

## Effects of Calcium and Vitamin-D Supplementation on Bone Quality in an Ovariectomized Rat Model

Blanca Torrubia<sup>1</sup>, Marta Martín-Fernández<sup>1</sup>, Mercedes Rubert<sup>2</sup>, Marina Gómez-Chinchón<sup>1</sup>, Manuel Sosa<sup>3</sup>, Adolfo Díez-Pérez<sup>4</sup>, Paz Recio Visado<sup>5</sup>, Concepción de la Piedra<sup>1\*</sup>

<sup>1</sup>Biochemistry Research, Instituto de Investigación Sanitaria Fundación Jiménez Díaz, Madrid, Spain

<sup>2</sup>Hospital Support Team Palliative Care, Hospital Universitario de Móstoles, Móstoles, Madrid, Spain

<sup>3</sup>Osteoporosis Research Group, Instituto de Investigación Biomédica, University of Las Palmas de Gran Canaria, Las Palmas de Gran Canaria, Spain

<sup>4</sup>Department of Internal Medicine and Infectious Diseases, Hospital del Mar-IMIM, Universidad Autónoma de Barcelona, Barcelona, Spain

<sup>5</sup>Department of Physiology of Pharmacy University, Complutense University of Madrid, Madrid, Spain

Running title: Effects of calcium and vitamin D on osteoporosis

**\*Corresponding author:** Concepción de la Piedra, Instituto de Investigación Sanitaria Fundación Jiménez Díaz Bioquímica Investigación, Avenida Reyes Católicos 2, 28040 Madrid, Spain

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### Abstract

**Introduction:** Many osteopenic patients or patients with osteoporosis are treated only with calcium and vitamin D (vit D) as a preventive or therapeutic regimen. All the clinical trials about the use of osteoporosis treatments administered calcium and vit D to both control and intervention groups.

**Aim of the study:** To determine if treatment with calcium and vit D is useful in the treatment of osteoporosis. With this purpose we study the effects on bone quality produced by long-term treatment with calcium and vit D in ovariectomized rats.

**Methods:** Forty-five 6-month-old female Wistar rats were grouped as follows: SHAM (n=15), simulated intervention; OVX (n=15), ovariectomized; and OVX+(Ca+vit D) (n=15), ovariectomized treated with calcium and vit D (10 mg of calcium and 6.7 IU of vit D/kg/day by oral gavage). The rats were sacrificed 8 months after treatment. We measured femoral (F) and lumbar (L) Bone Mineral Density (BMD), biochemical markers of bone turnover, and trabecular microstructure, irregular structures through fractal dimension analysis in 2D (D2D) and 3D (D3D) and biomechanical testing by finite elements analysis.

**Results:** FBMD and LBMD decreased in OVX rats, with no changes observed in treated rats. Ovariectomy produced an increase in osteocalcin values and carboxyterminal telopeptide/tartrate resistant acid phosphatase, producing no differences in aminoterminal propeptide of procollagen I; These variations did not change after treatment. Ovariectomy produced a decrease in bone microstructural parameters maintained under treatment with calcium and vitamin D. D2D decreased significantly in the OVX group, while no changes were evidenced on D3D; no variations were seen after treatment with calcium and vit D. The apparent elastic modulus in the x, y, and z direction was significantly decreased in the OVX group, with no differences after treatment. Ovariectomy significantly increased the weight of the rats. Treatment with calcium and vit D prevented this increase.

**Conclusion:** Long-term treatment with calcium and vit D did not revert alterations in bone quality caused by ovariectomy.

