.P., M. Teotia (1973) Secondary hyperparathyroidism in patients with endemic skeletal fluorosis. *Br Med J* 1: 637–640.
- Teotia, M., S.P. Teotia, K.P. Singh (1998) Endemic chronic fluoride toxicity and dietary calcium deficiency interaction syndromes of metabolic bone disease and deformities in India: year 2000. *Indian J Pediatr* 65: 371–381.
- Torres, P.A., D.P. De Brauwere (2011) Three feedback loops precisely regulating serum phosphate concentration. *Kidney Int* 80: 443–445.
- Turner, C.H., Y.F. Hsieh, R. Muller, M.L. Bouxsein, D.J. Baylink, C.J. Rosen, M.D. Grynpas, L.R. Donahue, W.G. Beamer (2000) Genetic regulation of cortical and trabecular bone strength and microstructure in inbred strains of mice. *J Bone Miner Res* 15: 1126–1131.
- Turner, C.H., Y.F. Hsieh, R. Muller, M.L. Bouxsein, C.J. Rosen, M.E. McCrann, L.R. Donahue, W.G. Beamer (2001) Variation in bone biomechanical properties, microstructure, and density in BXH recombinant inbred mice. *J Bone Miner Res* 16: 206–213.
- Vogel, G.L., C.M. Carey, L.C. Chow, J. Ekstrand (1990) Fluoride analysis in nanoliter- and microliter-size fluid samples. *J Dent Res* 69: 522–528, discussion 556–527.
- Wang, Y., Y. Yin, L.A. Gilula, A.J. Wilson (1994) Endemic fluorosis of the skeleton: radiographic features in 127 patients. *AJR Am J Roentgenol* 162: 93–98.
- Whitford, G.M. (1994) Intake and metabolism of fluoride. *Adv Dent Res* 8: 5–14.
- Whyte, M.P., W.G. Totty, V.T. Lim, G.M. Whitford (2008) Skeletal fluorosis from instant tea. *J Bone Miner Res* 23: 759–769.
- Wongsurawat, N., H.J. Armbricht (1987) Comparison of calcium effect on in vitro calcitonin and parathyroid hormone release by young and aged thyroparathyroid glands. *Exp Gerontol* 22: 263–269.
- Xu, H., Q.Y. Liu, J.M. Zhang, H. Zhang, G.S. Li (2010) Elevation of PTH and PTHrP induced by excessive fluoride in rats on a calcium-deficient diet. *Biol Trace Elem Res* 137: 79–87.
- Yan, D., A. Gurumurthy, M. Wright, T.W. Pfeiler, E.G. Loba, E.T. Everett (2007) Genetic background influences fluoride's effects on osteoclastogenesis. *Bone* 41: 1036–1044.
- Yuan, J.S., A. Reed, F. Chen, C.N. Stewart Jr (2006) Statistical analysis of real-time PCR data. *BMC Bioinformatics* 7: 85.
- Zierold, D., M. Chauviere (2012) Hydrogen fluoride inhalation injury because of a fire suppression system. *Mil Med* 177: 108–112.