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Prolonged increase in dietary phosphate intake alters bone mineralization in adult male rats☆

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Abstract

Excessive intake of dietary phosphate without the company of calcium causes serum parathyroid hormone (s-PTH) concentration to rise. We investigated the effect of a modest but prolonged increase in dietary intake of inorganic phosphate on the bone quantitative factors of mature male rats. Twenty Wistar rats were divided into two groups and fed a high-phosphate diet (1.2% phosphate) or a control diet (0.6% phosphate) for 8 weeks. In the beginning and at the end of the study period, femur and lumbar bone mineral density (BMD), bone mineral content and area were measured using DXA, s-PTH was analyzed from the blood sample, and after sacrifice, right femur was cut loose and processed into paraffin cuts. Bone diameter, inner diameter and cortical width was measured from the hematoxylin- and eosin-dyed femur cuts. Tibias were degraded and calcium and phosphate content was analyzed by inductively coupled plasma–mass spectrometer. Femoral BMD increased significantly more in the control group than in the phosphate group ($P=.005$). Lumbar BMD values decreased in both groups, and the fall was greater in the control group ($P=.007$). The phosphate group had significantly higher s-PTH values ($P=.0135$). Femoral histomorphometric values or tibial mineral contents did not differ between groups. In conclusion, increase in dietary phosphate intake caused s-PTH to rise and hindered mineral deposition into cortical bone, leading to lower BMD. The effect on trabecular bone was opposing as mineral loss was less in the lumbar spine of phosphate group animals. These results are in concurrence with the data stating that skeletal response to PTH is complex and site dependent.

Keywords: Dietary phosphate; Bone densitometry; PTH; Section modulus; Aging

1. Introduction

Phosphorus functions as a limiting nutrient in the biosphere. A common feature of such nutrients is their efficient absorption; phosphate absorption is 60% to 80%, remaining unchanged even at high intakes [1]. In human nutrition, phosphate is widely distributed in many foods [2] and is, thus, no longer a limiting nutrient. The recommended dietary allowance for phosphate (700 mg/day) is exceeded by many people by two- to threefold [3]. The intake of inorganic phosphate (P_i) increases easily with the consumption of foods rich in phosphate additives, such as meat products. A habitual diet can influence bone health, with phosphate intake being one of the nutritional factors under active debate [4].

Phosphate homeostasis is regulated principally by the kidneys. Renal proximal epithelial cells express sodium-dependent phosphate (Na/P_i) cotransporter. Activity of the cotransporter is regulated by dietary P_i intake and such hormones as 1,25-dihydroxyvitamin D (vitamin D) and parathyroid hormone (PTH). Tubular reabsorption is increased by vitamin D and low dietary intake of P_i, whereas high intakes of P_i and PTH decrease reabsorption, causing phosphate to be excreted in urine [5–7].

Adequate phosphate intake is essential for many biological processes, including skeletal mineralization, but excessive intake can have deleterious effects on bone. Phosphate can directly cause osteoblast apoptosis [8] as well as a rise in PTH concentration. Phosphate has a direct action on the parathyroid gland, although the mechanism remains somewhat obscure. High-phosphate concentration inhibits arachidonic acid production in the parathyroid tissue, which in

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turn stimulates PTH secretion [9]. A high-phosphate diet induces parathyroid cell proliferation [10] and PTH secretion, causing serum PTH levels to rise [11–15]. Intermittent administration of human PTH increases bone mass in humans and rats [16,17], but continuously, high levels of PTH reduce bone mineral density (BMD). In vitro studies on cell cultures indicate the deleterious effects of PTH on bone to be mediated by increases in the number and activation of osteoclasts [18–20] and increased osteoblast apoptosis [21,22]. In the end, bone mineral content (BMC) will be affected if the number or function of mineral-depositing osteoblasts is impaired.

The aim of this study was to clarify the effect of a modest but prolonged increase in dietary intake of P_i on bone quantitative factors in mature male rats. Changes in BMC and structural characteristics in an intact male rat model were measured. The results contribute to a greater understanding of the role of dietary phosphate in bone health and the site-dependent effect of PTH.