**Keywords:** Calcium; Ovariectomy; Vitamin D

## Introduction

Calcium is the most important nutrient for achieving optimal bone mass as well as preventing age-related loss of bone mass [1]. In addition, calcium plays an essential role in the normal functioning of a wide variety of body tissues and physiological processes, including muscle contraction, blood coagulation, nerve transmission, and in the role of second messenger, controlling the activity of various hormones [2]. In the United States, the recommended calcium intake is 1000 mg/day up to age 50 and 1200-1500 mg thereafter [3]. In Europe, the recommended intake is somewhat lower, at 700-800 mg/day at any age and 800 mg/day for women aged 50-65 years [4]. Worldwide, however, the daily dietary intake of calcium is below these recommendations. In recent years there has been a renewed interest in the effects of vitamin D status on health. Although deficiencies in vitamin D status have been classically associated with bone pathology, the discovery that nearly all body tissues and cells have Vitamin D Receptors (VDR) and an enzyme system to synthesize  $1,25(\text{OH})_2\text{VitaminD}$  ( $1,25(\text{OH})_2\text{D}$ ) has made it clear that vitamin D carries out other functions, such as inhibition of cellular proliferation, angiogenesis, and renin production, the stimulation of cellular maturation, and the regulation of immune response. For this reason, low levels of vitamin D have been associated with mortality, cancer, cardiovascular disease, autoimmune diseases, diabetes, psychiatric illness, and even respiratory disease in addition to the previously known effects on bone metabolism (osteomalacia, secondary hyperparathyroidism, osteoporosis, and fracture) [2,5].

In the general population, there is a high prevalence of vitamin D deficiency [6], creating a need for vitamin D supplementation. Osteoporosis is presently the most common metabolic bone disease and one of the most pressing global public health problems due to its huge social and economic burden. All the recommendations for the treatment of osteoporosis recommended therapy with antiresorptive agents, or anabolic agents such as PTH 1-84 or PTH 1-34 (7). At this point, it is important to note that all the clinical trials upon which these recommendations are based administered calcium and vitamin D to both control and intervention groups [7]. Due to economic reasons, many osteopenic patients or patients with osteoporosis, especially in their initial stages in primary attention, are treated only with calcium and vitamin D as a preventive or therapeutic regimen in order to avoid the development of the disease and future fractures. The aim of this work is to determine the effectiveness of calcium and vitamin D in the treatment of osteoporosis. For that, we studied an experimental model of postmenopausal osteoporosis consisting of ovariectomized rats treated with calcium and vitamin D over a prolonged time period (8 months) assessing the effects of these two substances on Bone Mineral Density (BMD), bone microstructure, bone turnover, and

biomechanical parameters.

## Materials and Methods

### Animals

Forty-five 6-month-old female Wistar rats weighing  $328 \pm 50$  g (mean  $\pm$  SD) were used in this study. The animals were kept under constant living conditions (22°C, 12-hour light-dark cycles per day), and food (standard laboratory chow) and water were available ad libitum. The animals were randomized into the following groups: SHAM (n=15), simulated intervention; OVX (n=15), ovariectomized; OVX+(Ca+vit D) (n=15), ovariectomized and treated with calcium and vitamin D (25 mg/kg/day of Natecal D Flash®, Italfarmaco, Milan, Italy; by oral gavage) over 8 months. Natecal D Flash® is composed of calcium carbonate and cholecalciferol. Twenty-five mg of Natecal D Flash® is equivalent to 10 mg of calcium and 6.7 IU of vitamin D. Treatment began first day after ovariectomy. The diet of the rats, SAFE A04 (Augy, France), contained 7.3 g/kg of calcium and 1000 IU/kg of vitamin D3. No animals died during the study. One day after the last treatment, the experimental animals were weighed and killed by exsanguination under isoflurane (Florane®) anesthesia. Blood samples were obtained by cardiac puncture and serum samples were immediately frozen at -80°C as aliquots until determination of biochemical markers of bone turnover. Once the blood was collected, the animals were frozen at -20°C until determination of BMD in previously thawed animals. Prior to BMD analyses, the left femurs were excised and cleaned of adjacent tissue for BMD determination. Right femurs were also excised and cleaned of adjacent tissue and used for computerized micro tomography (micro-CT) and biomechanical testing. Lumbar spine BMD was determined in situ. It has been shown that the use of repeated freeze-thaw cycles does not influence the structural properties of bone [8]. All procedures were carried out in accordance with European Community standards on the care and use of laboratory animals and after approval of the Ethics Committee of Instituto de Investigación Sanitaria Fundación Jiménez Díaz.

