ZOLEDRONATE FOR TREATMENT OF HUMERAL OSTEOPOROSIS IN FEMALE RATS. A PROSPECTIVE RANDOMIZED TRIAL

EVANDRO PEREIRA PALACIO[1], EDUARDO MARTINELLI JACOB[1], THIAGO BALTHAZAR CAMPI[1], SÉRGIO SWAIN MÜLLER[2]

ABSTRACT

Objective: To evaluate the clinical, biomechanical and histomorphometric effects of zoledronate in the humerus of ovariectomized rats. Methods: Forty female rats (Rattus norvegicus albinus) were prospectively evaluated. Within 60 days, animals were randomized into two groups: ovariectomy (O) (n=20) or sham surgery (P) (n=20). Within 90 days, groups "O" and "P" were subdivided into four groups, according to the administration of zoledronate (AZ) or deionized water (AD): OAZ (n=10), OAD (n=10), PAZ (n=10) and PAD (n=10). Within 15 months, animals were sacrificed. Body weight was used for clinical study, axial compression tests for the biomechanical study and cancellous bone area for the histomorphometric study. Results: Ovariectomized groups had greater body weight gain than the sham groups (p=0.005). Zoledronate groups had greater body weight gain compared to the deionized water groups (p=0.68). Maximum load support was greater in the groups treated with zoledronate (p=0.02). An increase in cancellous bone area was noted in the groups treated with zoledronate (p=0.001). A positive correlation was shown in the evaluation of cancellous area and maximum load (p=0.04; r=0.95). Conclusions: Zoledronate had no effect on animals' body weight. Groups treated with zoledronate had increased maximum load support and cancellous bone area.

Keywords: Osteoporosis. Menopause. Therapeutics. Biomechanics.

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INTRODUCTION

Osteoporosis is defined as a bone tissue disorder, characterized by the implication of its physical resistance. In actual fact, it is a metabolic disease, in which there is gradual loss of bone mass, with consequent deterioration of its microarchitecture,[1] increasing the risk of fractures.

Bone homeostasis is the main balance and renewal mechanism of this tissue. In the growth phase, the balance of this renewal is positive, reaching equilibrium in maturity and, after the age of 40, on average, becoming negative. The perpetuation of this negative balance over the years is the main cause of primary osteoporosis. The advent of the menopause accelerates this negative metabolic recycling, causing the so-called postmenopausal osteoporosis. On average, at the age of 65, a woman has already lost about 25% of her bone mass.[2,3] Studies estimate that in the United States, the costs generated by fractures resulting from osteoporosis amount to about 12.6 billion dollars/year.[4]

Like in all medical areas, the treatment of osteoporosis presents a much more preventative than curative characteristic, mainly through change of living habits and through physical activity. However, the pharmaceutical industry offers wide varieties of medications on an annual basis for the treatment of this ailment, such as the bisphosphonates, substances that increase bone mass, or before, decrease its resorption. There are also many adverse effects caused by the continuous administration of these drugs, which decreases the adhesion of patients to treatment on a large scale.

Based on this information, the aim of this study was to analyze the effects of zoledronate, a new and powerful anti-resorptive drug, in a single annual dose, in humeruses of ovariectomized rats, through clinical, biomechanical and histomorphometric studies.

METHODS

All the procedures were approved by the Committee of Ethics in Animal Experimentation under n° 622/2007. The study group consisted of 40 sexually mature and virgin female Wistar rats (*Rattus novergicus albinus*) from the Central Biotherium of Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP) - Campus of Botucatu.

All the authors declare that there is no potential conflict of interest referring to this article.

1 – Department of Orthopedics and Traumatology of Faculdade de Medicina de Marília/Famema – Marília. SP.
2 – Department of Surgery and Orthopedics of Faculdade de Medicina de Botucatu/Unesp – Botucatu. SP.

