

Effect of a calcium-rich diet on mineral and bone metabolism in rats

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Díaz-Tocados JM^{1,2,3}, Rodríguez-Ortiz ME^{1,2,3}, Almadén Y^{2,4,5,6}, Carvalho C^{7,8,9}, Frazão JM^{8,9,10}, Rodríguez M^{1,2,3,11}, Muñoz-Castañeda JR^{1,2,3,11}

1 Maimonides Institute for Biomedical Research of Cordoba (IMIBIC). Calcium Metabolism and Vascular Calcification Unit. Cordoba (Spain)

2 University of Cordoba. Cordoba (Spain)

3 Nephrology Service. Reina Sofia University Hospital. Cordoba (Spain)

4 IMIBIC. Lipids and Atherosclerosis Unit. Cordoba (Spain)

5 Internal Medicine Service. Reina Sofia University Hospital. Cordoba (Spain)

6 Center for Biomedical Research Network on the Physiopathology of Obesity and Nutrition (CIBEROBN). ISCIII. Madrid (Spain)

7 Hospital of Braga. Nephrology Department. Braga (Portugal)

8 Institute for Research and Innovation in Health (I3S). University of Porto. Porto (Portugal)

9 National Institute of Biomedical Engineering (INEB). University of Porto. Porto (Portugal)

10 Department of Nephrology. São João Hospital. Porto (Portugal)

11 Renal Research Network (REDinREN). ISCIII. Madrid (Spain)

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Summary

Objective: A diet rich in calcium has generally been recommended to maintain adequate bone health. However, recent studies have sparked controversy over its benefits. In this sense, most of the existing studies in animal models are carried out with diets deficient in vitamin D. In this study, the effect of a diet rich in calcium on mineral metabolism and bone histomorphometry in rats is evaluated. In addition, in UMR-106 cells, the direct effect of calcium supplementation on the expression of osteogenic genes is assessed.

Material and methods: A group of male wistar rats of approximately 3 months of age was fed a normal calcium content diet (0.6%) while another group received a high calcium content diet (1.2%). After 20 days urine samples were collected 24h, blood for biochemical analysis and the femur for bone histomorphometry study. *In vitro*, the gene expression of *Runx2*, *Osterix* and *Osteocalcin* was studied in UMR-106 cells cultured under conditions of high calcium content.

Results: The ingestion of a diet rich in calcium reduced the concentration of PTH and calcitriol in plasma, increased calciuria and decreased phosphaturia. At the bone level, a drastic decrease in osteoblastic activity was observed, consistent with the decrease in PTH. However, the trabecular volume remained similar in both groups. *In vitro*, calcium supplementation did not decrease the expression of osteoblastic markers in UMR-106, indicating that the *in vivo* effects are mostly indirect and due to the decrease in PTH.

Conclusions: A high-calcium diet reduces the concentration of PTH and calcitriol in plasma, which results in a decrease in osteoblastic activity.

Key words: Calcium, PTH, calcitriol, bone histomorphometry.

INTRODUCTION

The body's main reservoir of calcium is bone, where about 99% of total calcium is stored in the form of hydroxyapatite. Thus, the calcium content present in extracellular fluids only represents a small fraction of total calcium.

In healthy individuals, the concentration of calcium in the blood varies between 8.6 and 10.4 mg/dl, with around 40% being bound to proteins and 6% to phosphate, citrate or bicarbonate salts. The metabolic activity

of calcium is attributed to ionic calcium, which represents 54% of total calcium in the blood and is very precisely regulated so that plasma values remain in a range between 4.4 and 5.4 mg/dl (1.1-1.35 mM)¹.

With regard to bone health, the benefits of a diet rich in calcium on bone homeostasis are under debate². Thus, for example, the calcium supplement has been commonly recommended for the maintenance of bone health and for preventing osteoporosis. Nevertheless, meta-analysis studies have shown that this calcium



Correspondence: Juan Miguel Díaz Tocados (b22ditoj@uco.es)

supplement does not always have a positive effect. In a general adult population, it has been observed that neither the supplement with vitamin D, calcium nor the combination of both are associated with a decrease in the risk of fracture³. Thus the controversy concerning the effectiveness of these supplements has increased. In the same sense, in a prospective longitudinal study carried out in Sweden in which the incidence of fractures and osteoporosis in adult women was studied over 19 years, calcium intake was estimated by means of a questionnaire, it concluded that a higher intake was not associated with a reduced risk of fracture or osteoporosis⁴.