### Bone Mineral Density

BMD was determined in situ in the lumbar spine (L2, L3, and L4) and in the entire right femur by Dual-Energy X-Ray Densitometry (DEXA) using a Piximus densitometer (HOLOGIC QDR-1000 TM) with small-animal software. Intra- and inter-assay coefficients of variation (CV) were  $<0.53\%$  and  $<1.2\%$ , respectively. The scans of the femur were analyzed to determine the BMD of the whole femur. Scans of the L2, L3, and L4 vertebrae were analyzed for BMD and the results were expressed as the mean of the values obtained.

### Biochemical markers of bone turnover

Serum Bone Gla Protein (BGP) was measured by ELISA for

specific quantitative determination of rat osteocalcin levels (Rat-MID Osteocalcin, IDS, UK). The sensitivity of this assay was 50 ng/ml. Intra- and inter-assay coefficients of variation of the method were <5.0% and <6.6%, respectively. Serum procollagen type I N-terminal propeptide (PINP) was measured by a specific ELISA for both rat and mouse PINP (Rat/Mouse PINP EIA, IDS, UK). The sensitivity of this assay was 0.7 ng/ml. The intra- and inter-assay coefficients of variation were <7.4% and <8.0%, respectively. Serum 5b isoenzyme of tartrate-resistant acid phosphatase (TRAP) was measured by an ELISA specific for rat TRAP (RatTRAP Assay, IDS, UK). The sensitivity of the assay was 0.1 U/L. The intra- and inter-assay variation coefficients for the method were <5.0% and <5.5%, respectively. Serum C-Telopeptide of Type I Collagen (CTX) was measured using a specific ELISA assay for rat CTX (RatLaps ELISA, IDS, UK). The sensitivity of the assay was 2.0 ng/ml. Intra- and inter-assay variation coefficients of the method were <5.6% and <10.5%, respectively.

### Trabecular Microarchitecture Analysis of Femur

Trabecular bone microarchitecture was performed using the QUIBIM SL system (Quantitative Imaging Biomarkers in Medicine, Valencia, Spain) with computerized micro tomography. We used a GE system for *in vitro* samples (eXplore Locus SP<sup>®</sup>, General Electric, USA), which includes technology for plane detection by means of cone-beam CT. The spatial resolution of the acquired images was isotropic, with voxels of 50×50×50 μm. Although the voxel had a limited spatial resolution of 50μm, this was considered as adequate for the study of trabecular thickness assuming that the measurement would take into account partial volume effect and that previous studies have used larger voxels (290μm) to measure human trabecular thickness (80-150μm), obtaining significant correlation with mechanical properties [9]. Other studies have used an isotropic voxel of 180μm interpolated to 90μm [10]. Primary spongiosa was excluded in the analysis. Segmentation of the μCT samples was performed by placing a rectangular region of interest and automatically propagating this position to the rest of the slices, verifying that all the regions were segmented in each femur, that is, femoral head, trochanter, and distal femoral metaphysis (near the knee). Otsu's method was used for thresholding intensities [11]. The following morphometric parameters of trabecular bone were determined: Bone Volume/Total Bone Volume (BV/TV), Trabecular Thickness (Tb. Th), Trabecular Number (Tb. N), and (Tb. Sp). We also calculated irregularity through fractal dimension analysis in 2D (D2D) and in 3D (D3D), expressing the degree of complexity of the contour of a structure in the fill of a surface or a volume, respectively [12].

