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The effect of supplementation of calcium, vitamin D, boron, and increased fluoride intake on bone mechanical properties and metabolic hormones in rat

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Abstract

Evidence indicates that optimal nutrition plays a role in bone formation and maintenance. Besides major components of mineralization such as calcium, phosphorus, and vitamin D, other nutrients like boron and fluoride have beneficial role, too. In this study, 34 male Wistar rats were divided into five groups: control diet, fluoride, fluoride + boron, fluoride + calcium + vitamin D, and flouride + boron + calcium + vitamin D. Boron equal to 1.23 mg, calcium and vitamin D equal to 210 mg + 55 IU and fluoride equal to 0.7 mg/rat/day was added to their drinking water for 8 weeks. Plasma blood samples and bones were collected. Findings are evidence that fluoride + boron intake revealed significant positive effects on bone mechanical properties and bone metabolic hormones. These findings suggest that combined intake of these two elements has beneficial effects on bone stiffness and breaking strength comparing to even calcium + vitamin D supplementation. This evidence dealing with health problems related to bone and skeletal system in humans should justify further investigation of the role of boron and fluoride with other elements in relation to bone.

Keywords

Fluoride, boron, calcium, vitamin D, hormones, bone mechanical properties

Introduction

The role of nutrition on the development of bone tissue has long been under investigation. The necessary components to develop strong bones with the normal metabolic functions have been provided by the proper consumption of the known major nutrients, such as calcium, phosphorous, magnesium, fluoride, vitamin D, and trace elements, such as zinc, copper, and boron which are reported to intervene with the bone function, mass and strength (Hirota and Hirota, 2011; Palacios 2006).

The role of vitamin D in relation to calcium and bone metabolism is known as essential to build adequate bones and has long been under investigation. Recently, the vitamin D insufficiency was defined as serum 25-hydroxy vitamin D less than 20 ng/ml (50 nmol/l) as it relates to bone (Sai et al., 2011). Calcium metabolism is affected by nutritional factors like

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calcium and vitamin D as well as by endocrine factors. Because calcium deficiency could deteriorate bone metabolisms and cardiovascular systems, adequate intake of calcium and vitamin D is an important for enhancing skeletal health (Anderson et al., 2011).

Among dietary factors, evidence exists that boron may have antioxidants and anti-inflammatory properties (Armstrong and Spears, 2003; Ince et al., 2010; Mahabir et al., 2008; Nielsen, 2000) and play a role in bone formation and maintenance.

An increase level of steroid hormones after boron consumption and its potential to influence the bone metabolism has been reported, previously (Samman et al., 1998; Sheng et al., 2001; Naghii et al., 2011).

Better measurements of bone mechanical properties were observed for boron supplementation, and additional and longer studies for further determination of the effects of supplemental boron with different calcium levels and possibly other minerals were suggested (Naghii et al., 2006). Boron deficiency resulted in altered bone healing because of a marked reduction in osteogenesis (Gorustovich et al., 2008a) and altered periodontal alveolar bone modeling and remodeling by inhibiting bone formation (Gorustovich et al., 2008b).

Existing data indicate that fluoride is an essential and useful element for humans and animals. The main source of fluoride for humans is drinking water and is required for mineralization of bone and teeth (Chandrajith et al., 2011). It is well established that prolonged use and excessive intake of more than 8 ppm/day for many years can cause skeletal fluorosis (Dhar et al., 2009). In general, the optimal health benefit of this element is dependent on the optimal level of intake.

Short-term administration of boron for rapid detoxification in fluorosis (Franke et al., 1985) and beneficial effect of boron on serum minerals and alkaline phosphatase (Alp) in animals fed high fluoride ration (Bharti et al., 2008a), and its antagonizing effect on the absorption and retention of fluoride and consequently improvement in the feed intake (Bharti et al., 2008b) are indications of boron acting as an antidote in fluoride intoxication.

Overall, all these nutrients and probably others have a specific and independent role and may act in a synergistic way to maintain bone structure.

The aim of this study was to investigate the comparative effect of consumption of fluoride individually or in a combined effect or interaction with the selected nutrients (calcium, vitamin D, and boron) on bone mechanical properties and relevant metabolic hormones in rats.