In animal models, most of the studies on the bone effects associated with calcium are developed in models in which vitamin D levels are reduced, either through diets deficient in vitamin D or in knock out animal models for the vitamin receptor D (VDR). In male wistar rats fed a diet deficient in vitamin D from prenatal stages, a vitamin D-deficient diet has been shown to decrease bone mineral density, femur length and cause histological changes such as osteoid accumulation, increased osteoblastic activity or decreased osteoclastic activity. When these rats with a diet deficient in vitamin D were fed with a calcium supplement in the diet, the bone mineral density was partially recovered, as well as the length of the tibia, the volume of osteoid decreased and the osteoblastic activity, while the number of osteoclasts, which produced a decrease in trabecular bone volume⁵. In another similar study, animals fed a diet deficient in vitamin D decreased the calcium content in the bone, which was reestablished with the infusion of calcium and phosphorus, indicating that the effects of vitamin D on the bone must be mainly indirect and derived from its function on the regulation of mineral metabolism⁶.

On the other hand, in knock out mice for the vitamin D receptor and for 25-hydroxyvitamin D 1- α -hydroxylase, which are hypocalcemic, an increase in bone formation, bone volume and the number of osteoblasts was observed, associated with the consequent increase in parathyroid hormone (PTH) levels even though the animals were fed a lactose-free diet with a high calcium content. However, the number of osteoclasts was not associated with PTH levels in these animals and remained similar to that of mice with wild-type phenotype and normal PTH levels. When the animals were fed a rescue diet (2% calcium, 1.25% phosphorus, 20% lactose, and 2.2 units/g of vitamin D) it was possible to prevent hypocalcemia, hyperparathyroidism and, consequently, the number of osteoblasts, the mineral apposition rate and bone volume were reduced⁷.

Based on these premises, the objective of this study was to investigate the effect of a diet with a high calcium content on bone histomorphometry in rats, as well as on the osteogenesis of UMR-106 cells.

MATERIALS AND METHODS

All experimental procedures carried out in this study were approved by the Research Ethics and Animal Welfare Committee of IMIBIC/University of Cordoba in accordance with the provisions of Directive 2010/63/EU of the European Parliament and the Council of Europe of the September 22, 2010, the institutional guidelines for the care and use of laboratory animals and the Declaration of Helsinki, protocol authorization number 03/14/2018/026.

Experimental design

Male wistar rats with approximately 3 months of age were used to avoid interactions related to sex since in rats there is a sexual dimorphism in the bone phenotype that appears to be multifactorial⁸. The animals were fed with diets of normal content (0.6% Ca; n=6) or high content of calcium (1.2% Ca; n=9) and both diets had a phosphorus content of 0.2%. After 20 days, the rats were placed in metabolic cages to collect the 24-hour urine. The following day the animals were sacrificed by puncture of the abdominal aorta and exsanguinated under general anesthesia with sevoflurane. The blood was processed to separate the plasma and the right femur was placed in 70% ethanol for subsequent inclusion in methylmethacrylate.

Biochemistry in blood and urine

The blood samples were collected in heparinized tubes (BD Vacutainer; Franklin Lakes, NJ, USA) and centrifuged at 2000 x g, for 10 minutes at 4°C to separate the plasma that was stored at -80°C until the biochemical determinations were carried out. The 24h urine samples were centrifuged at 2000 x g, for 10 minutes at 4°C to discard the sediment and the aliquots were stored at -20°C until analysis. Colorimetric kits (BioSystems SA, Barcelona, Spain) were used to determine the content of phosphorus, total calcium and creatinine. The fraction of phosphorus excretion, expressed as a percentage, was calculated according to the formula: (urine phosphorus x plasma creatinine x 100)/(plasma phosphorus x urine creatinine). Ionic calcium quantification was performed in plasma just after sacrifice and before freezing in an ion analyzer (Spotlyte Ca²⁺/pH (Menarini Diagnostics, Barcelona, Spain). Circulating bioactive PTH contents were determined by ELISA (Immutopics, San Clemente, CA, USA) and intact FGF23 (Kainos Laboratories, Tokyo, Japan). Calcitriol concentration was measured by radioimmunoassay (Immunodiagnostic systems, Bordon, UK). All kits were used following manufacturer's instructions.

