EFFECTS OF PROTEIN AND CALCIUM SUPPLEMENTATION ON BONE METABOLISM AND THYROID FUNCTION IN PROTEIN AND CALCIUM DEFICIENT RABBITS EXPOSED TO FLUORIDE

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SUMMARY: This study was conducted to determine whether feeding a diet containing excess crude protein (CP) or calcium (Ca) for up to 120 days can ameliorate the effects of fluoride (F) intake on bone health and thyroid function observed in rabbits fed a CP- and Ca-deficient diet. Treatment with F increased serum bone Gla protein concentrations and effects of F were reversed within 60 days in animals fed a high Ca diet. Feeding either a high CP or high Ca diet for 60–90 days increased combined cortical thickness (CCT) and the ratio of CCT to medullary canal diameter (MCD) in the midshaft of the femur of F treated animals. Bone mineral content was also reduced in response to F treatment and effects of F were reversed in animals fed a high CP diet for 30 days. Feeding a high Ca diet reversed F-induced alterations in serum TSH concentrations on day 30 and day 120 and on serum T3 levels by day 90. In contrast, F-induced elevations in serum T4 at day 30 and day 60 were reversed in animals fed a high CP or high Ca diet. Although no effect of F treatment on serum free T3 levels was noted, free T4 was elevated on day 30 in response to F treatment and the F-induced elevation in free T4 was not observed in animals fed a high CP diet. These results demonstrate divergent protective effects of high CP and high Ca diets on adverse indices of bone metabolism and thyroid function induced by F treatment in rabbits fed a CP- or Ca-deficient diet.

Keywords: Bone development; Bone Gla protein; Calcium-deficient diet; Calcium supplementation; High fluoride diet; Malnourished rabbits; Protein-deficient diet; Protein supplementation; Thyroid hormones.

INTRODUCTION

Bone is a major site of fluoride (F) accumulation in the body.[1-3] Excessive F disrupts the balance of bone deposition and remodeling activities and is linked to skeletal disease, including osteoporosis, osteomalacia, and osteopetrosis.[4-6] Nutritional (e.g. protein,[7,8] calcium and phosphate [8-10]) and endocrine (e.g. thyroid hormone and growth hormone[11-13]) influences on bone metabolism are well established. Furthermore, thyroid function is highly sensitive to F intake.[14,15] Given the pronounced effects of thyroid hormone on bone turnover,[16] a link between F-induced thyroid dysfunction and poor skeletal health is plausible. Compared with a normal nutritional state, F exhibits more severe toxicity to bone when animals are subjected to crude protein (CP) or calcium (Ca) deficient diets.[17,18] However, potential therapeutic effects of nutrient (Ca and CP supplementation) on indices of skeletal health and thyroid function in animals with excessive F intake have not been investigated in detail. Hence, the objective of this study was to determine whether feeding a diet containing excess CP or Ca

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for up to 120 days can ameliorate the effects of F intake on bone metabolism and thyroid function observed in rabbits fed a CP and Ca deficient diet.

MATERIALS AND METHODS

Animals and experimental design: Eighty male and female New Zealand rabbits weighing 1.07±0.25 kg were randomly allocated equally (female: male = 1:1) to one of four treatment groups shown in Table 1. The study was approved by the Institutional Animal Care and Use Committee of China. Animals in the malnutrition control group (MC) received a CP and Ca deficient diet containing low F. Animals in the HiF group received a CP and Ca deficient diet containing high F. Animals in the HiF+HiCP group received a diet deficient in Ca that contained high F and high CP. Animals in the HiF+HiCa group received a diet deficient in CP that contained high F and high Ca. Diets were balanced for energy content (9.84–10.37 MJ/kg) and provided along with low F distilled water *ad libitum* for up to 120 days. All rabbits were caged individually and maintained at standard temperature (22–25ºC) and ventilation under hygienic conditions.

Table 1. F (mg/kg), % CP and % Ca in experimental diets

Dietary supplement	Malnutrition control (MC)	High fluoride (HiF)	High F and high CP (HiF+HiCP)	High F and high Ca (HiF+HiCa)
F	20.1	200	200	200
CP	8.58	8.58	18.41	8.35
Ca	0.49	0.49	0.46	2.23

Note: A standard rabbit diet contains 12–16% CP and 1% Ca.

