

EFFECTS OF ZOLEDRONIC ACID ON OOFORECTOMIZED RATS' TIBIAE: A PROSPECTIVE AND RANDOMIZED STUDY

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ABSTRACT

Objective: To investigate clinical, biomechanic and histomorphometric effects of zoledronic acid on osteoporotic rats' tibiae after bilateral ooforectomy. **Methods:** 40 female Wistar (*Rattus norvegicus albinus*) rats were prospectively studied. On the 60th day of life, the animals were randomized into two groups according to the surgical procedure: bilateral ooforectomy (O) (n=20) and sham surgery ("sham") (P) (n=20). After 30 days, the animals were divided into four groups, according to the administration of zoledronic acid (ZA) 0.1mg/kg or distilled water (DW): OZA (n=10), ODW (n=10), PZA (n=10) and PDW (n=10). After 12 months, the animals were sacrificed, and had their tibiae assessed. In the clinical study, animals' weight was considered; in the biomechanical study, compressive assays were applied and, in the histomorphometric analysis, the bone trabecular area was

determined. Results: "O" groups showed a significantly greater weight gain than "P" groups (p=0.005). Groups OZA and PZA showed an insignificant weight gain when compared to ODW (p=0.47) and PDW (p=0.68). The groups receiving zoledronic acid and distilled water were able to bear maximum load, similar (p=0.2), at the moment of fracture. In the groups receiving zoledronic acid, an insignificant increase of the bone trabecular area was found when compared to the groups receiving distilled water (p=0.21). There was a positive correlation between trabecular area and maximum load (p=0.04; r=0.95). Conclusion: Zoledronic acid did not significantly influence animals' weight. The results showed an insignificant increase both of the tibial shaft bone resistance and the bone trabecular area.

Keywords – Osteoporosis; Biomechanics; Tibia; Experimental epidemiology; Wistar rats; Prospective studies

INTRODUCTION

The technological changes, medical advances, and urbanization that have occurred in Brazil during the last century have led to an increased life expectancy of the general population allowing it to age (*)^(1,2). More so than demographics alone, the significant increase in the elderly portion of the population requi-

res new and efficient investments in public health. Chronic and degenerative diseases are becoming increasingly common, and among them, osteoporosis is playing an important role⁽³⁾.

Caused mainly by the loss of the homeostatic balance of bone tissue, osteoporosis is an asymptomatic disease. In most cases, the disease manifests itself

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initially by the occurrence of fractures, especially of the vertebrae, femur, and distal radius⁽⁴⁻⁶⁾.

Prevention is the treatment of choice. It is well-known that building bone reserves during youth is a primary factor for the onset of osteoporosis⁽⁵⁻⁷⁾. In some cases, drug therapy becomes the most effective measure to reverse or, more commonly, to prevent disease progression^(5,8,9). There are currently two major classes of drugs: inhibitors of bone resorption and bone formation stimulants⁽⁸⁾.

The most well established representatives of the class of inhibitors of bone resorption are bisphosphonates, which by blocking osteoclastic activity are especially useful in diseases that manifest by rapid bone turnover^(10,11). Among the drugs in this therapeutic class, those called the amino-bisphosphonates or third-generation bisphosphonates, are up to 10,000 times more potent than the best known bisphosphonates⁽¹²⁻¹⁴⁾.

Zoledronic acid (zoledronate), a new bisphosphonate, with a dose of 5 mg per year via intravenous infusion, is indicated for being able to reduce the side effects of the daily use of this pharmacological class and to eliminate the low adherence of patients to treatment^(15,16).

The few existing publications relate zoledronic acid to the prevention of osteoporotic fractures in the spine and proximal femur⁽¹⁷⁻¹⁹⁾. The aim of this study was to evaluate the effect of zoledronic acid in a single annual dose on the tibial diaphysis of oophorectomized rats through clinical, biomechanical, and histomorphometric studies.

METHODS

All procedures were approved by the Animal Experiments Ethics Committee of the Botucatu School of Medicine, SP, under the case number 622/2007. Forty sexually mature virgin Wistar rats (*Rattus norvegicus albinus*) from the Central Vivarium of the São Paulo State University (UNESP, Universidade Estadual Paulista "Julio de Mesquita Filho"), Botucatu Campus, were used.