Inclusion in methylmethacrylate and bone histomorphometry analysis

After the sacrifice, the right femur was removed from each animal and embedded in 70% ethanol. Subsequently, the femurs were dehydrated in alcohol, rinsed with xylene, and embedded in 75% methyl methacrylate, 25% dibutyl phthalate and 2.5% w/v benzoyl peroxide. Histomorphometry analysis was performed on 5 μ m sections without decalcification stained with Villanueva's modified Goldner trichrome method⁹. Briefly, bone sections were fixed with 50% ethanol under pressure for 24h at 37°C, then rehydrated and stained with 1:1 hematoxylin-ferric chloride, subsequently rinsed with 1% hydrochloric acid and blued with lithium carbonate saturated. After washing with water, sections were stained with Goldner trichrome stain for 20 minutes and then rinsed with 1% acetic acid. Subsequently, the samples were stained with a 1% w/v alcoholic saffron solution for 5 minutes, dehydrated with ethanol and mounted. Calcified tissue was stained green and areas stained red were considered osteoid. Bone histomorphometric parameters were evaluated at 200x in a Leica DM4000B optical microscope (Leica Microsystems Wetzlar, Germany) with an Olympus DP72 camera (Olympus, Tokyo, Japan) using OsteoMeasure Software (OsteoMetrics, Decatur, IL, USA). The distal part of the bone within the secondary cancellous was analyzed (1 mm away from

the growth plate and at a distance of approximately 0.25 mm from the endocortical bone). The bone histomorphometry parameters were calculated according to the American Society for Bone and Mineral Research (ASBMR) recommendations¹⁰.

***In vitro* experiments**

The effect of high calcium concentrations on the osteogenesis of UMR-106 cells was also evaluated. The cells were cultured with DMEM (Sigma-Aldrich) supplemented with 10% FBS (Lonza), 2 mM ultraglutamine (Lonza), 1 mM sodium pyruvate (Lonza), 20 mM HEPES (Sigma-Aldrich), 100U/ml penicillin and 100 mg/ml streptomycin. Once the cells reached approximately 90% confluence, the culture medium was changed to calcium-free DMEM (Gibco, Grand Island, NY) supplemented as indicated above, and a 0.1 M calcium chloride solution (Sigma-Aldrich) to increase the calcium concentration in the culture medium to 1.25 mM (normal concentration in blood) and 1.8 mM (equivalent to hypercalcemia). After 48h, cells were lysed and processed for total RNA isolation. 3 independent experiments were carried out with 4 replications for each group.

RNA isolation and RT-PCR

Total RNA was extracted with Trizol (Sigma-Aldrich) and the final concentration was quantified by spectrophotometry (ND-1000, Nanodrop Technologies). RNA samples were post-treated with DNase (Sigma-Aldrich) and real-time PCR was performed with 50 ng of DNase-treated RNA with the SensiFAST SYBR No-ROX One-Step Kit (Bioline). The primers used were: *Runx2* (Sense 5'CGG-GAA-TGA-TGA-GAA-CTA-CTC3' 'Antisense 5'CGG-TCA-GAG-AAC-AAA-CTA-GGT3'), *Osterix* (Sense 5'GTA-CGG-CAA-GGC-TTC-GCA-TCT-GA3' 'Antisense 5'TCA-AGT-GGT-CGC-TTC-GGG-TAA-AG3'), *Osteocalcin* (Sense 5'TCT-GAG-TCT-GAC-AAA-GCC-TTC-ATG3' 'Antisense 5'TGG-GTA-GGG-GGC-T GG-GGC-TCC3') and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* (Sense 5'AGG-GCT-GCC-TTC-TCT-TGT-GAC3' 'Antisense 5'TGG-GTA-GAA-TCA-TAC-TGG- AAC-ATG-TAG3'). The RT-PCR amplification was carried out in a Lighcycler 480 (Roche Molecular Biochemicals). The expression of the target genes was normalized by method $2^{-\Delta\Delta Ct}$ using *GAPDH* as constitutive.

Statistic analysis

Values are shown as mean $\pm$ standard error. The differences between the two groups were studied using the non-parametric Mann-Whitney U test. The groups were considered significantly different for a $p < 0.05$. Statistical analyzes and graph editing were performed with the GraphPad program (GraphPad Software, La Jolla, CA, USA).