2. Materials and methods

2.1. Rats and diets

Twenty approximately 15-week-old Wistar male rats (HsdBrlHan:WIST) with an average body weight of 399±37 g were randomized by weight into two groups: a control group and a phosphate group. Male rats were used because they are reported to be relatively insensitive to diet-induced nephrocalcinogenesis, which could produce inter-individual variation in results [23]. Groups were offered either a control (phosphate content, 0.6%) or a high-phosphate (phosphate content, 1.2%) diet of 20 g/day for 8 weeks. The calcium content in both diets was 0.6%. Food composition is shown in Table 1. Diets were provided by the same supplier (Harlan Teklad, Madison, WI, USA). Access to water was ad libitum. The rats were housed in groups of two to three in plastic cages at 21°C, 60±10% humidity, and with a 12-h light/dark cycle. The study was approved by the Ethics Committee for Animal Experimentation at the University of Helsinki.

Table 1
Composition of experimental diets

	Control diet (0.6% phosphate) (g/kg)	High-phosphate diet (1.2% phosphate) (g/kg)
Casein	200.0	200.0
DL-Methionine	3.0	3.0
Sucrose	503.41	476.89
Corn starch	150.0	150.0
Corn oil	50.0	50.0
Cellulose	50.0	50.0
AIN-76A vitamin mix	10.0	10.0
Ethoxyquin	0.01	0.01
Mineral mix	13.37	13.37
$CaHPO_4$	20.21	20.21
KH_2PO_4		15.38
$NaH_2PO_4H_2O$		11.14

Dietary treatments were based on AIN-76A to maintain Ca/P ratio of 1:1 and 1:2 with minimal changes in other mineral contents.

Table 2
Body weight (g), bone area (cm^2) and BMC (g) of the femur and spine of rats after 8 weeks of feeding control or phosphate diet

	Controls ($n=10$)	Phosphate ($n=10$)
Body weight (g)		
Initial	395±37	402±40
Final	476±55[a]	471±60[a]
Femur area (cm^2)		
Initial	1.8±0.08	1.7±0.11
Final	1.9±0.15[a]	2.0±0.13[a]
Femur BMC (g)		
Initial	0.44±0.03	0.46±0.04
Final	0.53±0.06[a]	0.53±0.03[a]
Femur BMC/body weight (g/kg)		
Initial	1.13±0.11	1.14±0.08
Final	1.12±0.07	1.13±0.12
Lumbar (L4–6) area (cm^2)		
Initial	1.82±0.13	1.87±0.17
Final	1.96±0.14[a]	1.99±0.15[a]
Lumbar (L4–6) BMC (g)		
Initial	0.33±0.04	0.36±0.04
Final	0.34±0.04	0.38±0.07
Lumbar BMC/body weight (g/kg)		
Initial	0.82±0.06	0.89±0.12
Final	0.71±0.06[a,b]	0.80±0.12[a]

Data are means±S.D.
[a] Significant difference ($P<.05$) compared with initial values, paired sample t test.
[b] Significant difference ($P<.05$) compared with the other group, independent sample t test.

2.2. Densitometry of the femur and lumbar spine

At the onset of the study as well as at the end of the 8-week study period, rats were put under light anesthesia (Hypnorm-Dormicum 1:1, 0.15–0.2 ml/100 g). Right femur and spine (L4–6) BMC and BMD were determined in vivo with a dual-energy X-ray bone densitometry device (Lunar PIXImus, GE Medical Systems, WI, USA), and analyses of the areas were carried out with the image of the animal on the screen using a region of interest for the segments. Following the final measurement, blood samples were drawn by cardiac puncture for serum PTH (s-PTH) assessment with a Rat-intact PTH ELISA Kit (Immutopics, San Clemente, CA, USA), after which, the rats were sacrificed by cervical dislocation.