After being clinically assessed and weighed, the animals were housed in groups of five in eight polypropylene cages with metal screen covers and lined with autoclaved pine shavings. These were cleaned

daily and kept in a cool dry place with 24°C controlled temperature and 12 hour light/dark cycles. Animals were offered rodent feed (Labina®, Nestle Purina PetCare Company[©]) and water *ad libitum*.

At 60 days, after reaching sexual maturity, rats were identified by perforations on the right and left auricular region and randomized, by drawing of sealed opaque envelopes, into two groups according to the surgical procedure to which they would be submitted: the oophorectomy group (O) (n = 20) and the sham surgery group (P) (n = 20).

The castration procedure was performed after intraperitoneal anesthesia, using 30 mg/kg of sodium pentobarbital 3% and bilateral trichotomy just below the last rib, dorso-laterally. The animals underwent washing with soap and water, antisepsis with a tincture of polyvinylpyrrolidone-iodine (PVPI-tincture), and were positioned on the operating table in lateral recumbency. After placement of a sterile field eyepiece, a longitudinal incision approximately 1.5 cm in length was made between the last rib and the hip joint. With the aid of tweezers, the peritoneal cavity was exposed, surpassing the muscular plain by divulsion, allowing access to the ovary surrounded by adipose tissue (Figure 1).

The ovary was ligated with 3.0 cotton thread and sectioned distal to the ligation. The musculature and skin were approximated with 4.0 nylon suture, and the same procedure was repeated on the contralateral side to remove the other ovary. The animals in group P underwent the same surgical procedures described, excepting the time of surgical ligation and sectioning of the ovaries.

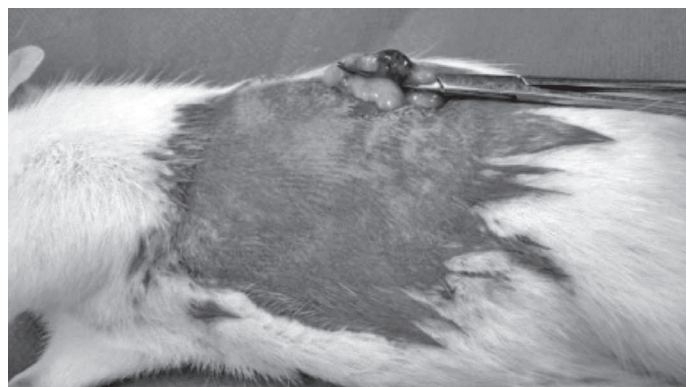


Figure 1 – Appearance of the clamping of the ovary in the middle of adipose tissue prior to ligation and section.

1. (*) Brazilian Institute of Geography and Statistics (IBGE, Instituto Brasileiro de Geografia e Estatística). Projection of the Brazilian population. Brazil already has more than 180 million inhabitants. [Social communication, August 30, 2004]. IBGE 2004.

At 90 days of life and after a new randomization, groups O and P were divided into four subgroups according to the intraperitoneal administration of either the recommended dose of 0.1 mg/kg^(17,20) zoledronic acid (ZA) (Aclasta™, Novartis® Biosciences Inc.) or distilled water (DW), as follows: OZA (n = 10), ODW (n = 10), PZA (n = 10) and PDW (n = 10). A sterile insulin syringe was used to administer the substances, with Injex Stilly Line® fixed needles (1ml/cc, 0.30 x 12.7 mm – 30G 1/2” needle).

Twelve months after the administration of zoledronic acid or distilled water, the animals were euthanized with a lethal intraperitoneal dose of 80 mg/kg of sodium pentobarbital 3%.

After euthanasia, the tibiae of the animals were disarticulated at the proximal (knee) and distal (ankle) regions, with the soft tissues (muscles, tendons, and ligaments) removed. For biomechanical testing, the right tibiae were wrapped in aluminum foil, labeled, and frozen for 24 hours in a domestic refrigerator at a temperature of -20°C. The left tibiae were placed in clean and properly identified glass containers, and fixed in 10% formaldehyde solution for histomorphometric study.

Clinical study: performed by analysis of the animals' body mass(g). Measurements were made monthly and always on the same day throughout the experiment, using a digital scale with a capacity of six kilograms-force and a range of 5 g. The scale was calibrated quarterly by trained personnel.