Measurements of combined cortical thickness, medullary canal diameter and bone mineral content: For determination of treatment effects on combined cortical thickness (CCT), medullary canal diameter (MCD) and bone mineral content (BMC), four rabbits from each treatment group were euthanized on days 30, 60, and 90 following initiation of treatments, and eight animals per group were euthanized on day 120. The femurs were immediately collected for analysis and infused in a stream of cold physiological saline to remove the marrow. The CCT and MCD were measured at the midshaft of the femur using a vernier caliper with a precision of ± 0.02 mm. Points of measurement (A-H) for CCT and (or) MCD are depicted in Figure 1. The CCT was calculated as an average of the thickness AB, CD, EF, and GH. The internal diameter of the medullary canal was calculated as the mean of the length BD and FG. All measurements were performed in duplicate. The bone samples were dried at 60ºC for 48 hr, then weighed and ashed individually at 550ºC for 48 hr. The

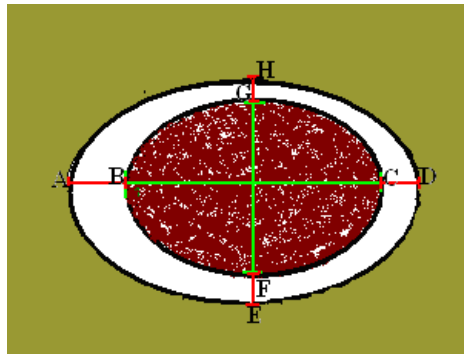

Figure 1. Pictorial representation of the midshaft of a rabbit femur illustrating points of measurement used for determining CCT and MCD.

calcined femur samples were then reweighed and BMC calculated as the ratio of calcined bone weight to dried bone weight.

Measurements of serum bone Gla protein and thyroid function: On days 30, 60, 90, and 120 of treatment, eight rabbits per group were randomly selected and deprived of food for 12 hr. They were then weighed and blood samples collected. Serum was harvested, after which it was centrifuged at 3000 rpm for 10 min and stored at –70^{o}C until use. Serum bone Gla protein (BGP), thyroid stimulating hormone (TSH), triiodothyronine (T3), free triiodothyronine (FT3), thyroxine (T4), and free thyroxine (FT4) concentrations were measured using standardized radioimmunoassay (RIA) kits provided by the Chinese Institute of Atomic Energy, Beijing, China.

Statistical analysis: Effects of treatments on indices of skeletal health and thyroid function were determined by analysis of variance (ANOVA) with differences between treatment means determined by Fisher's protected least significant difference test. Numerical results are expressed as mean±SEM with $P<0.05$ considered significant.

RESULTS

Effects of CP or Ca supplementation on indices of skeletal health in F treated rabbits: The effects of treatments on serum BGP concentrations at days 30, 60, 90, and 120 following initiation of treatment are shown in Figure 2, which show that feeding a high F diet (HiF) resulted in increased serum BGP concentrations on these days relative to the CP- and Ca-deficient (MC) animals. Feeding a high Ca diet (HiF+HiCa) ameliorated the effects of F on serum BGP within 60 days resulting in serum BGP levels similar to the MC animals on days 60, 90, and 120. Effects of the high F diet were not reversed in animals fed excess CP (HiF+HiCP).

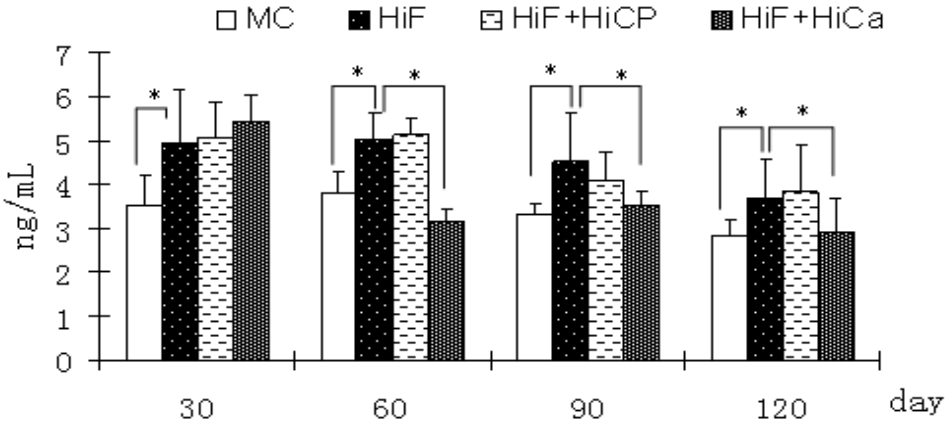

Figure 2. Effects of treatments on serum bone Gla protein (BGP) concentrations. Serum BGP concentrations are depicted as mean±SEM. *p<0.05 as compared to HiF group.