Materials and methods

The research was approved by the University research and ethics committee. Male Wistar rats weighing 140–180 g were obtained from the Animal House of Physiology Group, Baqiyattallah University of Medical Sciences. Rats were matched by body weight and separated randomly into control and four treatment groups, placed in polycarbonate cages in a controlled environment with a 12-h light–dark cycle and a constant temperature (22°C) and humidity (55–65%) for 8 weeks. Animals were provided clean cages twice weekly. Rats in all groups were fed with standard chow from Pars Animal Food Co. (Tehran, Iran) and water *ad libitum* throughout study. According to the manufacturer, it contained 650 mg Ca/100 g food and 80 IU vitamin D3/100 g food. The boron content was not analyzed, but it is reported to be kept at 70 μg/kg of the food and sodium fluoride is kept at 2.0 mg/kg of the food (Gorustovich et al., 2008b).

In the experiment, rats in the treatment groups were supplemented daily with 0.7 mg fluorine/d (group 2), 0.7 mg fluorine + 1.23 mg boron/d (group 3), 0.7 mg fluorine + mix of calcium (210 mg), and vitamin D (55 IU)/d (group 4), and 0.7 mg fluorine + 1.23 mg boron + mix of calcium (210 mg) and vitamin D (55 IU)/d (group 5) in their water. Boric acid and sodium fluoride (Merck, Germany) was used as the source of boron and fluoride, and calcium + vitamin D3 tablets (Darou paksh Co., Tehran, Iran) was used as the source of Ca and vitamin D. Overall supplementation provided 0.7 mg fluorine, 1.23 mg boron, 210 mg calcium, and 55 IU vitamin D/rat/d.

Eight weeks after treatments, rats from all groups were anesthetized for the collection of blood by cardiac puncture with a syringe and needle. Rats were restrained from food for 12 h but had access to drinking water. Since, some parameters such as steroid hormones are subject to circadian rhythm, therefore blood samples were collected at the peak time in the afternoon between 14.0 and 16.0 p.m., and the plasma samples were stored frozen until analysis.

Commercially available assay kits were used to determine the blood parameter levels. Plasma calcium concentrations were determined by atomic absorption spectrophotometry (*Chemtech Analytical, CTA-2000 AAS, Kempston, UK*). Alp activity in rat plasma was measured by kinetic photometric test using a kit from

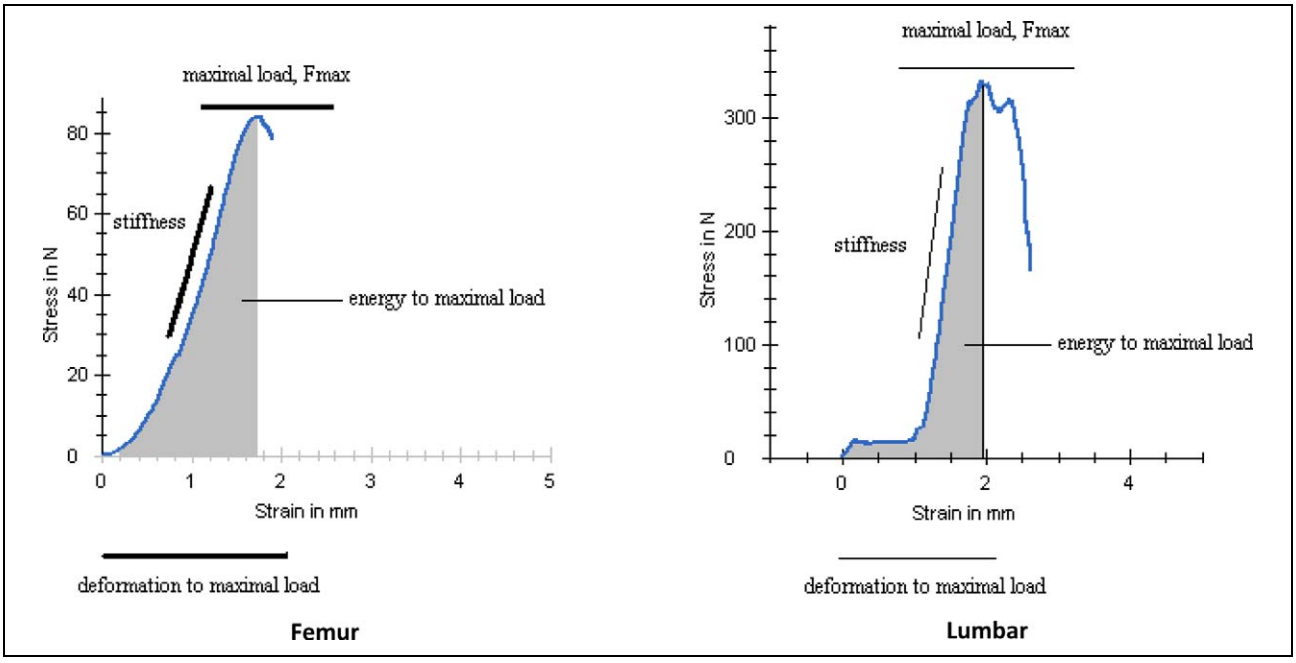

Figure 1. A typical graph of the application of strength to the femur or lumbar bone can be divided into three parts. In the first part, it rises linearly. This describes the elastic deformation of the bone. In the second part, the slope declines until the maximum load is reached. This is the first plastic deformation with microfracturing of the bone. In the last part, the graph declines because of multiple fracturing until complete failure of the bone occurs. The machine automatically stops the breaking test.