Study conducted in the Department of Surgery and Orthopedics of Faculdade de Medicina de Botucatu. UNESP. Botucatu.SP.
Mailing address: Faculdade de Medicina de Marília/Famema, Departamento de Ortopedia e Traumatologia, Rua Monte Carmelo, 800. Fragata, 17519-030, Marília. SP, Brazil.
Email: palacio@famema.br

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Upon reception and after clinical evaluation and weighing, the animals were housed in groups of five, in eight polypropylene cages with metal grill cover and lined with autoclaved pine wood shavings. These were cleaned daily and kept in a dry and well ventilated place, with room temperature controlled at 24º C and light/dark cycle of 12 hours. The animals were offered rodent feed (Labina®, Nestlé Purina PetCare Company©) and water *ad libitum.*

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

The castration procedure was performed after intraperitoneal anesthesia, with 30 mg/kg of 3% sodium pentobarbital, and bilateral trichotomy, just below the last rib, dorsolaterally. They were submitted to asepsis with soap and water, antisepsis with polyvinylpyrrolidone-iodine tincture (PVPI-tincture) and positioned on the operating table, in lateral decubitus. After the application of an ocular sterile drape, a transversal incision was made in the skin measuring approximately 1.5 centimeters in length, between the last rib and the hip joint. The peritoneal cavity was exposed with the assistance of forceps, surpassing the muscular plane by divulsion, allowing access to the ovary coated with adipose tissue. (Figure 1)

Figure 1 – *Aspect of the clamping of the ovary in the middle of fatty tissue before its ligature and sectioning.*

This procedure was followed by the ligature of the ovary with 3.0 cotton thread and its sectioning distally to the ligature. The musculature and the skin were sutured together with 4.0 nylon thread, repeating the same procedures on the opposite side for removal of the other ovary. The animals from group P were submitted to the same surgical procedures described, with the exception of the surgical time of ligature and section of the ovaries.

At the age of 90 days and after further randomization, groups "O" and "P", were split into four subgroups according to the intraperitoneal administration of 0.1mg/kg[5,6] of zoledronate (AZ) (Aclasta™, Novartis® Biociências S.A.) or distilled water (AD), as follows: OAZ (n=10), OAD (n=10), PAZ (n=10) and PAD (n=10). The substances were administered using an Injex Stilly Line® sterile syringe for insulin, with fixed needle (1ml/cc, needle 12.7mm x 0.30 - 30G 1/2'').

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

After euthanasia, the animals' humeruses were disjointed in the proximal (shoulder) and distal (elbow) regions, with the removal of the soft parts (muscles, tendons and ligaments). For the biomechanical assay, the right humeruses were packed in aluminum paper, identified and frozen for 24 hours, in a domestic refrigerator, at a temperature of -20ºC. The left humeruses were containerized in glass recipients, clean and properly identified, and fixed in a solution of 10% formaldehyde, for the performance of the histomorphometric study.

Clinical study

The clinical study was carried out through an analysis of the animals' body mass (g). The measurements were taken monthly and always on the same day, throughout the whole experiment, using digital scales with a capacity of six kilograms-force and variation of five grams. The scales were calibrated quarterly by technical staff.

Biomechanical study

Axial compression assays were conducted for determination of the mechanical properties of the humeruses, using the Universal Mechanical Testing Machine EMIC, model DL 10.000, with precision of (0.018 + F/3700)KN, determined within the specifications of standards ABTN, NBR6156 and NBR6674. The machine, which operates in conjunction with a computer under the *Windows*™ 2000 operating system, uses the Mtest program version 1.00 for the comparison of results. Twelve hours before the biomechanical assay, the right humeruses were thawed and kept in compresses soaked in 0.9% saline.