### Biomechanical Analysis

Samples of trabecular bone reconstructed from μCT images were subjected to simulation of mechanical compression by the

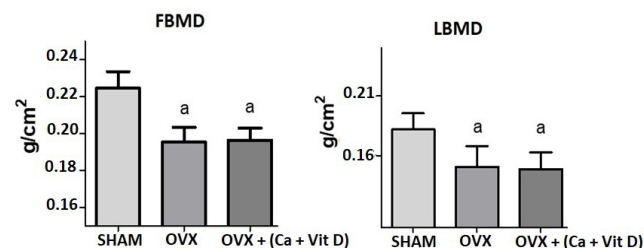
method of finite elements (FEA)0. Static simulations of uni-axial compression in the 3 axes were performed on the different virtual samples obtained by tension-deformation assay. Properties of the basic material were defined from those of compact bone (Young module E=10 GPa; Poisson coefficient, σ=0.3). In the simulations of compression assays, null displacement on nodes from one side, and a displacement of 1% of the edge length of the opposite side was applied. Using the resolution of the equation systems, the apparent elastic modulus of the structure was obtained in three directions (Ex, Ey, Ez) [13].

### Statistical Analysis

The results of the experiments were expressed as the mean ± SD of the different parameters. The Mann-Whitney test (Medcalc Software Program, Belgium), a nonparametric method, was used to compare the different treatment groups. A p-value <0.05 was accepted as denoting a significant difference.

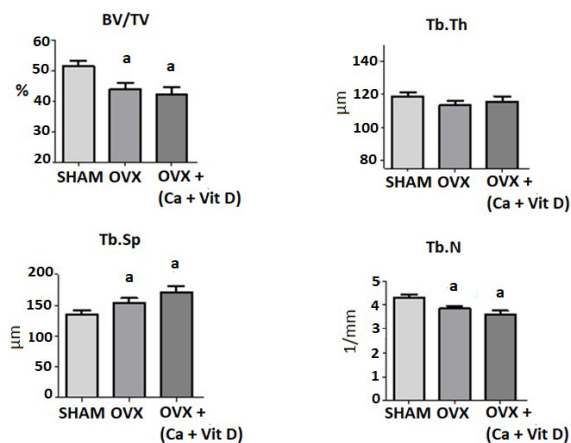
### Results

Compared to the SHAM group, FBMD and LBMD were significantly decreased in OVX rats (p<0.001). Administration of Ca+Vit D to OVX rats did not produce any significant change in FBMD or in LBMD with respect to the untreated OVX group (Figure 1).



**Figure 1:** FBMD and LBMD in a group of SHAM operated, ovariectomized, and ovariectomized rats treated with calcium and vitamin D (Ca+ Vit D) over a period of 8 months. Data are expressed as mean ± SD. Statistical significance p<0.001: (a) vs. SHAM.

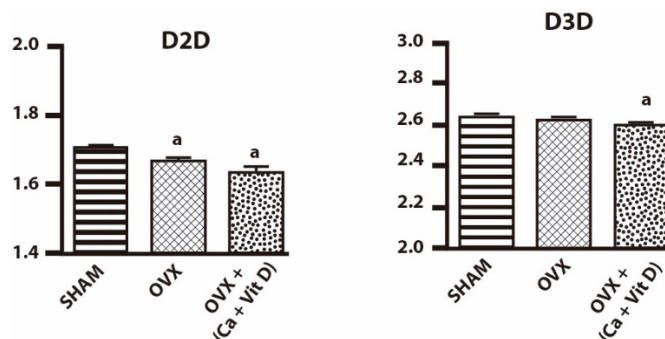
Figure 2 shows bone microstructural parameters. Eight months after surgery, most of the micro-CT variables characterizing bone structure were significantly different in the OVX group relative to the SHAM group. OVX rats presented a significant decrease in BV/TV in the femora (p< 0.01). This decrease in BV/TV was due to a significant increase in Tb. Sp (p<0.05) and a decrease in Tb. N (p<0.01), without differences in Tb.Th. Administration of Ca+VitD did not correct the state of OVX rats, and no significant differences were found with regard to the untreated OVX group (Figure 2).