Biomechanical study: to determine the mechanical properties of the tibiae, bending tests were conducted at three points, using an EMIC® universal testing machine model DL 10,000, with an accuracy of (0.018 + F/3700)KN, ascertained in accordance with ABTN, NBR6156, and NBR6674 standards. The machine operates in conjunction with a computer under the Windows™ 2000 operating system, under which the Mtest version 1.00 program was used for measuring the results. In the 12 hours prior to biomechanical testing, the right tibiae were thawed and kept in bandages soaked in 0.9% saline. The bones were individually supported horizontally at their ends on two wooden blocks. The standard distance between the two points of support was set at 2/3 the length of the specimen. The blade was positioned halfway between the ends on the concave face of the tibiae (Figure 2). To determine the maximum load supported by the body, the blade

was set into motion at a speed of 30mm/min^(21,22). The calculation of the maximum load was performed automatically by the program (Chart 1).



Figure 2 – Detail of the specimen at rest and the load application blade positioned halfway between the ends.

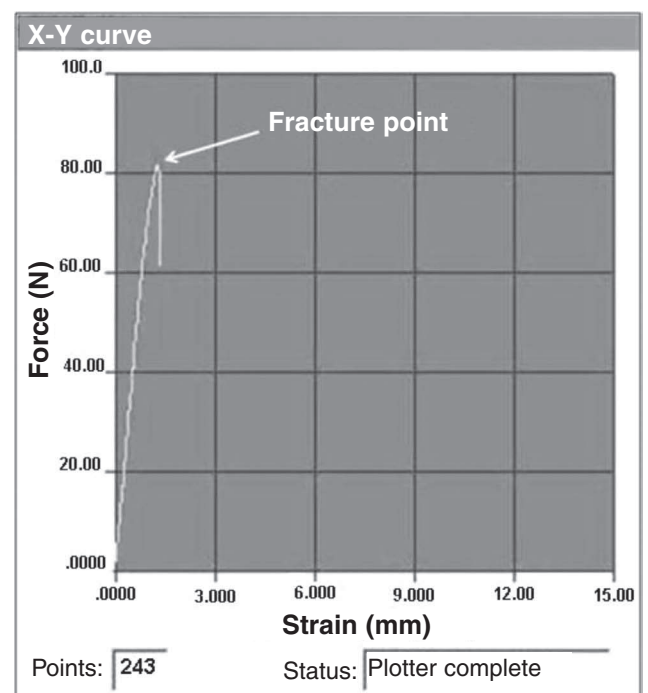


Chart 1 – Load-strain diagram obtained during the bending test.

Histomorphometric study: after decalcification, dehydration, diaphanization, and embedding in paraffin, the left tibiae were sliced transversally in the middle third and stained with hematoxylin-eosin (HE)⁽²³⁾. The slides were placed under a microscope (Laica®) coupled to a video monitor with a resolution of 1024x768 pixels, which sent the digital images to a computer. The trabecular bone area (µm²) was calculated using a 5X lens and the Image Pro Plus® image analysis program (Media Cybernetics, Silver Spring, Maryland,

USA) in two standard fields of the central region of the tibial diaphysis, after manual delimitation of the perimeter of the trabeculae (Figure 3). The calculation of the total area was carried out automatically by the program⁽²⁴⁾.

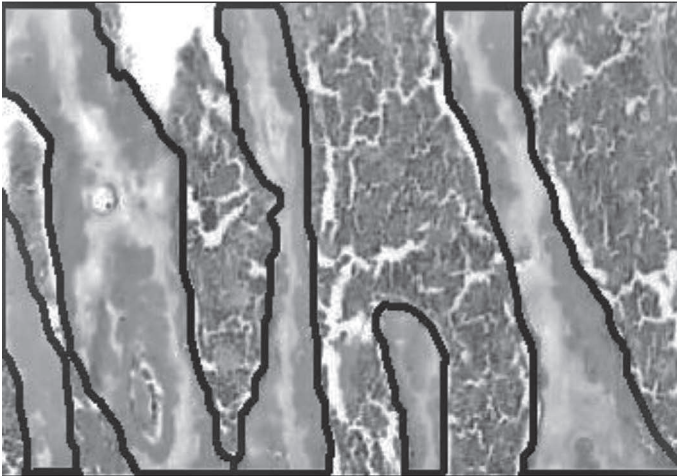


Figure 3 – Appearance of the histological cross-tibial diaphysis slice (HE, 20X), after manual delimitation of the perimeter of trabeculae.