2.3. Histomorphometry of the femur

For tissue collection, the right leg was disarticulated at the hip, knee and ankle. The femur and tibia were cleaned of soft tissue. The femur was fixed for 24 h with 10% formalin (Formal-Fixx, Thermo Shandon, UK). After fixation, the femur was decalcified (TBD-2, Thermo

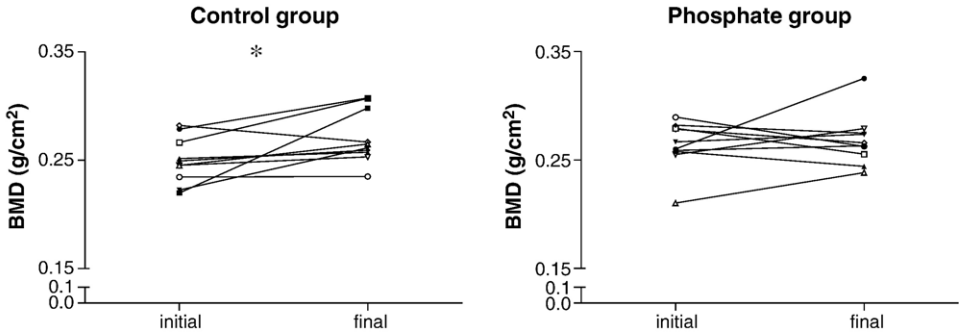

Fig. 1. Femur BMD over the study period, $n=10$ for both groups. Differences between the initial and final BMD values were significant in the control group but not in the phosphate group. *$P=.0135$.

Shandon), dehydrated in graded ethanols and acetone, and then embedded in paraffin. Three 5-μm-thick paraffin-embedded horizontal bone sections were cut from the proximal end of the diaphysis and dyed with a hematoxylin–eosin stain. Bone diameter, inner bone diameter and cortical width of the sections were measured using a 2× objective and a Nikon micrometer on a Nikon Eclipse E400 microscope. Results represent an average of three separate measurements. In addition to these measurements, section modulus, an expression of bending or torsional resistance, was calculated. Section modulus is expressed as $z=\pi/4r_o^3[1-(r_i/r_o)^4]$, where r_o is the periosteal radius and r_i is the endosteal radius.

2.4. Calcium and phosphate analysis of the tibia

Tibias were washed with MilliQ water and dried overnight at +70°C. They were then weighed and dissolved in 70% HNO_3. The organic matrix was degraded by a wet digestion apparatus (Foss Tecator Digestion System 40, Controller 2000, Sweden). Calcium and phosphate content was determined with an inductively coupled plasma–mass spectrometer (ICP/MS, Perkin-Elmer Sciex Elan 6000, USA) using quantitative analysis techniques. Quantitation was performed using external standards (Merck VI, Multi Element Standard for ICP). Results represent an average of two parallel samples. Standard reference material (NIST 1567a wheat flour, National Institute of Standards and Technology, Finland) and in-house control sample (flour) were used as quality controls. The coefficient of variation (CV%) for analysis of standard reference material was 0.03% for calcium and 0.5% for phosphate, and for in-house control samples, 0.8% and 0.3%, respectively.

2.5. Statistical analyses

All values are expressed as means±S.D. in tables and as means±S.E.M. in figures. Comparisons between groups for BMD were tested by analysis of covariance in which baseline bone mineral values and changes in body weight were used as covariates. Paired sample t test was used to analyze changes within a group over the study period. Independent sample t test was used to compare s-PTH concentration between groups. The correlation coefficient was tested by Pearson's correlation. Results were considered significant at a 95% significance level ($P<.05$). Analyses were carried out using SPSS for Windows (version 12.0).

3. Results

3.1. Rat weight

Initial body weight, weight gain and final body weight did not differ between groups (Table 2). Body weight of rats