**Figure 2:** Femoral trabecular microarchitecture analysis: Bone volume/total volume (BV/TV), Trabecular Thickness (Tb. Th), Trabecular Separation (Tb. Sp), and Trabecular Number (Tb. N). SHAM operated, ovariectomized, and ovariectomized rats treated with calcium and vitamin D (Ca+ Vit D) OVER a period of 8 months. Data are expressed as mean  $\pm$  SD. Statistical significance  $p < 0.001$ : (a) vs. SHAM. Data are expressed as mean  $\pm$  SD. Statistical significance:  $p < 0.01$  (a) vs. SHAM. OVX vs. Ca+Vit D: BV/TV  $p < 0.01$ ; Tb.Sp  $p < 0.05$ ; Tb N  $p < 0.01$ .

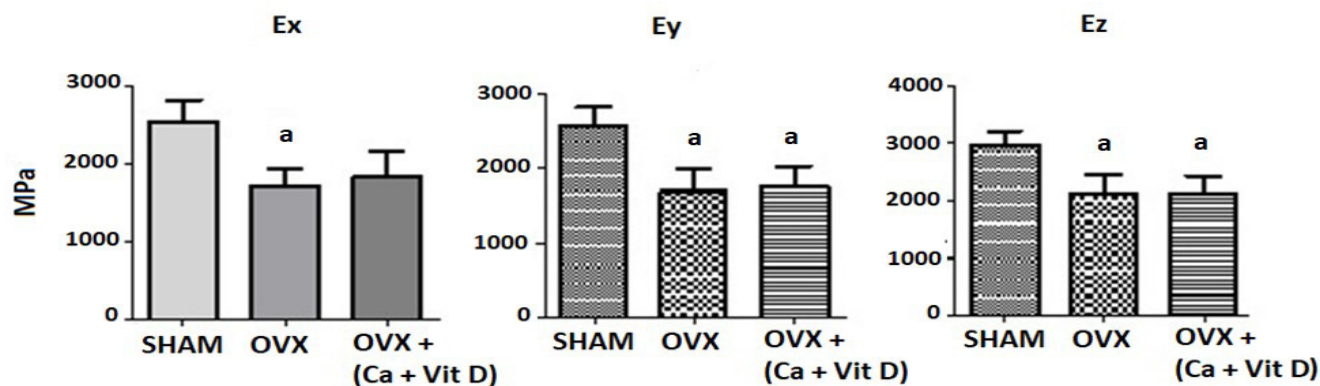
D2D decreased significantly ( $p < 0.05$ ) in the OVX group compared to the SHAM group, without significant changes in

D3D. OVX rats treated with Ca+Vit D showed the same decrease in D2D and a significant decrease with respect to the SHAM and OVX groups on D3D (Figure 3).



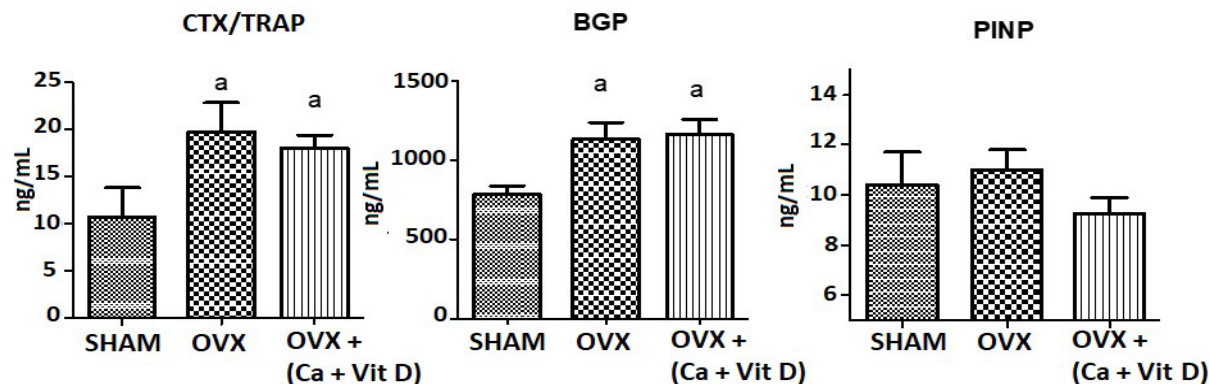
**Figure 3:** Fractal dimension in 2D (D2D) and in 3D (D3D) in a group of SHAM operated, ovariectomized, and ovariectomized rats treated with calcium and vitamin D (Ca+ Vit D) over a period of 8 months. Data are expressed as mean  $\pm$  SD. Statistical significance  $p < 0.05$ : (a) vs. SHAM.

