Bone 68 (2014) 85–91

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

Peak bone strength is influenced by calcium intake in growing rats

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ARTICLE INFO

Article history:
Received 25 November 2013
Revised 21 July 2014
Accepted 25 July 2014
Available online 4 August 2014

Edited by: David Fyhrie

Keywords:
Calcium
Bone strength
Bone microarchitecture
Bone material properties
Growing rat
Bone turnover

ABSTRACT

In this study we investigated the effect of supplementing the diet of the growing male rat with different levels of calcium (from low to higher than recommended intakes at constant Ca/P ratio), on multiple factors (bone mass, strength, size, geometry, material properties, turnover) influencing bone strength during the bone accrual period. Rats, age 28 days were supplemented for 4 weeks with high Ca (1.2%), adequate Ca (0.5%) or low Ca level (0.2%). Bone metabolism and structural parameters were measured. No changes in body weight or food intake were observed among the groups. As anticipated, compared to the adequate Ca intake, low-Ca intake had a detrimental impact on bone growth (33.63 *vs.* 33.68 mm), bone strength (−19.7% for failure load), bone architecture (−58% for BV/TV) and peak bone mass accrual (−29% for BMD) due to the hormonal disruption implied in Ca metabolism. In contrast, novel, surprising results were observed in that higher than adequate Ca intake resulted in improved peak bone strength (106 *vs.* 184 N/mm for the stiffness and 61 *vs.* 89 N for the failure load) and bone material properties (467 *vs.* 514 mPa for tissue hardness) but these effects were not accompanied by changes in bone mass, size, microarchitecture or bone turnover. Hormonal factors, IGF-I and bone modeling were also evaluated. Compared to the adequate level of Ca, IGF-I level was significantly lower in the low-Ca intake group and significantly higher in the high-Ca intake group. No detrimental effects of high Ca were observed on bone modeling (assessed by histomorphometry and bone markers), at least in this short-term intervention. In conclusion, the decrease in failure load in the low calcium group can be explained by the change in bone geometry and bone mass parameters. Thus, improvements in mechanical properties can be explained by the improved quality of intrinsic bone tissue as shown by nanoindentation. These results suggest that supplemental Ca may be beneficial for the attainment of peak bone strength and that multiple factors linked to bone mass and strength should be taken into account when setting dietary levels of adequate mineral intake to support optimal peak bone mass acquisition.

Introduction

During growth it is important to maximize peak bone mass and strength to reduce the risk of osteoporosis later in life. A low calcium (Ca) intake in children and animals impairs bone mass acquisition whereas the influence of high Ca intake on bone mass remains unclear particularly during periods of bone mineral accrual. Furthermore, although several studies have already investigated the effect of various dietary Ca levels on bone metabolism in growing animals, in most of them, the Ca/P ratio was not kept constant across the different groups which compromised the analysis of the role of calcium intake on bone growth. Indeed, it is important to keep a constant Ca/P ratio to avoid the induction of a secondary hyperparathyroidism. Epidemiological studies have shown an association between a reduced Ca/P intake with a lower BMD [1] and an excess intake of Ca without appropriate phosphorus supplementation with reduced BMD in post-menopausal women [2]. Furthermore, high dietary phosphate intake reduces bone strength and impairs bone growth in growing rats even when Ca intake is sufficient [3,4]. This effect is at least partly mediated by PTH. Thus it can be concluded that adequate Ca/P balance is important to support optimal peak bone mass development.

Studies in children and adolescents during periods of bone accrual have shown that supplementation in Ca (1000–1200 mg/day) above the usual dietary intakes (600–800 mg/day depending on studies) enhances their bone mineral content (BMC) and areal bone mineral density (BMD) gain [5–12]. Recently, a meta-analysis of 19 randomized controlled trials including 2859 children aged 3 to 18 years, showed a positive effect of Ca supplementation, with doses ranging between 300 and 1200 mg/day, on total body BMC and upper limb BMD with

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http://dx.doi.org/10.1016/j.bone.2014.07.029
8756-3282/

an effect-size of 0.14 standard deviation for both [13]. However, the effect of increasing Ca intake above the usual dietary intakes has been less investigated on bone turnover or bone geometry in children and in adolescents. The effect of high Ca intake on bone formation and bone resorption markers is still controversial: Dibba & Jonhston [6,7] reported that supplementation in Ca decreased bone formation whereas Rozen et al. [12] showed no change in osteocalcin level but a significant increase in bone-specific alkaline phosphatase concentrations in girls aged 14 years. Furthermore in prepubertal children, high Ca intake had no effect on bone resorption [7,12,14]. More recently, thanks to the development of new imaging techniques such as pQCT, Matkovic et al. [15] found beneficial changes on the geometry of the proximal radius of adolescent Caucasian girls. Girls who were supplemented in Ca had a slightly higher cortical to total area ratio compared to the low Ca intake group. Thus, Ca exerted its action on bone accretion during growth primarily by influencing volumetric bone mineral density and consequently bone strength. Thus, all these data showed that the BMD and also several bone quality parameters respond to Ca intake.

Regulation of peak bone mass acquisition is largely an endocrine process. The growth spurt is paralleled by a rapid increase in circulating IGF-I, during the period when peak calcium accretion occurs. IGF-I acts in an autocrine/paracrine fashion to increase bone formation and it is also important for linear and appositional bone growth, bone mineralization and increase in cortical thickness at puberty in human and rodents [16]. Low plasma IGF-I levels are associated with increased fracture risk [17,18].