No evidence of effects of treatments on MCD was observed in the present studies (Figure 3). High F (HiF) treatment did not impact the CCT or the CCT/MCD ratio relative to the MC animals (Figure 4). The CCT/MCD ratio (Figure 5) was elevated on day 30, 60, and 90 (relative to HiF animals) in animals supplemented with excess dietary CP (HiF+HiCP) or Ca (HiF+HiCa).

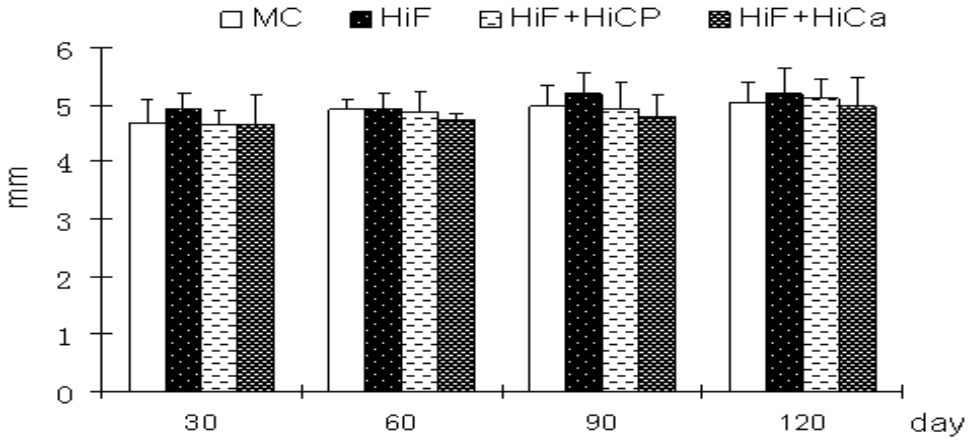

Figure 3. Effects of treatments on medullary canal diameter (MCD). The MCD is depicted as mean±SEM.

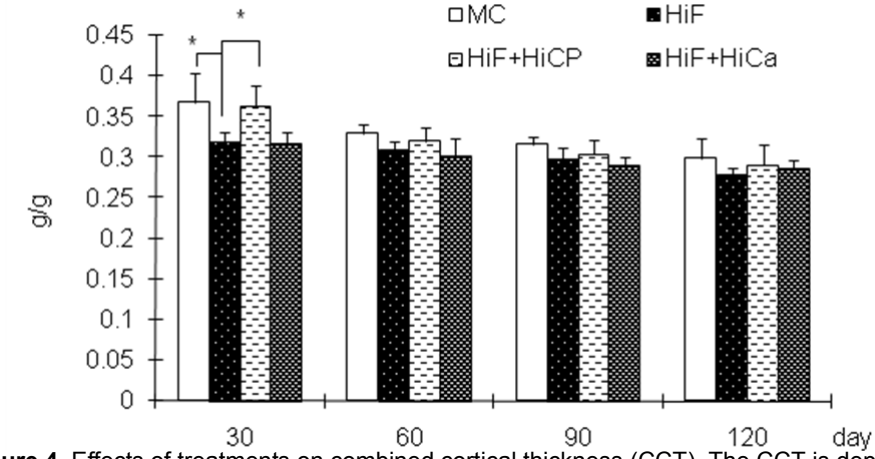

Figure 4. Effects of treatments on combined cortical thickness (CCT). The CCT is depicted as mean±SEM. *p<0.05 as compared to HiF group.