The apparent elastic modulus in the three spatial directions (Ex, Ey, and Ez) was significantly decreased in OVX rats with respect to the SHAM group ( $p < 0.01$ ). OVX animals treated with Ca VitD had Ex values that did not differ significantly when compared to the SHAM or OVX groups (Figure 4).



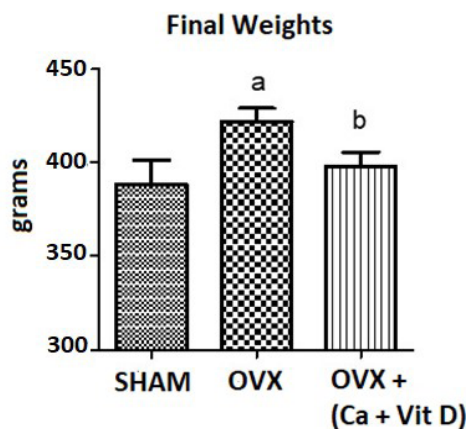
**Figure 4:** Apparent elastic modulus in the x, y, and z spatial direction (Ex, Ey and Ez) in a group of SHAM operated, ovariectomized, and ovariectomized rats treated with calcium and vitamin D (Ca+ Vit D) over a period of 8 months. Data are expressed as mean  $\pm$  SD. Statistical significance  $p < 0.05$ : (a) vs. SHAM.

Ovariectomy produced an increase in BGP values ( $p < 0.01$ ) and CTX/TRAP ( $p < 0.001$ ) without causing differences in PINP levels. Treatment with Ca+VitD did not produce any difference in the OVX rat group with respect to the OVX group (Figure 5).



**Figure 5:** Biochemical markers of bone turnover: serum osteocalcin (BGP), telopeptide carboxyterminal of collagen/isoenzyme  $\beta$  of tartrate resistant acid phosphatase (CTX/TRAP) and propeptide carboxyterminal of procollagen I (PINP) in a group of SHAM operated, ovariectomized, and ovariectomized rats treated with calcium and vitamin D (Ca+ Vit D) over a period of 8 months. Data are expressed as mean  $\pm$  SD. Statistical significance:  $p < 0.001$  (a) vs. SHAM; OVX vs. OVX + Ca+VitD: BGP  $p < 0.01$ ; CTX/TRAP  $p < 0.001$ .

Ovariectomy significantly increased the weight of rats ( $p < 0.05$ ), though treatment with Ca+VitD avoided this increase in OVX-treated animals (Figure 6).



**Figure 6:** Body weight in a group of SHAM operated, ovariectomized, and ovariectomized rats treated with calcium and vitamin D (Ca+ Vit D) over a period of 8 months. Data are expressed as mean  $\pm$  SD. Statistical significance  $p < 0.05$ : (a) vs. SHAM, (b) vs. OVX.