RESULTS

Biochemistry in blood and urine

The group of animals fed a diet rich in calcium showed plasma levels of calcium and phosphorus similar to those of the group fed normal calcium (figure 1a and b respectively). However, the high calcium diet produced both a decrease in plasma concentrations of intact PTH (figure 1 c) and calcitriol (figure 1d). The intact FGF23 levels remained similar in both groups (figure 1e). As expected, the 24-hour urinalysis showed an increase in calciuria and a decrease in phosphaturia in rats fed a calcium-rich diet (figure 1f and g respectively).

Bone histomorphometry

The volume of trabecular bone and the volume of osteoid in the rats fed the calcium-rich diet remained similar to that of the rats fed a normal calcium diet (figure 2a and b), while a significant reduction in the osteoid surface area of the calcium was observed (figure 2c) in the group of animals fed a diet rich in calcium, which was consistent with a decrease in the bone surface covered by osteoblasts (figure 2 d). Both the resorption surface and the bone surface covered by osteoclasts were similar in both groups (figure 2e and f respectively). At the level of trabecular micro-architecture, no differences were observed with respect to trabecular thickness, trabecular separation and number of trabeculae (figure 2 g, h, i).

Effect of calcium on the osteogenesis of UMR-106

To study *in vitro* the direct effect of calcium on osteoblasts we used the rat osteoblast cell line UMR-106. The culture medium of these cells was supplemented with calcium until reaching a concentration of 1.8 mM and it was compared with cells cultured with culture media with normal calcium content (1.25 mM). After 48h of treatment, it was observed that the high levels of calcium did not modify the expression of osteogenic genes such as *Runx2*, *Osterix* or *Osteocalcin* (figure 4 a, b and c respectively).

DISCUSSION

In this study, a Ca-rich diet for 21 days did not promote significant differences in plasma levels of Ca or P at the expense of increasing calciuria and decreasing phosphaturia which had a direct impact on plasma PTH and calcitriol levels. This effect could be due to a transient hypercalcemia at the beginning of the experiment, resulting in a subsequent decrease in PTH production, hypercalciuria and calcitriol synthesis, since it seems consistent with an activation of the calcium receptor (CaSR) in the parathyroid glands. and in the kidney, which has been widely described to result in a decrease in PTH and an increase in urinary calcium excretion^{11,12}. The levels of calcium in plasma, which remained similar in both groups, could also be due to this excessive calciuria or to an adaptation of the body to the prolonged high intake of calcium in the diet. With respect to bone, animals fed a diet high in calcium showed a reduction in osteoblastic activity associated with the decrease in PTH and a tendency to decrease trabecular bone volume. It is interesting to note that in animals fed a diet deficient in vitamin D, osteosclerosis occurs at the trabecular level and the infusion of calcium and phosphorus results in a decrease in osteoblastic activity¹³. These observations support our results suggesting that increased calcium loading reduces osteoblasts on the bone surface. Furthermore, in our study, osteoclastic activity did not show significant differences compared to the group fed a diet with normal calcium content despite the decrease in PTH, suggesting that other mechanisms must be involved.

A limitation of this *in vivo* study is that no calcein-type marking with specific fluorochromes was performed in these animals. Therefore, formation and mineralization kinetic parameters could not be determined.

In this study the intake of a diet rich in calcium during 3 weeks did not produce significant changes in various parameters bone histomorphometry (volume trabecular

bone, volume osteoid separation trabecular and number of trabeculae) despite a decline significant of the osteoblastic activity and a similar osteoclastic activity.

In vitro, UMR-106 cells that were treated with high levels of calcium did not show changes in the gene expression of *Runx2*, *Osterix* and *Osteocalcin*. This suggests that the decrease in osteoblastic activity observed in bone is not directly influenced by a high calcium concentration, but could be more closely related to the decrease in PTH concentration. The rat osteosarcoma cell line UMR-106 is a widely used model with an osteoblastic phenotype in which the response to extracellular calcium and PTH has been well characterized^{14,15}. Previously, our group has published that the activation of CaSR by a calcimimetic increases osteogenesis and bone remodeling, therefore, presumably, its natural activators such as ionic calcium and others should have a similar effect¹⁶. In this previous study, the effect of calcimimetic on UMR-106 with a very low concentration of calcium (0.5 mM) was examined, so that treatment with the drug produced a more significant response. Therefore, in this *in vitro* experiment with UMR-106 with a normal calcium concentration (1.25 mM), in which the CaSR would be in a high degree of activation based on that described in parathyroid glands¹⁷, a high calcium (1.8 mM) should not produce a significant additional activation and therefore a significant increase in the expression of osteogenic genes would not be observed. Probably 1.8 mM calcium causes only a slight increase in CaSR activation with respect to 1.25 mM calcium, which only produces tendencies to increase *Osterix* and *Osteocalcin* as observed in this study.