The distal extremities of the humeruses were fixed vertically, in plastic recipients, with a 35 ml capacity, containing 25 ml of self-polymerizing acrylic resin (Jet®). After 2 hours, the pieces were sent for the biomechanical tests. The bone cleaver, whose extremity is concave, was positioned axially to the humerus, adjusting perfectly to its head. (Figure 2) For determination of the maximum load borne by the body, the bone cleaver was put into movement at a speed of 30mm/min.[7,8] The calculation of the maximum load was performed automatically by the program. (Figure 3)

Histomorphometric study

The left humeruses, after decalcification, dehydration, diaphanization and paraffin embedding, were cut transversally in the proximal metaphyseal area (surgical neck) and dyed with Hematoxylyn-eosin

Figure 2 – *Detail of the test specimen at rest and of the load application bone cleaver, positioned axially to the humeral head.*

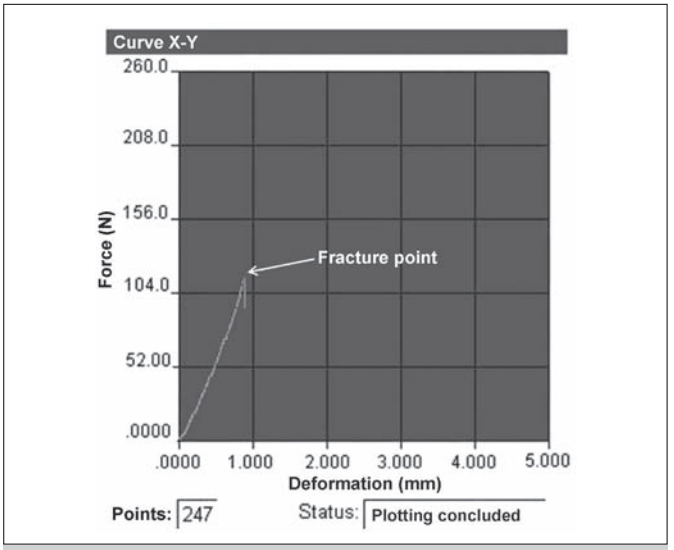

Figure 3 – *Load-deformation diagram obtained during the axial compression assay.*

(HE).[9] The slides were placed under a microscope (Leica®) coupled to a video monitor with resolution of 1024x768 pixels, which sent the digital images to a computer. The cancellous bone area (μm^2) was calculated using the 5X objective and the Image Pro plus image analysis program (Media Cybernetics, Silver Spring, Maryland, USA), in two standard fields of the central region of the humeral surgical neck, after manual delimitation of the trabecular bone perimeter. (Figure 4) The calculation of the total area was performed automatically by the program.[10]

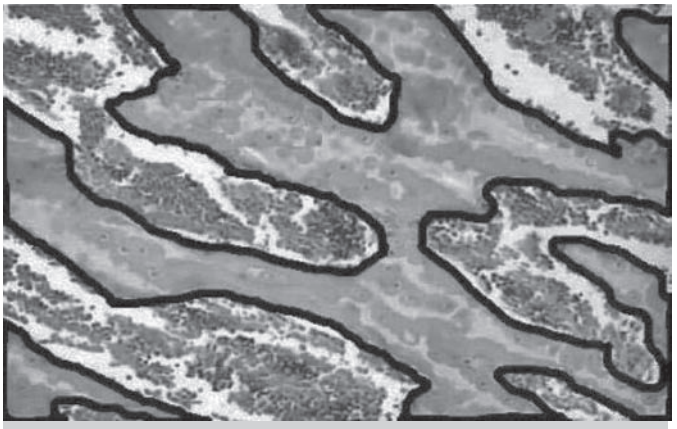

Figure 4 – *Aspect of the transversal histological cut of the humeral surgical neck (HE, 20X), after manual delimitation of the trabeculae perimeter.*

Statistical Analysis

The statistical analysis was performed through variance analysis (parametric or non-parametric), in the entirely casualized model, supplemented with the respective multiple comparison tests, using for this purpose the programs SigmaStat® version 3.5 (Systat Software Inc., 2006) and Minitab® version 15 (Minitab Inc., 2007). The parametric option occurred when the variable presented Gaussian behavior (Student's t-test, ANOVA test associated with Holm-Sidak's multiple comparison test, and Pearson's correlation test), otherwise, the non-parametric option was indicated (Mann-Whitney's U-test and the Kruskal-Wallis test associated with Dunn's multiple comparison test). A significance level of 5% was used for all the calculations.