Statistical analysis: was performed by analysis of variance (parametric or nonparametric), in a completely randomized model, complemented by multiple comparison tests, using SigmaStat[®] version 3.5 (Systat Software Inc., 2006) and Minitab[®] version 15 (Minitab Inc., 2007). Parametric tests (Student's t-test and Pearson correlation) were adopted when the variable presented Gaussian behavior, otherwise, nonparametric tests were indicated (Mann-Whitney U and Kruskal-Wallis test combined with Dunn's multiple comparisons test). A significance level of 5% was used for all calculations.

RESULTS

Clinical analysis: in general, all groups increased body mass regardless of the substance administered without, however, presenting any significant difference ($p = 0.05$) (Chart 2). Increased body mass was significantly higher in oophorectomized animals ($p = 0.005$) (Chart 3).

Taking into account the substance administered, within group O, the subgroup that received zoledronic acid (OZA) was found to have more body mass than the subgroup that received distilled water (ODW), without, however, a statistical difference ($p = 0.47$)

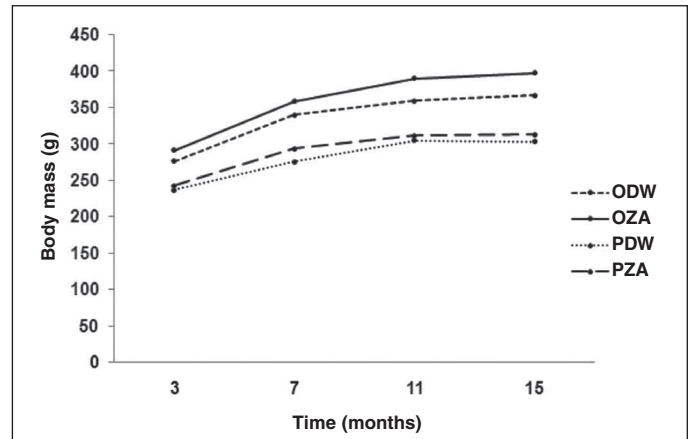


Chart 2 – Mean body mass of groups throughout the experiment ($p = 0.05$).

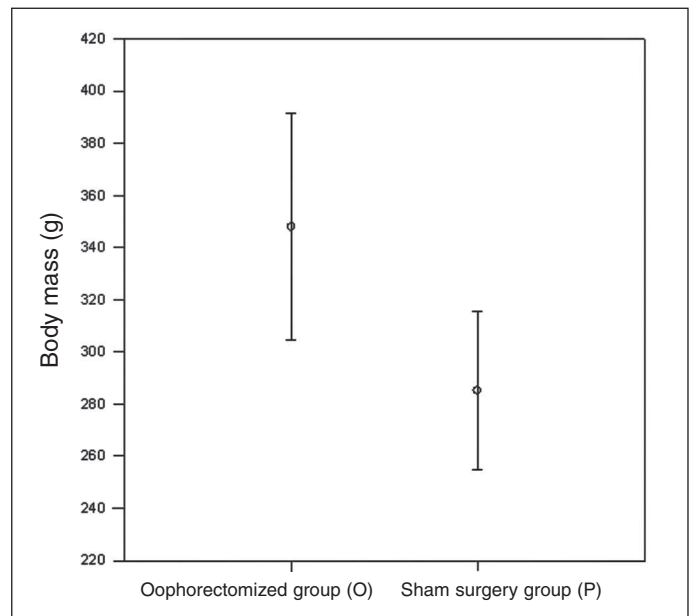


Chart 3 – Mean body mass in groups O and P throughout the experiment ($p = 0.005$).

(Chart 4). The same occurred with the sham surgery group (P) ($p = 0.68$) (Chart 5).

Biomechanical analysis: when comparing all four groups, we found that those who received zoledronic acid supported a greater compression force than those who did not use it, although without statistical difference ($p = 0.20$) (Chart 6).