In a study with young and healthy volunteers, the effects of the acute administration of 400 mg of oral calcium were evaluated and after 10 hours it was observed that the serum

Figure 1. Biochemistry in plasma and urine. Bars represent mean $\pm$ standard error. U of Mann-Whitney test. * $p < 0.05$ vs 0.6% Ca. ** $p < 0.01$ vs 0.6% Ca

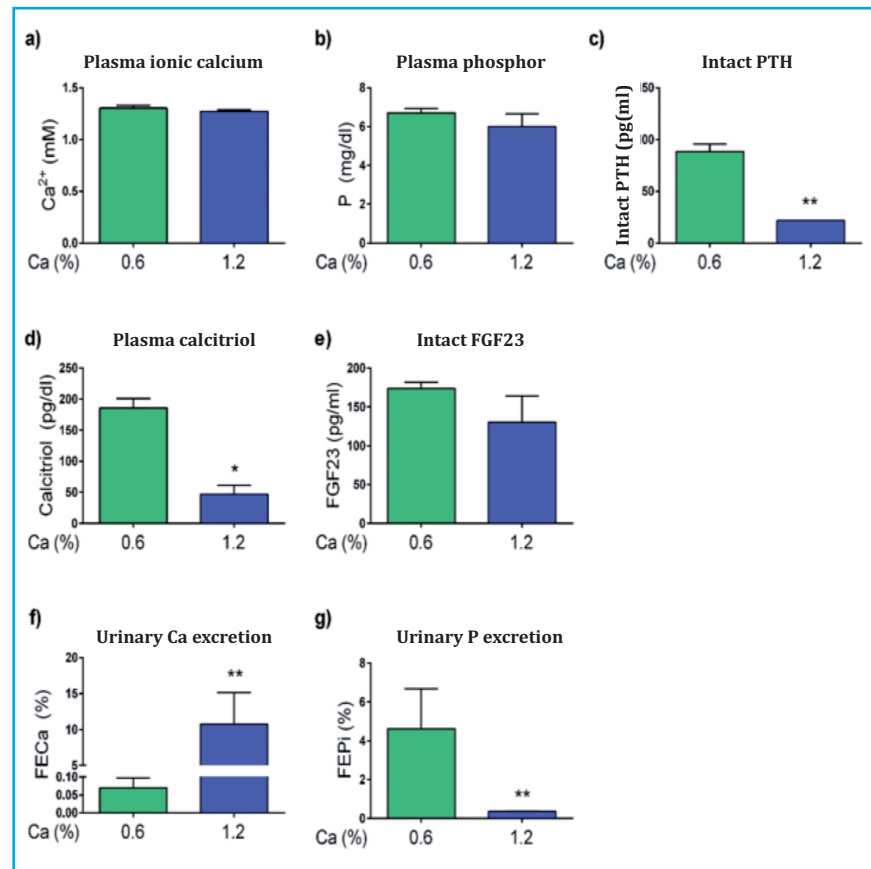


Figure 2. Bone histomorphometry analysis. Bars represent mean $\pm$ standard error. U of Mann-Whitney test. * $p < 0.01$ vs 0.6% Ca