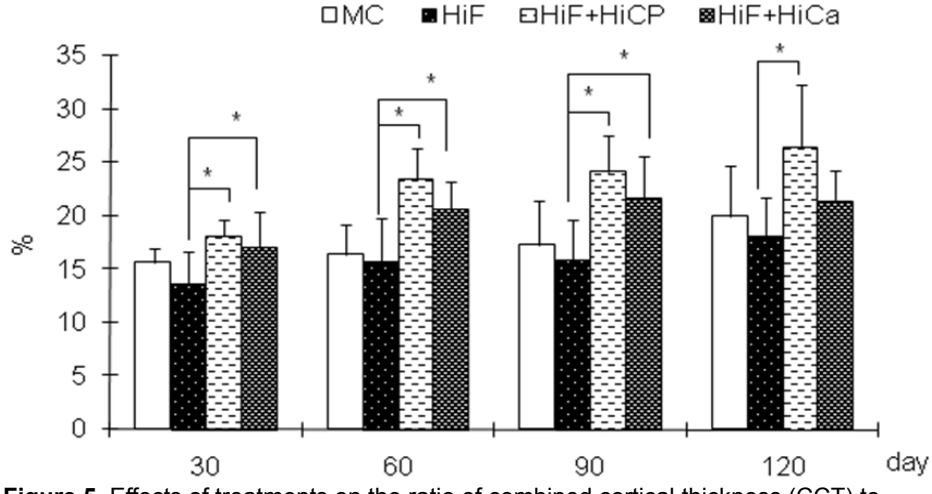

Figure 5. Effects of treatments on the ratio of combined cortical thickness (CCT) to medullary canal diameter (MCD). The CCT/MCD ratio is depicted as mean±SEM. *p<0.05 as compared to HiF group.

Effects of treatments on BMC are depicted in Figure 6. These were transient and observed only on day 30. HiF resulted in reduced BMC relative to the MC animals. The effects of HiF treatment on day 30 BMC were reversed in animals supplemented with excess dietary CP (HiF+HiCP), but not in animals supplemented with excess dietary Ca (HiF+HiCa).

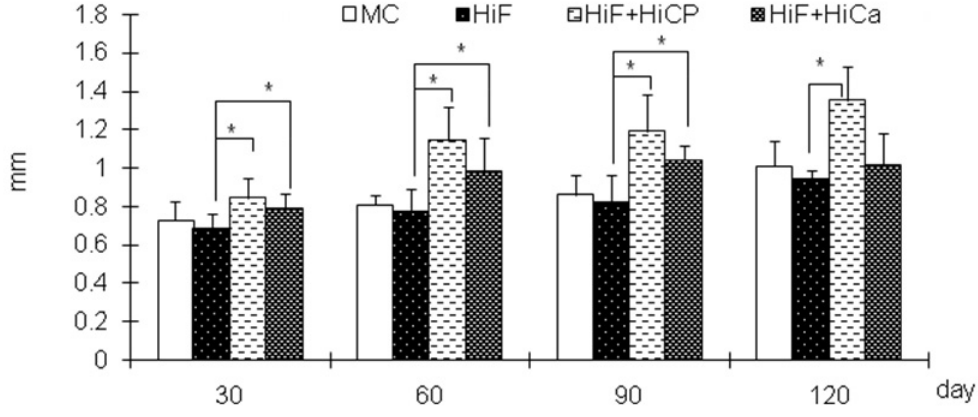

Figure 6. Effects of treatments on bone mineral content (BMC). BMC is depicted as the mean±SEM of g calcined bone/g dried bone. *p<0.05 compared to HiF group.

Effects of CP or Ca supplementation on indices of thyroid function in F treated rabbits: Effects of treatments on serum TSH, T3 and T4 and FT3 and FT4 concentrations are depicted in Tables 2-6, respectively. Serum TSH concentrations were altered by high F treatment on days 30 and 120, with increased TSH on day 30 and decreased TSH on day 120 observed in the HiF animals relative to the MC animals. Supplementation with excess dietary Ca (HiF+HiCa), but not excess CP (HiF+HiCP) ameliorated the effects of F treatment on serum TSH levels.

Table 2. Serum TSH levels (μIU/mL) in rabbits (mean±SEM; n=8)

Day	MC	HiF	HiF+HiCP	HiF+HiCa
30	3.495±0.622	4.295±1.490*	3.856±0.659	3.278±0.706†
60	3.479±0.508	3.479±0.726	2.710±0.923	2.744±0.859
90	2.771±0.239	3.023±0.700	2.670±1.353	2.838±0.687
120	2.558±0.445	1.561±0.886*	2.158±0.508	2.520±0.844†

*p<0.05 as compared to MC group; †p<0.05 as compared to HiF group.