The average of the maximum load supported by the OZA group ($109.2 \text{ N} \pm 14.9$) was higher than that of the ODW group ($98.6 \text{ N} \pm 15.4$), with no statistical difference between these values ($p = 0.14$) (Chart 7). However, it was confirmed that the average maximum load supported by the OZA group (109.2 ± 14.9) was statistically the same as the PDW group (109.36 ± 10.2)

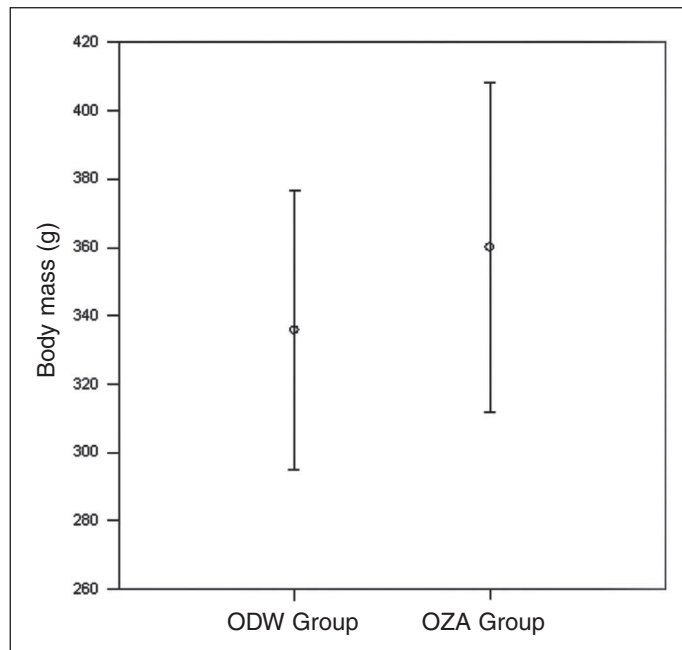


Chart 4 – Mean body mass in groups ODW and OZA throughout the experiment ($p = 0.47$).

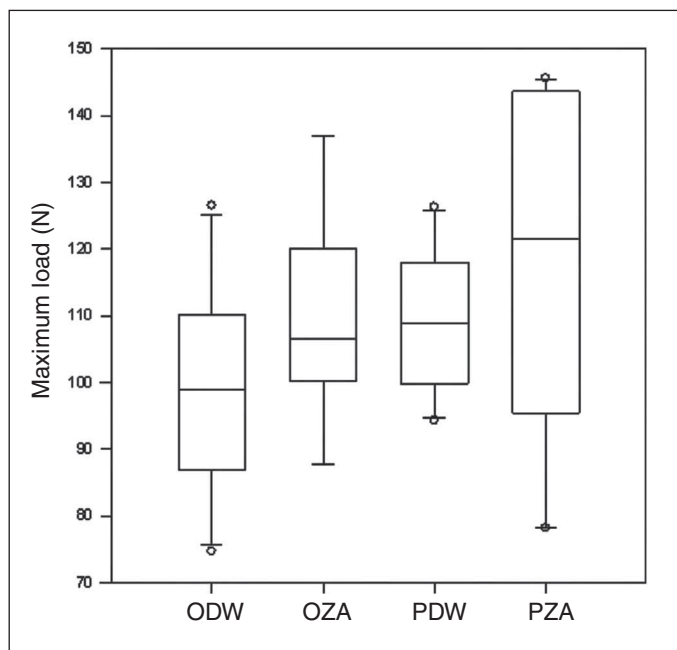


Chart 6 – Box plot of the median maximum load at the time of fracture in different groups ($p = 0.20$).