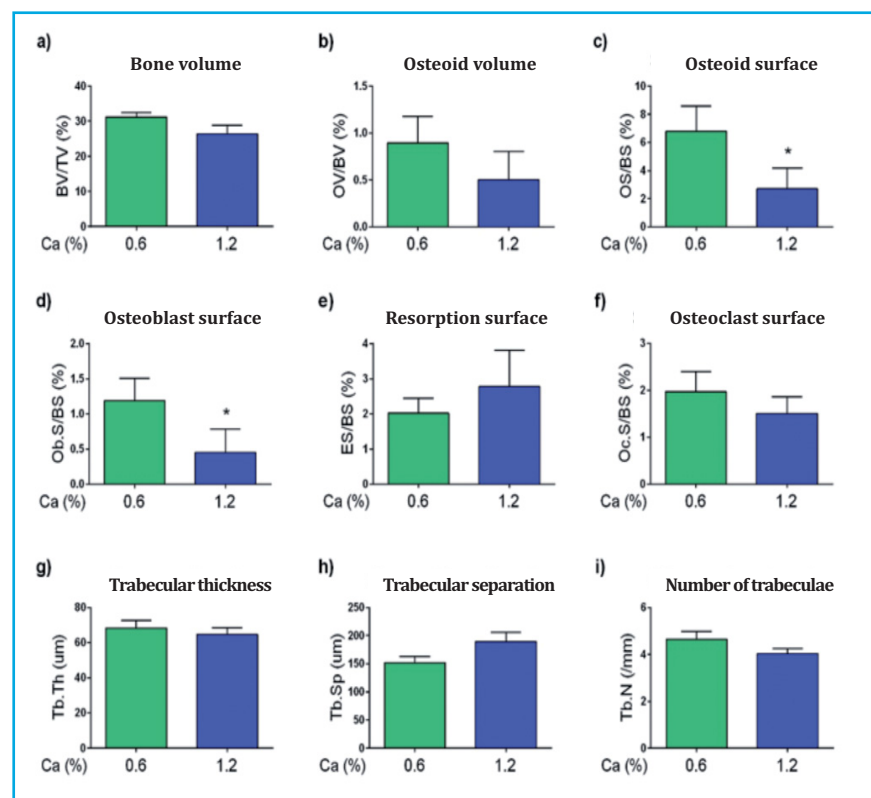
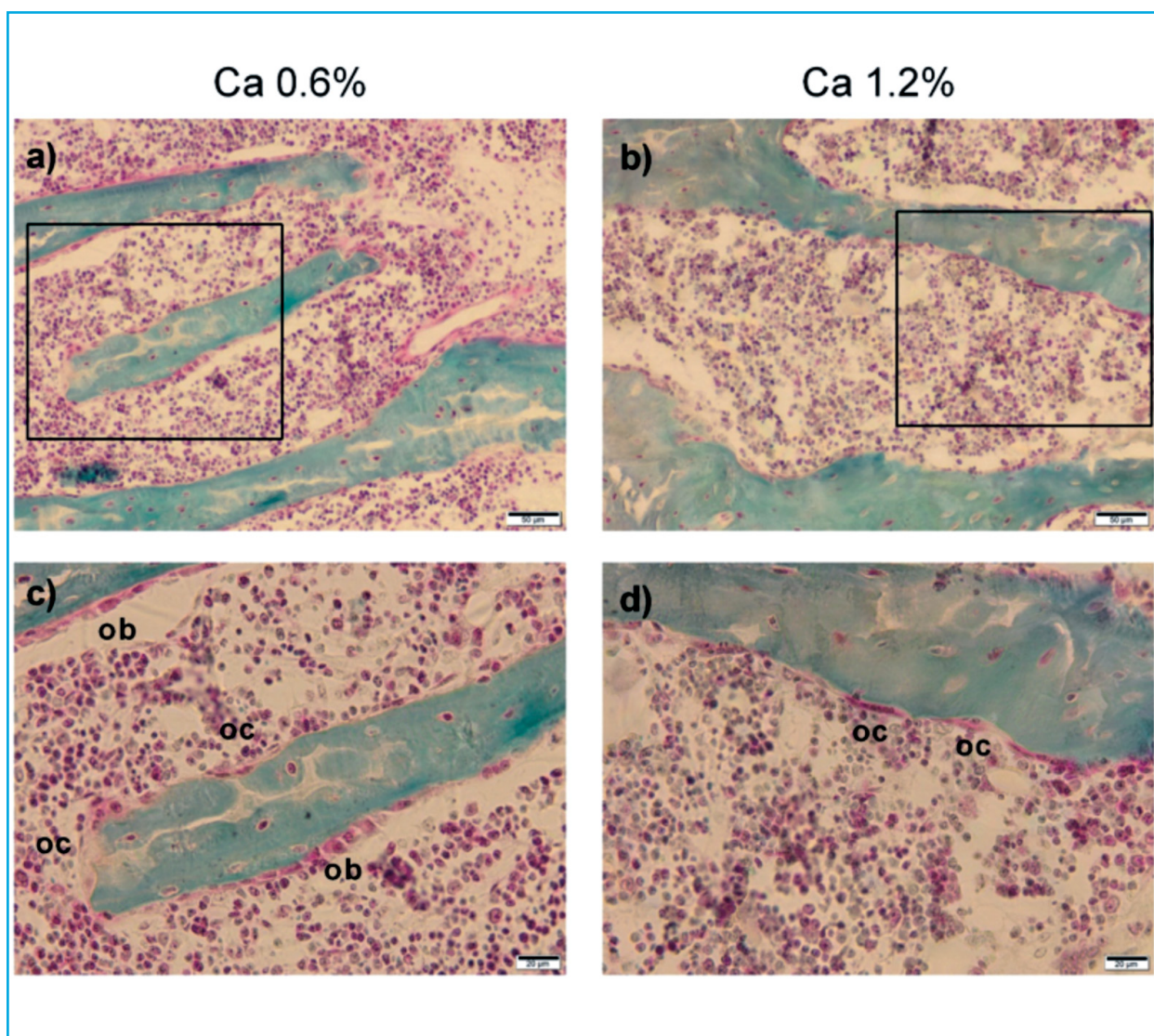