Pars Azmun, Tehran, Iran. Plasma 25-hydroxy vitamin D was measured by enzyme immunoassay method using reagent kit obtained from Immunodiagnostic System Ltd (IDS Ltd), Boldon, UK. Plasma rat calcitonin was measured by enzyme-linked immunosorbant assay (ELISA) method using reagent kit (Rat CT, USCN Life Science Inc. Wuhan, China). Plasma parathyroid hormone (PTH) was measured utilizing a two-site ELISA kit (Rat PTH, USCN Life Science Inc. Wuhan, China). The assays for plasma total testosterone (T), free testosterone (FT), and estradiol (E2) were performed by ELISA methods using reagent kits (Diagnostics Biochem Canada Inc., Ontario, Canada).

Immediately, after blood sampling, the left and right femurs, and the fifth lumbar vertebral bones were excised from all animals. The bones were kept in 0.9% saline. Mechanical properties of the fifth lumbar vertebral bones were determined with the axial compression test and bone mechanical properties of the left femurs by a three-point bending test using a Zwick materials testing-machine (Z2.5, Germany). For three-point bending, the span of the two support points was 20 mm, and the deformation rate was 1 mm/min.

All measures of mechanical properties of the bones were determined on load deformation curve and consist of the extrinsic material properties of the bone samples, including linear stiffness, the maximal load or breaking strength (Fmax), energy to maximal load, and deformation to maximal load (Figure 1).

Energy to maximal load was computed as the area under the load deformation curve. Stiffness was computed as the slope of the linear portion of the load deformation curve. The maximal load or the yield point is the point on the curve after which plastic or permanent damage occurs to the bone. Prior to the yield point, the slope of the line is linear, and the bone undergoes elastic deformation (Crenshaw et al., 1981).

Statistical analysis

Data are expressed as mean $\pm$ SD, and a Statistical Package for the Social Sciences ([SPSS 17.0], McGraw-Hill, New York, NY) was used to perform all comparisons. Analysis of variance (ANOVA) was used to evaluate the effects of training and treatments between the groups (determined by least significance difference [LSD] test). A $p < 0.05$ was considered significant for the differences.

Results

The rats in all groups adjusted to the treatments well and consumed food and water normally.

Table 1. Effects of nutrient supplementations on plasma parameters in the groups

Variables	Group 1, Control	Group 2, F	Group 3, F + B	Group 4, F + Ca + D	Group 5, F + B + Ca + D
Ca (mg/dl)	4.83 ± 0.30	4.61 ± 0.27[a]	4.94 ± 0.25	4.34 ± 0.39[b]	4.90 ± 0.30
D (nmol/l)	91.0 ± 19.0	93.0 ± 21.0	96.0 ± 17.0	101.0 ± 13.0	96.0 ± 19.0
Alp (U/l)	215.0 ± 19.0	229.0 ± 27.0	210.0 ± 24.0	207.0 ± 31.0	236.0 ± 43.0
CT (pg/ml)	80.0 ± 11.0	73.0 ± 8.0	77.0 ± 9.0	71.0 ± 10.0	82.0 ± 12.0
PTH (pg/ml)	36.0 ± 9.0	35.0 ± 6.0	34.0 ± 5.0	37.0 ± 6.0	33.0 ± 8.0
T (ng/ml)	1.85 ± 0.40[c]	2.02 ± 0.84	2.02 ± 0.67	2.60 ± 0.66	2.63 ± 1.00
Free T (pg/ml)	0.44 ± 0.19	0.53 ± 0.25	0.55 ± 0.14	0.58 ± 0.28	0.52 ± 0.14
E2 (pg/ml)	8.35 ± 1.32[d]	9.91 ± 1.67	9.68 ± 0.94	9.92 ± 0.89	9.70 ± 1.06

F: fluorine; Ca: calcium; D: vitamin D, B: boron; Alp: alkaline phosphatase; PTH: parathyroid hormone; T: testosterone; E2: estradiol; CT: calcitonin; Free T: free testosterone.
[a]Statistically significant with group 3.
[b]Statistically significant with groups 1, 3, and 5.
[c]Statistically significant with group 5.
[d]Statistically significant with groups 2, 3, 4, and 5.