RESULTS

Clinical analysis

In general, all the groups increased body mass regardless of the substance administered without, however, significant difference ($p=0.05$). (Figure 5) The increase of body mass was significantly higher in the ovariectomized animals ($p=0.005$). (Figure 6)

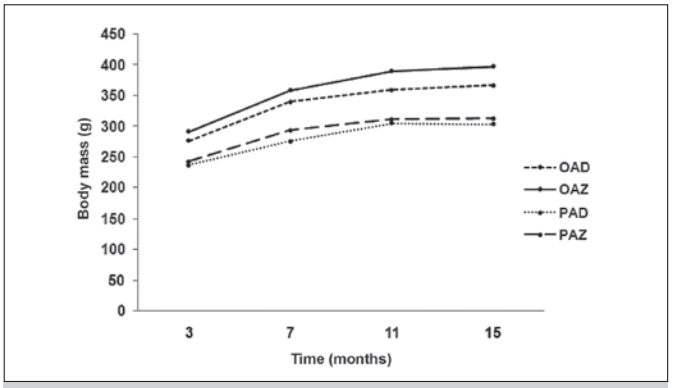

Figure 5 – *Mean body mass of the groups throughout the experiment (p=0.05).*

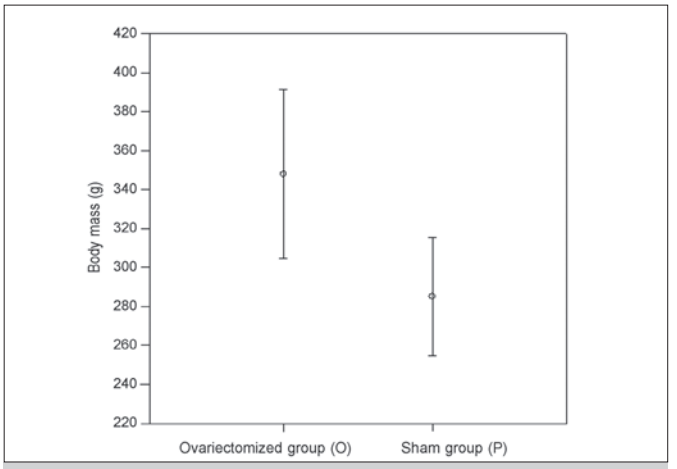

Figure 6 – *Mean body mass of groups "O" and "P" throughout the experiment (p=0.005).*

Taking into consideration the substance administered, it was verified, inside group O, that the group that received zoledronate (OAZ) increased its body mass more than the group that received distilled water (OAD), without, however, any statistical difference ($p=0.47$). (Figure 7) This also occurred with the sham group (P) ($p=0.68$). (Figure 8)

Biomechanical analysis

In comparing the four groups, it was observed that those that received zoledronate bore a statistically higher compression force than those that did not receive it ($p=0.02$). (Figure 9)

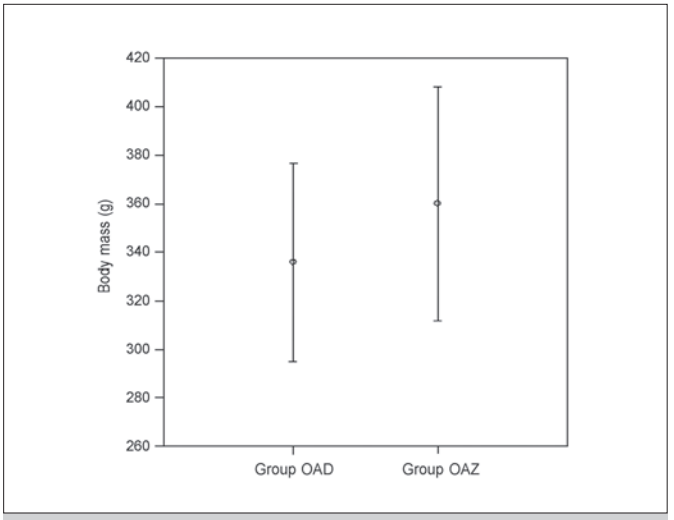

Figure 7 – *Mean body mass in groups OAD and OAZ throughout the experiment (*p*=0.47).*