## Discussion

Calcium plus vitamin D supplementation has been widely recommended to prevent osteoporosis and subsequent fracture [14]. However, considerable controversy exists about its efficacy. There is a large number of works about the effects of calcium and vitamin D in patients with osteoporosis. However, it is difficult in these cases to have a homogeneous population. This is the reason to make this study in an ovariectomized rat model, a reference pattern in the study of osteoporosis [15]. As expected, we found a decrease in FBMD and LBMD, a decrease in trabecular number, increased

trabecular separation, a decrease in D2D fractal dimension, and a decrease in the biomechanical parameters Ex, Ey, and Ez as a result of ovariectomy. Ovariectomy also produced an increase in bone remodeling as reflected in BGP and CTX/TRAP values. Although PINP levels were not changed, we could not rule out that they were previously elevated during the eight-month period following ovariectomy. The results of our work show that treatment with calcium and vitamin D over a long period of time did not produce any effect on BMD, bone microstructure, biochemical markers of bone turnover, or biomechanics in ovariectomized rats. These

results were analyzed at the end of treatment, and we cannot rule out the possibility that these changes took place in earlier stages of the disease.

Previous research supports the use of calcium and vitamin D to prevent fracture risk in osteoporosis. Weaver, et al. [16] conducted a meta-analysis of randomized controlled trials of calcium plus vitamin-D supplementation for fracture prevention in adults. The study shows that calcium plus vitamin-D supplementation produced a statistically significant 15% reduction in risk of total fractures and a 30% reduced risk of hip fracture. This study supports the use of calcium plus vitamin-D supplements as an intervention for fracture-risk reduction in both community dwelling adults and institutionalized middle-aged to elderly adults. Pascalis, et al. [17] analyze a number of bone-quality indices in patients who received long-term Vitamin-D (400-1200 IU) and calcium (1.0-1.5 g) supplementation versus those who did not. The study was a part of the HORIZON trial. The mineral/matrix ratio and glycosaminoglycan content were higher, while Nano porosity (a surrogate for tissue water content), mineral maturity/crystallinity, and pyridinoline content were lower in patients without long-term supplementation. The authors conclude that vitamin D and calcium administration were associated with altered mineral and organic properties.

Aloia, et al. [18] performed a study to evaluate the influence of calcium and vitamin D on PTH and bone turnover. One hundred fifty-nine healthy postmenopausal women participated in a 6-month, double-blind, placebo-controlled study in which patients were grouped as follows: a) double placebo, 2) 1200 mg/day calcium, 3) 100 µg, and 4) vitamin D<sub>3</sub> and calcium. Fasting PTH declined with vitamin D supplementation, and PTH declined after calcium intake. Dietary supplementation with 1200 µg calcium/day reduced CTX and PINP, whereas supplementation with up to 100 µg of vitamin D<sub>3</sub> did not. Avenell, et al. [19] reviewed 53 trials in which a total of 91 791 participants were included to determine the effects of vitamin D with or without calcium for preventing fracture in post-menopausal women and older men. High-quality evidence indicates that when administered alone and in the formats and doses tested, vitamin D is unlikely to be effective in preventing hip fracture. Quality evidence also suggests that vitamin D plus calcium results in a slight reduction in hip-fracture risk. It has also been found that vitamin D plus calcium is associated with a statistically significant reduction in the incidence of new non-vertebral fractures. However, they found only moderate-quality evidence of an absence of a statistically significant preventive effect on clinical vertebral fractures.

It is important to note that many studies that have investigated the effects of combined calcium and Vitamin-D supplementation on postmenopausal women have shown a reduction in fracture risk, provided sufficient patient compliance (75-85%) was reached

[14]. Lips, et al. [20] reviewed 19 randomized clinical trials of vitamin D administered with or without calcium. Vitamin-D supplementation of 800 IU/day in combination with calcium showed good compliance and may decrease the incidence of non-vertebral fractures, especially in older individuals, groups having low baseline Vitamin-D status and low calcium intake and also showing good compliance. However, analysis of vertebral fractures was negative in detecting reduction in all cases. Boonen, et al. [21] conducted a study in women and men ≥ 50 years who were at risk for hip fracture. They studied 9083 patients treated with vitamin D and 45 509 treated with vitamin D and calcium. Their results suggest that oral vitamin D reduces the risk of hip fracture when calcium supplementation is added.