Table 2. Measures of extrinsic biomechanical properties of femur in 3-point bending and lumbar vertebra in compression test in the groups

Variable		Group 1, Control	Group 2, F	Group 3, F + B	Group 4, F + Ca + D	Group 5, F + B + Ca + D
Stiffness (N/mm)	Femur	69.4 ± 11.0[a]	98.0 ± 5.0[b]	16.01 ± 23.0[c]	108.0 ± 20.0	92.0 ± 13.0
	Lumbar	332.0 ± 98.0	320.0 ± 61.0	341.0 ± 19.0	322.0 ± 94.0	349.0 ± 88.0
Maximal load	Femur	71.0 ± 8.0[b]	80.0 ± 13.0	86.0 ± 11.0	80.0 ± 10.0	79.0 ± 5.0
(Fmax) (N)	Lumbar	256.0 ± 39.0	302.0 ± 50.0[d]	291.0 ± 12.0	255.0 ± 24.0	290.0 ± 20.0
Energy to maximal	Femur	47.0 ± 12.0	45.0 ± 13.0	45.0 ± 10.0	38.0 ± 7.0	50.0 ± 10.0
load (N mm)	Lumbar	197.0 ± 64.0	157.0 ± 18.0	232.0 ± 122.0	185.0 ± 74.0	238.0 ± 69.0
Deformation to	Femur	1.90 ± 0.48	1.7 ± 0.25[c]	1.90 ± 0.14	0.03 ± 1.73	2.07 ± 0.32
maximal load (mm)	Lumbar	1.83 ± 0.27[e]	2.17 ± 0.30[c]	2.18 ± 0.20	2.06 ± 0.26[c]	2.46 ± 0.23

F: fluorine; Ca: calcium; D, vitamin D, B: boron.
[a]Statistically significant with groups 2, 3, 4, and 5.
[b]Statistically significant with group 3.
[c]Statistically significant with group 5.
[d]Statistically significant with groups 1 and 4.
[e]Statistically significant with groups 2, 3, and 5.

To study the effect of different treatments on the plasma variables (Table 1), a significant difference in the plasma concentration of calcium was noted between groups 2 and 3 (a), ($p \leq 0.05$), observing a higher concentration in the group 3. Also, a lower concentration in the group 4 was noted in comparison to the groups 1, 3, and 5 (b), ($p \leq 0.05$).

A significant higher plasma T concentration was observed in the group 5 compared to the group 1 (control) (c), ($p \leq 0.05$). Moreover, E2 concentration was significantly higher in all the treatment groups compared to the control (d), ($p \leq 0.05$). Although, a nonsignificant higher level for the plasma-free T concentration was noted in all the treatment groups, generally no other major changes were noted for other plasma variables.

Measures of the bone mechanical properties are presented in Table 2.

Femur bone

The results in Table 2 show that in the bone mechanical parameters, significant differences were observed for femur stiffness in all the groups compared to the control (a), ($p \leq 0.05$); and also in comparison between the groups, femur stiffness was higher in the group 3 compared to the group 2 (b) and the group 5 (c), ($p \leq 0.05$), indicating a higher femur strength in that group.

The breaking strength (Fmax) of the femur bone was higher in all the treatment groups, but a significant difference was only present in the group 3 in

comparison to the control group (b), ($p \leq 0.05$). Finally, significant lower deformation to maximal load (DFmax) in the group 2 was noted compared to the group 5 (c), ($p \leq 0.05$).

Lumbar vertebra bone

The breaking strength (Fmax) of the lumbar vertebra bone was higher in group 2 compared to all the groups and was statistically significant compared to group 4 (d), ($p \leq 0.05$).

A significant lower deformation to maximal load (DFmax) in the control group was noted compared to all treatment groups and was significant compared to the groups 2, 3, and 5 (e), also a lower deformation in groups 2 and 4 in comparison with group 5 was noted (c), ($p \leq 0.05$).

Discussion

In the current study, a simultaneous combination effect of the known dietary factors consisting of different minerals and vitamin D on bone strength has been determined. It is known that dietary changes and other lifestyle alterations can lower the risk of developing bone diseases. Healthy diets have been proven to reduce bone risks significantly. Findings are evidence that fluorine + boron intake revealed significant effects on bone mechanical properties and bone metabolic hormones. These findings suggest that the combined intake of these two elements produces beneficial effects on bone stiffness and breaking strength compared to calcium + vitamin D supplementation.