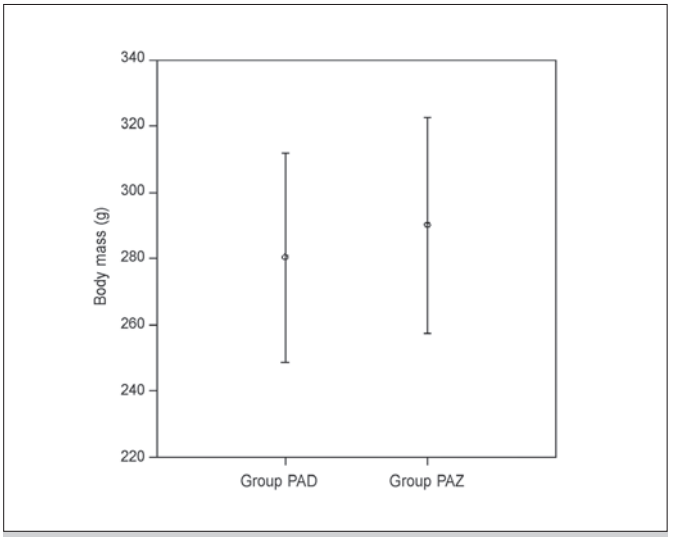

Figure 8 – *Mean body mass in groups PAD and PAZ throughout the experiment (*p*=0.68).*

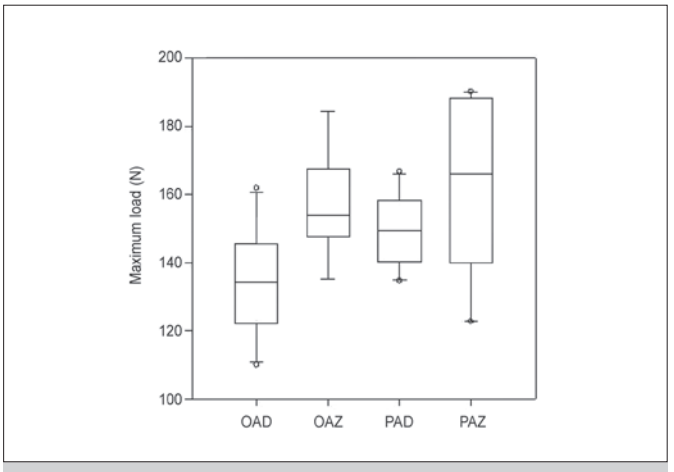

Figure 9 – *Box plot of the median of maximum load at the time of the fracture in the different groups (*p*=0.02).*

The mean maximum load sustained by group OAD (133.9N ±14.2) was statistically lower than that of group OAZ (156.5N ±14.9) (*p*=0.005) (Figure 10). In comparing the mean maximum load in groups OAD (133.9 ±15.4) and PAD (149.7 ±10.3), a significant difference was verified between them (*p*=0.01). (Figure 11) On the other hand, the mean maximum load sustained by group OAZ (156.5 ±14.9) was, statistically, the same as group PAD (149.6 ±9.8) (*p*=0.25). (Figure 12)