Serum T3 concentrations were elevated on day 90 and reduced on day 120 in the HiF animals relative to the MC animals (Table 3). Effects of HiF on day 90 serum T3 concentrations were reversed by supplementation with high dietary Ca (HiF+HiCa) but not with high CP (HiF+HiCP).

Feeding a high F diet resulted in elevated serum T4 concentrations on days 30, 60 and 90 of treatment in HiF animals relative to MC animals (Table 4). Feeding a high CP (HiF+HiCP) or high Ca (HiF+HiCa) diet resulted in reduced serum T4

concentrations relative to HiF animals on days 30 and 60, but not day 90 of treatment.

Table 3. Serum T3 levels (nmol/L) in rabbits (mean±SEM; n=8)

Day	MC	HiF	HiF+HiCP	HiF+HiCa
30	1.350±0.129	1.540±0.168	1.349±0.139	1.252±0.064
60	1.453±0.165	1.538±0.131	1.445±0.120	1.180±0.097†
90	1.276±0.075	1.959±0.168*	2.028±0.339	1.291±0.141†
120	1.579±0.121	1.186±0.124*	1.304±0.200	1.080±0.124

*p<0.05 as compared to MC group; †p<0.05 as compared to HiF group.

Table 4. Serum T4 levels (nmol/L) in rabbits (mean±SEM; n=8)

Day	MC	HiF	HiF+HiCP	HiF+ HiCa
30	35.673±8.143	55.588±7.881*	24.563±1.976†	33.090±7.477†
60	32.111±10.140	41.380±5.793*	23.363±5.215†	10.262±1.935†
90	21.210±3.074	34.000±5.055*	36.494±4.749	33.676±7.845
120	21.649±2.607	25.371±3.982	21.692±2.882	69.580±12.942†

*p<0.05 as compared to MC group; †p<0.05 as compared to HiF group.

No effects of F treatment (HiF) on FT3 concentrations were observed relative to MC animals (Table 5). However, serum FT3 concentrations were elevated on days 30, 60, and 90 in the F treated animals fed a high CP diet (HiF+HiCP) and in animals fed a high Ca diet (HiF+HiCa) on day 60 relative to HiF animals fed a CP and Ca deficient diet.

Table 5. Serum free T3 (FT3) levels (pmol/L) in rabbits (mean±SEM; n=8)

Day	MC	HiF	HiF+HiCP	HiF+ HiCa[2]
30	2.669±0.400	2.961±0.218	6.318±1.941*	2.557±0.665
60	2.159±0.400	1.621±0.320	5.264±0.810*	3.285±0.873*
90	2.316±0.325	2.134±0.528	4.784±0.978*	2.844±0.637
120	2.700±0.355	2.666±0.738	3.220±0.701	3.353±1.443

*p<0.05 as compared to HiF group.

Feeding a HiF diet resulted in elevated FT4 concentrations on day 30 relative to the MC animals (Table 6). Supplementation with excess dietary CP (HiF+HiCP), but not high Ca (HiF+HiCa) abated the F-induced elevation in FT4 on day 30 and caused elevated FT4 levels on day 90 relative to HiF animals maintained on a CP and Ca deficient diet.

Table 6. Serum free T4 (FT4) levels (pmol/L) in rabbits (mean±SEM; n=8)

Day	MC	HiF	HiF+HiCP	HiF+ HiCa
30	3.020±0.467	5.232±0.622*	3.586±0.498†	4.055±0.819
60	5.276±0.959	4.252±0.420	4.038±0.512	3.322±0.864
90	4.208±0.571	3.337±.0773	5.732±0.315†	2.137±0.325
120	2.816±0.557	2.657±1.027	4.198±0.987†	3.196±0.519

*p<0.05 as compared to MC group; †p<0.05 as compared to HiF group.