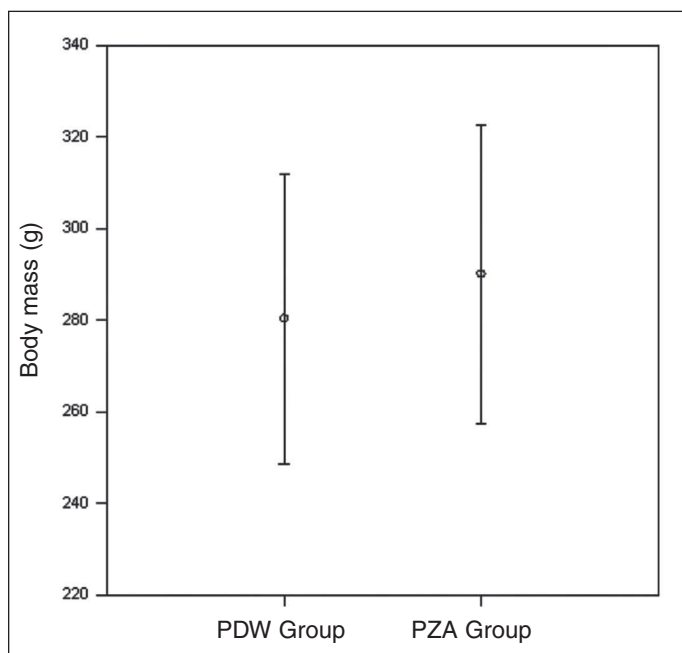


Chart 5 – Mean body mass in groups PDW and PZA throughout the experiment ($p = 0.68$).

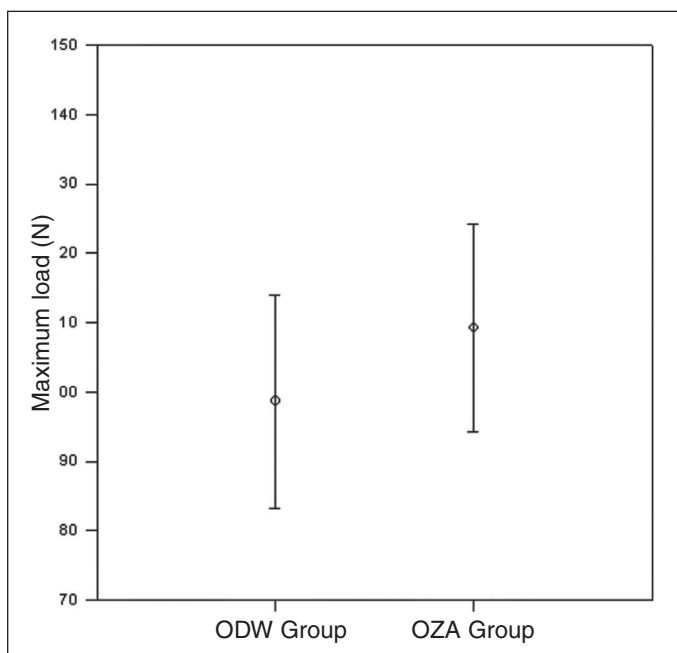


Chart 7 – Mean maximum load at the time of fracture in the ODW and OZA groups ($p = 0.14$).

($p = 0.98$) (Chart 8), when comparing the ODW and PDW groups, there is a tendency toward a difference ($p = 0.08$) (Chart 9).

Histomorphometric analysis: comparing the four groups, there is an increase in the median trabecular bone area in the groups that received zoledronic acid, although without statistical significance ($p = 0.21$) (Chart 10).

No difference was found when comparing the median tibial trabecular area of the ODW ($153,923.5 \mu\text{m}^2$) and OZA ($243,002 \mu\text{m}^2$) groups ($p = 0.15$). However, it appears that the median of the trabecular area of the OZA group ($243,002 \mu\text{m}^2$) was statistically the same as group PDW ($176,189 \mu\text{m}^2$) ($p = 0.90$).

Linear regression analysis: performed using Pearson's coefficient showed a strong positive

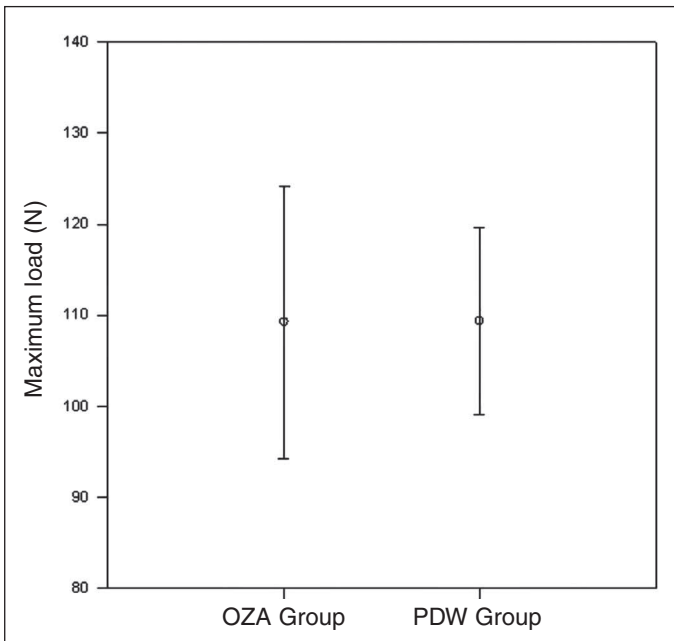


Chart 8 – Mean maximum load at the time of fracture in the OZA and PDW groups ($p = 0.98$).