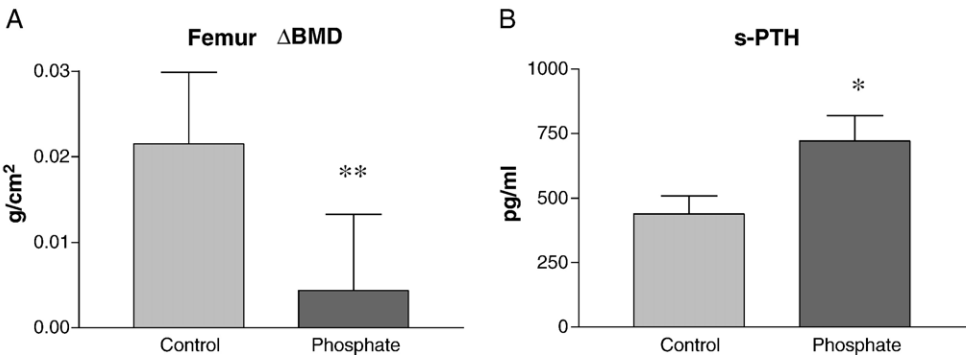

Fig. 2. (A) The increase (Δ) in femur BMD was greater in the control group than in the phosphate group, when initial femur BMD and weight gain were used as a covariates. **$P=.005$. (B) Serum parathyroid hormone was significantly higher in the phosphate group at the end of the study. *$P=.03$.

in both groups significantly increased over the 8-week study period (controls, 80.6±20.4 g, $P<.001$; phosphate group, 69.3±28.6 g, $P<.001$).

3.2. Densitometry of the femur and lumbar spine and serum PTH concentration

Femur BMD increased significantly in the control group but not in the phosphate group. Fig. 1 presents femur BMD changes over the study period. The increase (Δ) in femur BMD was greater ($P=.005$) in the control group than in the phosphate group when initial BMD and weight gain were used as covariates (Fig. 2A). Lumbar (L4–6) BMD decreased in both groups (phosphate group, −0.0026± 0.024 g/cm^2, and controls, −0.0059±0.024 g/cm^2). Despite the large S.D., inclusion of covariates (initial lumbar BMD and weight gain) resulted into statistical difference between groups ($P=.007$). Decrease in lumbar BMD was less in the phosphate group. Serum PTH was significantly higher ($P=.0135$) in the phosphate group at the end of the study (Fig. 2B).

Femur area increased concurrently with BMC in both groups, but neither value differed between groups. A similar increase in area was found in the lumbar spine, but not in the BMC. When lumbar BMC was corrected for body weight, the increase in BMC became significant within groups as well as between groups (Table 2).

3.3. Histomorphometry of the femur

Table 3 shows morphometric parameters of the femur midshaft. No significant difference was present between groups in any of the values measured; however, some interesting correlations are worth noting. Femur Δarea and spine Δarea had a negative correlation ($r=-.478$, $P=.033$), and femur Δarea and femur cortex width had a positive correlation ($r=.498$, $P=.025$). Section modulus, periosteal and endosteal radius correlated with the following variables only within control animals: final femur area ($r=.846$, $P=.004$), femur Δarea ($r=.854$, $P=.003$) and cortex ($r=.747$, $P=.021$). Values correlating negatively within the phosphate group only were lumbar spine Δarea with both femur diameter ($r=-.690$, $P=.027$) and marrow diameter ($r=-.740$, $P=.014$).

Table 3
Morphometric parameters of the femur midshaft in control and phosphate animals

	Controls ($n=9$)	Phosphate ($n=10$)
Bone diameter (mm/kg)	6.0±2.1	6.9±0.9
Marrow diameter (mm/kg)	4.6±1.7	5.4±0.7
Cortex (mm/kg)	0.34±0.15	0.47±0.15
Section modulus (cm^3)[a]	2.07±0.40	2.10±0.51

Data are means±S.D.
Bone diameter, marrow diameter and cortex width were corrected by animal weight for bone size correlates strongly with animal size. There was no statistical difference between groups.
[a] An expression of bending or torsional resistance.

Table 4
Tibia BMD, BMC, calcium and phosphate content after 8 weeks of feeding control or phosphate diet

	Control ($n=10$)	Phosphate ($n=9$)
Tibia BMD (g/cm^2)	0.18±0.011	0.18±0.012
Tibia BMC (g)	0.39±0.059	0.37±0.063
Area (cm^2)	2.2±0.32	2.0±0.36
Dry weight (g)	0.60±0.13	0.55±0.12
Length (cm)	4.06±0.12	3.97±0.14
Total Calcium (g)	0.12±0.020	0.11±0.026
Total Phosphorus(g)	0.070±0.012	0.063±0.015

Data are means±S.D.
The values did not differ between groups.