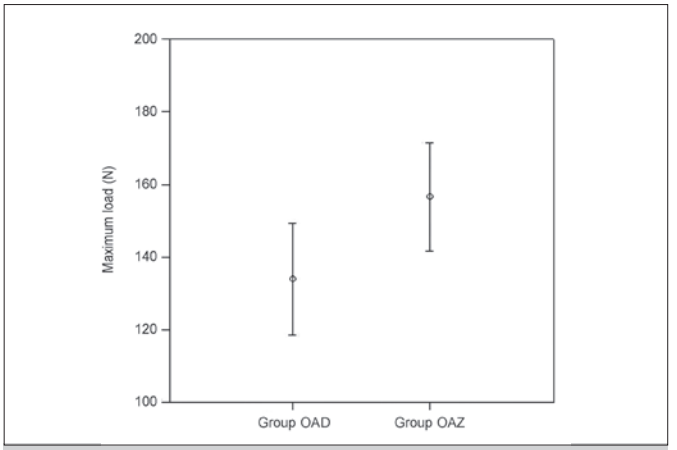

Figure 10 – *Mean maximum load at the time of the fracture in groups OAD and OAZ (*p*=0.005).*

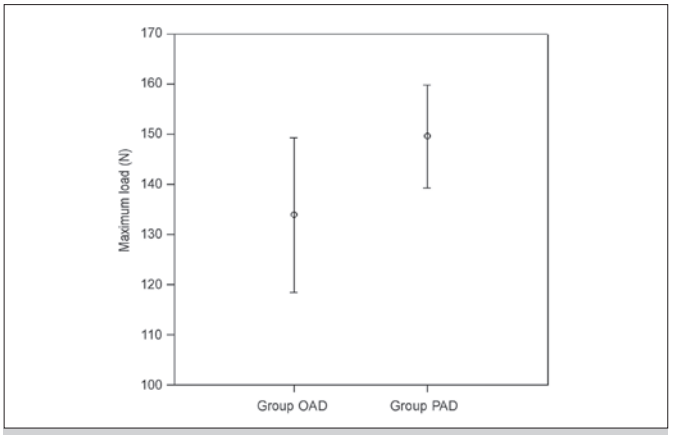

Figure 11 – *Mean maximum load at the time of the fracture of groups OAD and PAD (*p*=0.01).*

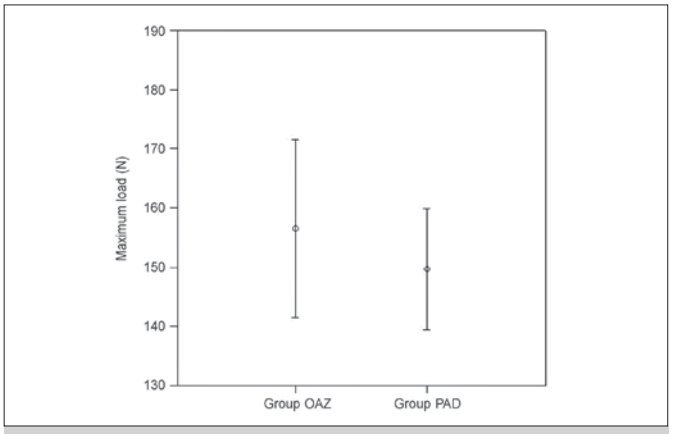

Figure 12 – *Mean maximum load at the time of the fracture in groups OAZ and PAD (*p*=0.25).*

Histomorphometric analysis

In comparing the four groups, they verified the significant increase of the median of the cancellous bone area in the groups that made use of zoledronate ($p=0.001$). (Figure 13)

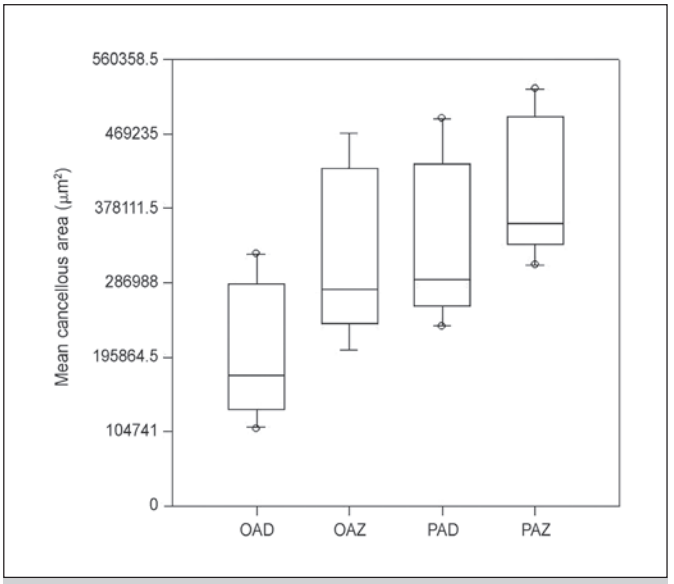

Figure 13 – *Box plot of the median of the cancellous bone area in the different groups (*p*=0.001).*