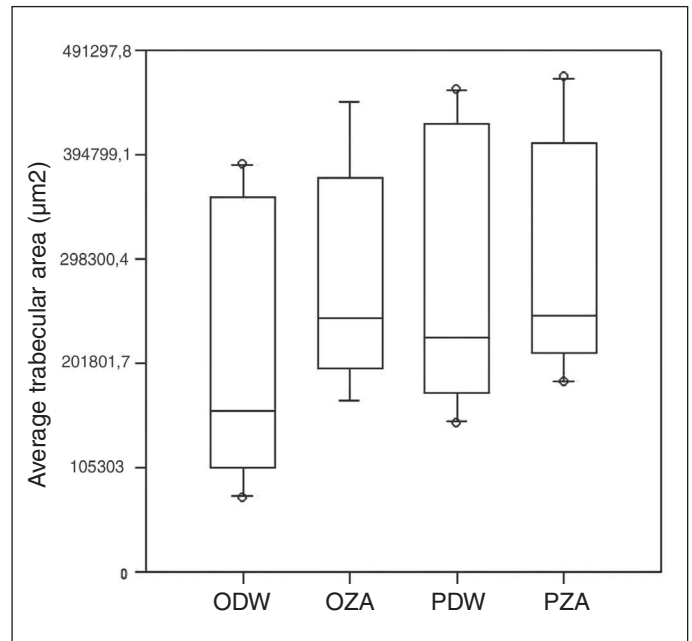


Chart 10 – Box plot of median trabecular bone area in the different groups ($p = 0.21$).

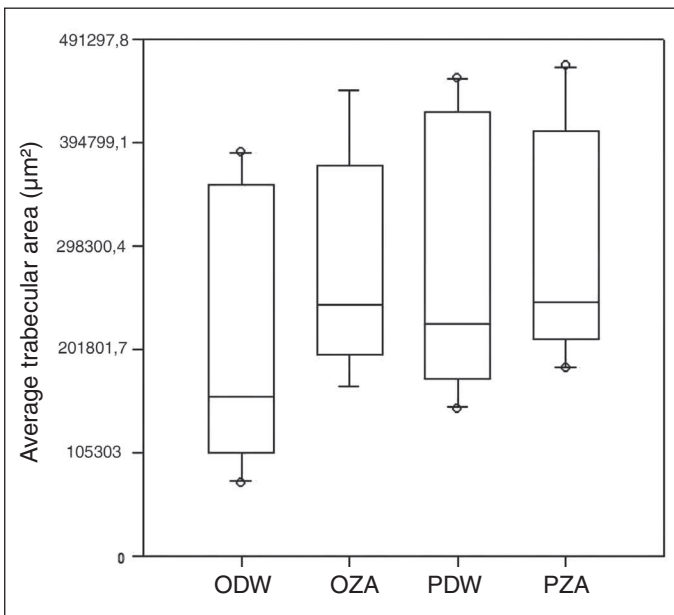


Chart 9 – Mean maximum load at the time of fracture in the ODW and PDW groups ($p = 0.08$).

correlation between the trabecular bone area and the maximum load supported by the specimen ($p = 0.04$, $r = 0.95$) (Chart 11).

DISCUSSION

Zoledronic acid, a powerful new bisphosphonate, inhibits osteoclast action and, consequently, decreases bone resorption^(10,11). Trabecular bone is the main site of drug action, maintaining thickness and density of