The Women's Health Initiative (WHI) carried out a double-blind, placebo-controlled trial [22] in which 36 282 postmenopausal women living in the US were randomly assigned to a regimen consisting of 1000 mg of elemental calcium carbonate plus 400 IU of vitamin D daily or placebo. This study, which reports an average intervention period of 7 years, found that long-term use of calcium and vitamin D appears to cause a possibly significant reduction in the risk of hip fracture among postmenopausal women. However, there are other reports in which treatment with calcium and vitamin D do not seem to provide any benefit in the number of bone fractures. Michaelson, et al. [23] studied 60 689 women aged 40-74 years over a 4-year period. During follow-up, the authors observed that 1535 women had sustained hip fractures. Women with an estimated calcium intake below 400 mg/day, intermediate calcium intake, and calcium intake above 1200 mg/day had a similar age-adjusted hip-fracture risk, and Vitamin-D intake was not associated with fracture risk. Grant, et al. [24] studied 5292 people aged 70 years or older who had experienced a low-trauma fracture. Study patients were randomly assigned to daily administration of vitamin D, 1000 mg/day of calcium, both, or placebo. The groups did not differ in the incidence of all-new fractures or hip fractures. Kahwati, et al [25] recently carried out an extensive review that included 47 676 patients regarding primary prevention of fractures in adults with vitamin D, calcium, or combined supplementation. The authors found that supplementation with vitamin D and calcium for 3 to 7 years had no statistically significant effect on total fracture incidence.

Here it is worth mentioning how treatment with calcium and vitamin D could influence the course of other diseases. Cesario, et al. [26] concluded that it is currently not possible to ensure that calcium supplements given with vitamin D do not cause adverse cardiovascular events or link these supplements to increased cardiovascular risk. However, other authors such as Kahwati, et al. [25], Kopecky, et al. [27], and Prentice [22] stated that vitamin D with calcium does not increase all-cause mortality from cardiovascular events. Kahwati, et al. [25] and Avenell, et al. [19] suggest that vitamin D with calcium is associated with

an increase in the incidence of kidney stones. Grant, et al. [24] observed that treatment with calcium and vitamin D had no effect on death, number of falls, or quality of life. In sum, there are two opposing lines of opinion: one supporting that supplementation with calcium and vitamin D should be indicated for osteoporotic women and those at risk of osteoporosis and the opposite position, that is that oral supplementation with calcium and vitamin D is not useful in the treatment of osteoporosis or prevention of fractures. In our work, we failed to find any positive effect of calcium and vitamin D on BMD, bone microstructure, biochemical markers of bone turnover, and biomechanics in ovariectomized rats. According to previous research produced by our group, the rats were osteopenic at least from three months after ovariectomy [28]. The only positive effect of treatment was the absence of excess weight presented in ovariectomized rats. We have no explanation for this result.

## Limitations of The Study

The study was done in rats and this could be a limitation. Ovariectomized rats is considered an adequate research model to study postmenopausal osteoporosis. We used rats in order to have a homogeneous population, very difficult to have in the case of a study in osteoporotic women.

## Conclusion

According to our results, calcium and Vitamin-D supplements do not have any positive effect on bone quality in ovariectomized rats. Due to this fact, we can affirm that calcium and vitamin D supplements are not useful in osteoporosis treatment and they are not necessary in all the cases. However, we have not studied the possible effects of these treatments on the many other metabolic pathways that are important for life in which calcium and vitamin D play a role. Moreover, a substantial percentage of the world population presents Vitamin-D deficit [29].

Recommendations: These findings do not support treatment with calcium and vitamin D in all the patients with osteoporosis. However, Vitamin-D treatment may be necessary in osteoporotic or non-osteoporotic patients with Vitamin-D levels below 24 ng/ml [30], mainly to prevent secondary hyperparathyroidism and a review of calcium intake in the general population is necessary to ensure enough intake.

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