Maximal strength is determined by bone microarchitecture, geometry, and material level properties. Consequently, any animal model used to analyze the effect of Ca intake on bone strength during growth needs to take these multiple parameters into account. Since calcium is an important contributor to bone strength [19], the purpose of this study was to test the effect of Ca at different doses (low, adequate, higher than recommended dietary intakes but remaining in physiological conditions with a constant Ca/P ratio) on these multiple factors affecting bone strength in young growing male rats and to understand to what extent higher than recommended Ca intakes was beneficial. Moreover, the effect of Ca restriction on these different parameters was also re-evaluated.

Material and methods

Animal study

The study was carried out according to a protocol approved by the Cantonal Veterinary Office of Switzerland. Three groups of 10 weaning 21-day-old male Sprague–Dawley rats (tolerance ± 3 days) were purchased from Charles River (France). The rats were housed individually under ambient conditions of 21 °C with relative humidity of 55% and under a 12 h:12 h light/dark cycle. Animals were allowed to acclimatize to the local environment for 1 week before study initiation. The rats were randomized at day 0 (*D0*) according to the body weight before allocating the rats in the 3 diet groups. All animals had access to diet (Kliba Nafag, Basel, Switzerland) and deionized sterile water *ad libitum* throughout the 4 week feeding study. Rats were fed an AIN-93G diet containing either high Ca (HCa: 1.2%), adequate Ca (ACa: 0.5%) or low Ca (LCa: 0.2%) levels. The molar Ca/P ratio remained constant (1.3) between the 3 different AIN-93G diets (Reeves et al. [20]). Food intake and body weight were measured three times weekly. At day 25 and day 28 before sacrifice, calcein (Sigma Aldrich), a fluorescent marker for bone labeling was injected intravenously (10 mg/kg body weight) into the rats. On day 30 (*D30*), animals were euthanized by isofloran inhalation and body length (nose–anus length) was measured. Blood samples were collected and stored at −80 °C until biochemical analysis. Femurs were separated from adjacent tissue, cleaned, and wrapped in saline-soaked gauze and stored at −80 °C until testing for physical measurements (bone mineral density, microarchitecture and mechanical testing).

Bone measurements

Bone imaging

Bone mineral density (BMD) of the whole femur was assessed by dual-energy X-ray absorptiometry (PIXImus2; GE-Lunar, Madison, WI). Furthermore, bone microarchitecture was analyzed by microcomputed tomography (μCT 20; Scanco Medical AG, Brüttisellen, Switzerland) with a 16 μm resolution. The following variables were measured: cortical thickness (Ct.Th; mm) measured at the diaphysis and the trabecular bone volume fraction (BV/TV, %), trabecular number (Tb.N #), thickness (Tb.Th, μm) and separation (Tb.Sp. μm) were quantified in the distal metaphysis femoral bone. In order to be in the secondary spongiosa, we chose an area of 0.8 mm (or 50 slices) above the growth plate. For the midshaft femur scans were 29 slices (or 0.464 mm) from the middle of the femur (total length divided by 2) and we chose this area as this was comparable to where biomechanical testing in a three point bending test would take place. The cortical area and the total area and the mineralization densities (mg HA/cm^3) were also assessed by microcomputed tomography.

Femoral length and 3-point bending mechanical testing

The length of the femur and the diameter of the femoral diaphysis were measured with a digital caliper. The midline of the shaft was determined and a line was drawn using a marker at this point. Femoral failure load was determined using a three-point bending test using a servo-controlled electro-mechanical system (Instron 1114; Instron Corp., High Wycombe, UK) with the actuator displaced at 2 mm/min. The direction of loading for the femoral 3-point bending tests was done in the posterior–anterior axis. Both displacement and load were recorded. Mechanical properties including femoral maximal failure load (N), stiffness (N/mm), deflection (mm), yield (N) and post yield load (N), failure load (N), post yield deformation (mm), elastic energy (N · mm), plastic energy (N · mm) and total energy (N · mm), were determined from the load–displacement curve. The post yield load is obtained by subtraction of the yield load to the failure load.

Nanoindentation

After sacrifice, cortical diaphysal femur bones were tested using a nanoindentation technique for bone material level properties. The same rat cortical femur bones were used for mechanical testing and the measurements of bone material level properties were performed close to the site of rupture around 5 mm above the condyles in order to preserve bone mechanical properties. Bones were embedded in polymethylmethacrylate and cut. Modulus, tissue hardness and working energy of the bone were determined on hydrated bone tissue samples. A nano-hardness tester (NHT, CSM Instruments, Peseux, Switzerland) was used as follows; force–displacement data of a pyramidal diamond indenter that is pressed into a material are recorded as previously described [21]. Briefly, the mechanical tests included five indentations in the cortical compartment. Indents were set to a 900 nm depth with an approximate speed of 76 mN/min for both loading and unloading. Full rehydration occurs at this distance and is necessary for the nanoindenter of less than 1 μm from the surface of the sample and is stable for up to a period of 60 h. At maximum load, a 5 s holding period was applied. The limit of the maximal allowable thermal drift was set to 0.1 nm/s.

Dynamic histomorphometry

Histomorphometry was carried out on the tibia. Bones were dehydrated with a sequential change of ethanol and then infiltrated and embedded without decalcification in methylmethacrylate. A longitudinal section of the tibia was cut at a thickness of 8 μm and dynamic