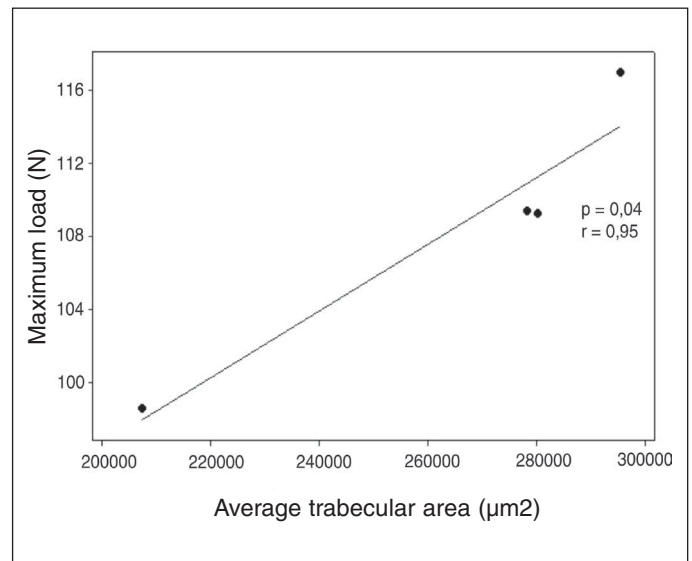


Chart 11 – Linear regression between the trabecular bone area and the maximum load ($p = 0.04$).

bone connections and increasing their resistance to fracture⁽¹²⁻¹⁴⁾. Patients with osteoporosis, especially primary type I (post-menopause) osteoporosis, who have high osteoclastic activity, can benefit from this third generation bisphosphonate by simply maintaining their trabecular bone mass⁽²⁵⁾.

The rat was chosen for this study because it has characteristics similar to those of human beings in relation to their musculoskeletal and hormonal systems, as well as their ease of handling, vivarium availability, and low cost⁽²⁶⁻²⁸⁾.

In terms of body mass, hormone-deprived animals, that is, oophorectomized (group O) animals had higher body mass gain than the sham surgery group (group P) ($p = 0.005$). Similar findings were obtained by other authors^(26,29,30). After menopause, due to changes in not only the distribution of adipose tissue caused by estrogen deficiency but also in the accumulation of peripheral fat, there is a change in the lipid profile and increased body mass⁽³¹⁾. In this study, we did not find zoledronic acid to increase body mass, a fact demonstrated by Reid et al.⁽¹⁶⁾, Hornby et al.⁽³²⁾, Otrrock et al.⁽³³⁾, and Gilfillan et al.⁽³⁴⁾. We conclude therefore that the increase in body mass was caused by the removal of the ovaries (oophorectomy) and not by the substances administered.

In the biomechanical analysis comparing the maximum load supported by the tibiae in the four groups, we observed that the groups that received zoledronic acid showed an increased load at the time of fracture compared to those who did not receive it, although not constituting a statistical difference ($p = 0.20$), suggesting an increase in trabecular bone mass in those groups. These results were repeated during the analysis of the OZA and ODW groups, without a significant difference between groups ($p = 0.14$), but with a substantial increase in the maximum load in the group submitted to zoledronic acid. We know today that bone resistance to fracture is due more to the quantity and quality of trabecular bone than cortical bone⁽¹⁷⁾. This fact could explain the results, since the diaphyseal portion of the tibia has a lower amount of trabecular bone in proportion to cortical bone.

When comparing the trabecular bone area in the four groups histomorphometrically, it was observed

that the groups that received zoledronic acid had a larger trabecular area, although without statistical significance ($p = 0.21$). Analyzing the trabecular bone area of the oophorectomized groups, we found that the presence of zoledronic acid did not significantly alter the trabecular area ($p = 0.15$). However, the median trabecular area of the OZA group was statistically the same as the PDW group ($p = 0.9$), demonstrating that zoledronic acid had a positive effect in maintaining the trabecular bone of that group. Patlas et al. analyzed the effect of oophorectomy on the diaphysis, metaphysis, and epiphysis of rats, observing a decrease in trabeculae only in the metaphyseal region. They concluded that oophorectomy exerts significant changes in the trabecular bone of long bones, especially in the extremities, and to a lesser extent in the middle region⁽²⁴⁾.

The dispersion analysis between the trabecular bone area and the maximum load supported by the body showed that these are positively correlated quantities, that is, the maximum load supported by the specimen varies in proportion to the area of trabecular bone. Therefore, the amount of trabecular bone can be considered a predictive factor for fractures from osteoporosis. Other studies are needed to demonstrate the efficacy of zoledronic acid in other regions of tibia.

CONCLUSION

Zoledronic acid had no significant influence on the body mass of animals. Results showed that zoledronic acid did not significantly increase bone strength of the tibial diaphysis or the trabecular area of diaphyseal bone.

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