DISCUSSION

Effects of CP or Ca supplementation on skeletal health indices of fluorotic rabbits: Serum gla protein (BGP) is a sensitive index of osteoblast activity and is known to be impacted by F.[19,20] In the present study, serum BGP concentrations were increased in CP- and Ca-deficient (MC) animals subjected to F treatment. In our previous studies,[18] expected corresponding changes in other serum

biochemical markers reflective of abnormal bone metabolism (alkaline phosphatase and tartrate resistant acid phosphatase) were observed in F-treated rabbits fed such an MC diet. Here we found that supplementation with excess dietary Ca, but not CP, reversed the effects of high F treatment on serum BGP concentrations. Elevated serum concentrations of BGP are associated with increased bone turnover, and BGP is thought to play a role in bone mineralization.[21] However, CP supplementation, rather than Ca, did reduce the acute negative effects of F treatment on BMC in the present study. Collectively, our results indicate that Ca supplementation could reduce the negative effects of high F intake on bone turnover, but the effects are not likely to be mediated by increased mineralization.

Previous studies have demonstrated that F stimulates (increases) both bone resorption and formation, especially in patients with skeletal fluorosis.[22-24] There are extensive evidences for F-induced skeletal abnormalities in other model systems, including decreased growth plate matrix volume and cartilage septae thickness in rats exposed to high doses of F.[25-26] We have previously shown that dietary CP and Ca supplementation in rabbits can help ameliorate F-induced toxicity by decreasing F digestion and/or absorption.[18,27] In the present study we found that CCT and the CCT/MCD ratio were elevated in the HiF rabbits fed excess dietary CP or Ca. Furthermore, an important relationship between Ca intake and the degree of skeletal changes during endemic fluorosis has been reported previously.[28] On the basis of the current and previous studies, we propose that excess dietary CP and/or Ca can help alleviate the negative effects of high F intake on skeletal health, potentially directly by affecting osteoblast and/or osteoclast function[18] and indirectly by regulation of F digestion and absorption.[27]

Effects of CP or Ca supplementation on indices of thyroid function in fluorotic rabbits: F is a potent regulator of the hypothalamic-pituitary-thyroid axis.[29,30] F acts as a TSH analogue,[29] and hence can modulate thyroid hormone production.[30] Furthermore, excessive F can damage the structure of the thyroid gland and induce thyroid disease.[14,15] Given the fact that thyroid hormones also have pronounced effects on bone turnover,[13] we also examined whether feeding excess dietary CP or Ca can ameliorate effects of high F intake on indices of abnormal thyroid activity observed in animals fed a CP and Ca deficient diet. In fact, we found divergent protective effects of dietary excess CP and Ca on indices of thyroid function impacted by high F treatment.

High F intake markedly increased serum TSH concentrations in the rabbits on day 30 of treatment, but the THS concentrations were reduced on day 120 of treatment. Observed temporal changes in TSH in response to F treatment may be attributed to feedback effects of corresponding changes in thyroid hormones on thyrotropin releasing hormone (TRH) at the hypothalamic level[24] and (or) direct effects of thyroid hormones on pituitary TSH synthesis and secretion.[31] Additionally, in the present study, high Ca but not high CP supplementation reversed the effects of high F intake on serum TSH levels.

Exposure to F is reported to have pronounced effects on serum concentrations of thyroid hormones, potentially due to the TSH agonist-like activity of F.[29] Decreases in serum T3 and T4 were found after administration of F to suckling pups[15,32] and also in cows with chronic fluorosis.[33] The present study also revealed negative effects of F on serum levels of T3 and T4. However, our investigation showed that the reversal of the effects of high dietary CP or Ca on F toxicity is inconsistent at different experimental time intervals. These divergent effects of dietary CP and excess Ca on indices of thyroid function in F exposed rabbits indicate potential differential sensitivity to F among various components of the hypothalamic-pituitary-thyroid axis or that beneficial effects of dietary treatments are not mediated solely by a reduction in F digestion and/or absorption. Previous studies have demonstrated that F can directly injure the thyroid gland,[34,35] disturb the synthesis and secretion of thyroid hormone, and interfere with the activity of enzymes that catalyze the conversion of T4 into the active thyroid hormone T3, thereby leading to perturbations in circulating thyroid hormone levels.[36-38]

In summary, results of the present study demonstrated pronounced negative effects of F on indices of activity of the hypothalamic-pituitary-thyroid axis and on indices of skeletal health. They also demonstrated divergent protective effects of excess dietary Ca and CP on F-induced changes in skeletal health and thyroid function. Although an important role for thyroid hormones in regulation of bone metabolism has been demonstrated,[39-41] further studies will be required to determine the extent to which F-induced changes in thyroid hormones contribute to the observed negative effects of high F intake on skeletal health.

ACKNOWLEDGEMENTS

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