Figure 3. Photomicrographs of the bone samples stained with Goldner's trichrome. oc: osteoclasts. ob: osteoblasts. (a and b) magnification: 100x. Scale bar: 50 μ m. The inset indicates the area magnified in the following photomicrographs. (c and d) magnification: 200x. Scale bar 20 μ m

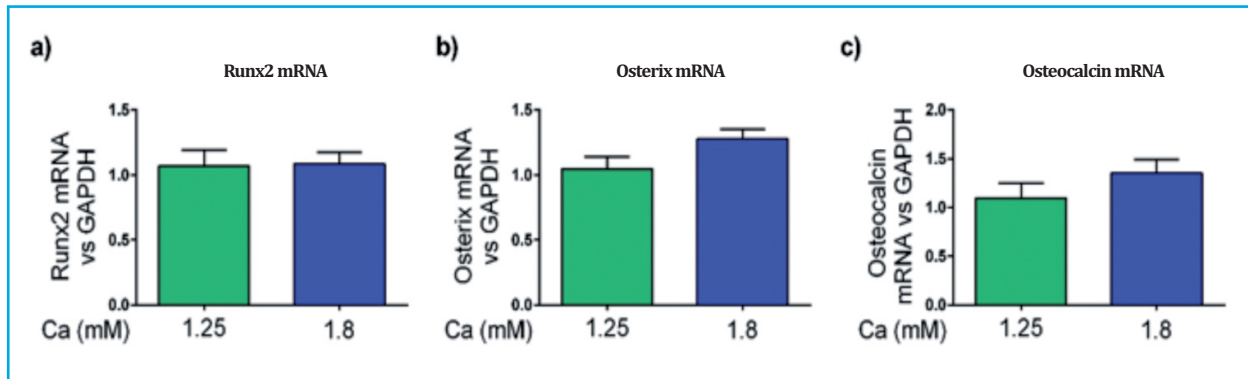


PTH concentration decreased, accompanied by a decrease in the serum levels of collagen telopeptides type I did not, however, show data related to bone formation¹⁸. This study supports our observations that dietary calcium supplementation reduces PTH levels, resulting in changes at the bone level. In this study acutely, the decrease in PTH produced a decrease in osteoclastic activity that we did not observe in our study with rats and that is probably due to prolonged treatment with a diet

rich in calcium. It is important to note that the expression of 25 (OH) D-1 α -hydroxylase is directly regulated by PTH¹⁹, and that therefore an increase in calcium intake would result in a decrease in calcitriol synthesis, consistent with the results obtained in our study.

In conclusion, a diet rich in calcium could lead to a reduction in osteoblastic activity due to a decrease in PTH production that would also result in a decrease in active vitamin D.

Figure 4. Expression of osteogenic genes in UMR-106 cells treated with high levels of calcium. Calcium chloride (0.1 M) was added until reaching levels corresponding to a situation of hypercalcemia (1.8 mM) and they were compared with normal levels (1.25 mM). Exposure to different levels of extracellular calcium was 48h. Bars represent mean $\pm$ standard error



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Conflict of interests: The authors declare no conflict of interest.

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