3.4. Calcium and phosphate analysis of the tibia

Chemical analysis of calcium and phosphate content in the tibia is shown in Table 4. No significant difference was found between groups. Tibia BMC/body weight correlated with section modulus ($r=.560$, $P=.016$) as well as with calcium and phosphate content/bone length ($r=.979$, $P<.001$, and $r=.974$, $P<.001$, respectively).

4. Discussion

Bone quality can be defined by qualitative and quantitative factors contributing to bone fragility. Qualitative factors include fatigue damage accumulation, architectural deterioration, increased bone turnover and osteocyte deficiency [24–26]. Quantitative factors include material properties (e.g., BMC) and structural characteristics (e.g., size, shape, cortical thickness and trabecular architecture) [27].

Bone mineral density measurements are valued in the diagnosis and risk-assessment of osteoporosis, as low BMD is associated with increased fracture risk. Bone mineral density reflects the end result of bone modeling and remodeling activities. Several bone densitometry studies suggest that PTH has site-specific effects on bone [28]. In rats, intermittent human PTH increases cortical mass and trabecular thickness [17,29], whereas continuous treatment induces cortical porosity [28]. Cortical porosity leads to a decrease in areal BMC. We found femur BMD to increase significantly in the control group over the study period, and when initial femur BMD and weight gain were used as covariates, this increase was significantly greater than in the phosphate group. At the same time, the phosphate group had a significantly higher s-PTH concentration. Lumbar areal BMD values were, however, reduced in both groups. The overall decrease in lumbar BMD values could be due to a physiological decrease in bone remodeling because bone turnover slows as rats age [24]. Lumbar BMD decreased less in the phosphate group, which could be explained by a possible increase in trabecular bone turnover due to high s-PTH. These areal differences in BMD indicate that PTH does have a site-specific function in bone, supporting the conclusions of Lotinun et al. [28].

Bone densitometric measurements also provided information on bone area (cm^2) and mineral content (g). The relative increase in femur area was 8.7% for the control group and 13.6% for the phosphate group, whereas the relative increase in BMC was 18.3% and 15.1%, respectively. Lumbar relative areal values increased (7.5% for controls and 6.7% for the phosphate group), but because the relative increase in BMC (4.5% for controls and 5.7% for the phosphate group) was less than the increase in size, the overall mineral density decreased.

Bone size, regarded as a critical element of bone strength [27,30,31], is affected by several determinants, including weight. After adjusting the morphometric parameters of the femur for weight, the phosphate group had wider bones than the control group. Our finding is in accord with studies reporting that continuous treatment with PTH increases the formation of bone at the outer surface (periosteal bone formation), resulting to wider bones [28]. Bone size reflects bone strength because the resistance of bone to bending is exponentially related to its diameter [31]. The greater width confers greater bone strength by placing the cortical mass further away from the neutral axis of the bone [30]. A geometric parameter, section modulus, can be calculated from bone diameter and used as an expression of bending or torsional resistance; this parameter does not, however, take into account any material properties. We calculated the section modulus for the control and phosphate groups and found out the phosphate group to have values 16% higher. Phosphate rats thus seem to compensate the lack of mineral deposition in femurs by an increase in size.

Combining the findings of the densitometric measurements and the morphometric measurements, we conclude that bone mineral deposition was unable to keep up with bone growth in the high-phosphate group, but long bones adapted to the situation by an increase in size through periosteal apposition. This periosteal apposition maintains the cross-sectional area of the bone and, thus, its strength [32], despite less mineral being deposited. A very likely candidate for producing this effect was the significant increase in s-PTH. Although we did not test bone mechanical properties, our results suggest unfavorable changes in bone modeling, resulting in fragility. Thus, an increase in dietary intake of phosphate does appear to affect negatively BMD. This could, at least in part, be due to an increase in s-PTH, but other mechanisms cannot be ruled out.

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