Consumption of boron and fluoride together demonstrated higher stiffness and maximal load (or breaking strength) in femur, and higher maximal load for lumbar vertebral bone strength, as a result of consumption of fluoride alone or along with boron. Better results on bone mechanical properties were found with the combination of fluoride and boron in comparison with the consumption of calcium and vitamin D. These findings require firm confirmation in studies with longer times and different doses of these nutrients. Applications of other elements, in addition to bone mass, histology, and histomorphometry studies, are recommended.

Boron has been shown in numerous studies to alter bone mechanical properties and mass in different animal species. In pigs, supplementation level of 5 mg/kg diet increased the bone bending moment in males (Armstrong et al., 2000). In male rats, dietary levels of 200 mg boron/kg diet did increase vertebral resistance to crush force (Chapin et al., 1997). In addition, supplementation of 15 mg of boron/kg diet increased ultimate shear stress force of the fibula (Armstrong and Spears, 2001) and the basal diet supplemented with 5 mg/kg diet increased the measures of intrinsic and extrinsic strength of the femur (Armstrong et al., 2002).

The mean maximal load or the total applied force at the yield point of the tibiae, femora, and lumbar vertebras in the boron treatment group was reported to be greater, indicating higher mechanical property or strength of the bones with boron supplementation (Naghii et al., 2006).

The calcium concentration was significantly higher as a result of fluoride and boron supplementation. Boron supplementation was reported to markedly reduce the urinary excretion of calcium and magnesium and elevate the serum concentrations of 17β-E2 and testosterone, suggesting a role in the prevention of calcium loss and bone demineralization in postmenopausal (Nielsen et al., 1987).

Boron as an antidote in acute fluoride intoxication in rabbits tends to increase the elimination of fluoride from the body, and calcium and phosphorous balance was normal. Boron given as a curative corrects secondary hyperthyroidism and increases sequestration of fluoride from bone and reduces cortical thickness caused by high fluoride intake (Elsair et al., 1980, 1982).

Fluoride as a minor element in bone rendered a suitable effect on bone formation *in vivo* (Inoue et al., 2005), and the intake level is advised to be kept within the limit of 0.5–0.65 mg/l to avoid toxic effects and fluorosis (Viswanathan et al., 2009). When the concentrations of fluoride in two different areas of the same village were 4 and 4.5 ppm, radiological finding showed fluorosis of skull bones and barrowing of long bones was reported (Rawlani et al., 2010). However, it is well documented that consumption of adequate quantity prevents dental caries, assists in the formation of dental enamels, and prevents deficiencies in bone mineralization (Jha et al., 2011).

The influence of the sodium fluoride in the acceleration of fracture healing is attributed to the accelerated chondrogenesis process in the area of insufficiently perfused bone, osteogenesis including temporary callus formation, and mineralization of the new bone, as well as remodeling into mature lamellar bone (Białecki, 1999).

In our study, no major differences were observed in the plasma concentrations of Alp, parathormone, and

calcitonin. The mean testosterone levels was higher in all the treatment groups, but a significant difference was found in group 5 compared to the control. The FT concentration increased in all groups and a significant higher concentration of E2 was found in all groups in comparison with the control. The above findings indicate that a positive interaction exists between minerals and steroids and finally on the bone quality.

The association between sex steroids, bone mineral density (BMD), and incident fractures in Chinese men with low serum E2 levels displayed elevated bone loss and increased risk of fractures in the lowest quartile of E(2) and bioavailable E(2) (Woo et al., 2011).

Additionally, steroids, in particular E2 production is reported to be influenced by boron supplementation (Naghii et al., 2011; Naghii and Samman, 1997; Nielsen et al., 1987). The higher level of steroids found in all treatment groups seems to be the result of fluoride and/or boron intake. The report of steroid production by boron (Naghii et al., 2011) is consistent with the finding of our study. Therefore, conducting further studies to explore possible associations between nutrients and steroid hormone productions and their impact on bone structure, quality, and growth is highly recommended.

In the current study, effect of the known factors consisting of fluoride, boron, and vitamin D plus calcium has been determined and overall, it appeared that combination of fluoride and boron has the most beneficial effects on bone structure. Further, it would be well advised to determine the influence of fluoride and boron on bone metabolism, mechanical properties, and strength, along with more histomorphological and bone mass studies. Conducting more studies on bone diseases, such as osteoporosis, and on ovariectomized animals might be of interest.

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