There was a significant difference in comparing the humeral mean cancellous area of groups OAD (202448 μm^2 ± 86247) and OAZ (334785.2 μm^2 ± 111205) ($p=0.01$). (Figure 14) In comparing the medians of groups OAD (175978.5) and PAD (304468), significant difference was verified between them ($p=0.01$). (Figure 15) At the same time, it was verified that the median value of the maximum load sustained by group OAZ (290925) was, statistically, the same as group PAD (304468) ($p=0.71$). (Figure 16)

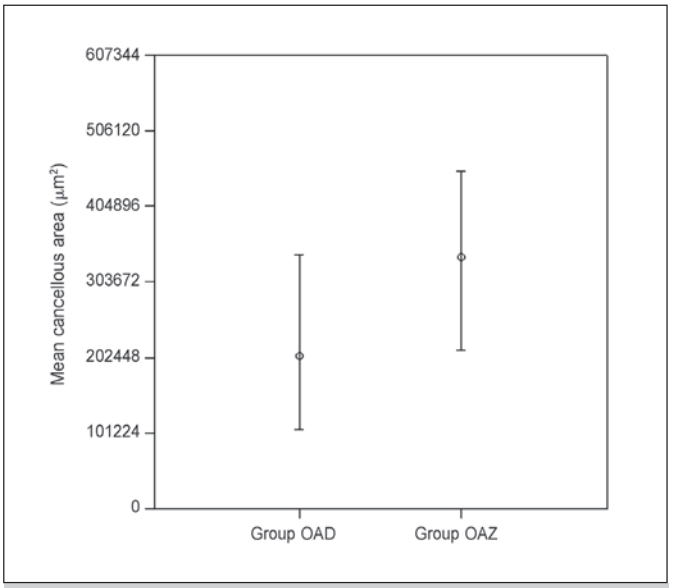

Figure 14 – *Mean cancellous bone area in groups OAD and OAZ (*p*=0.01).*

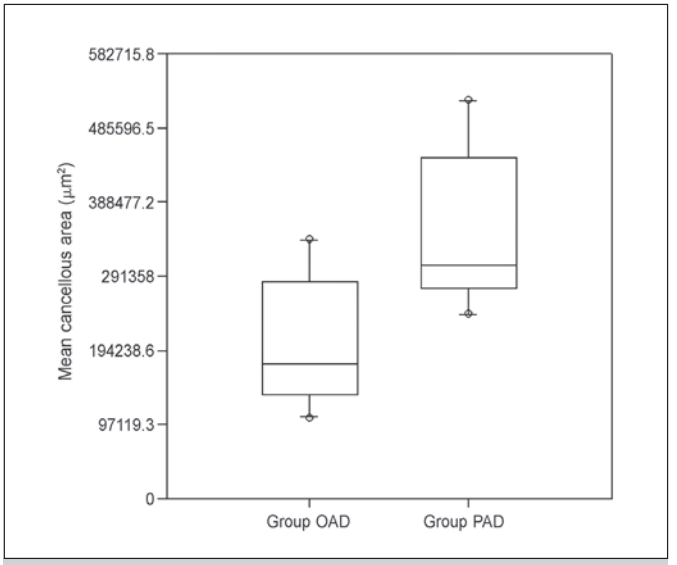

Figure 15 – *Box plot of the median of the cancellous bone area in groups OAD and PAD (*p*=0.01).*

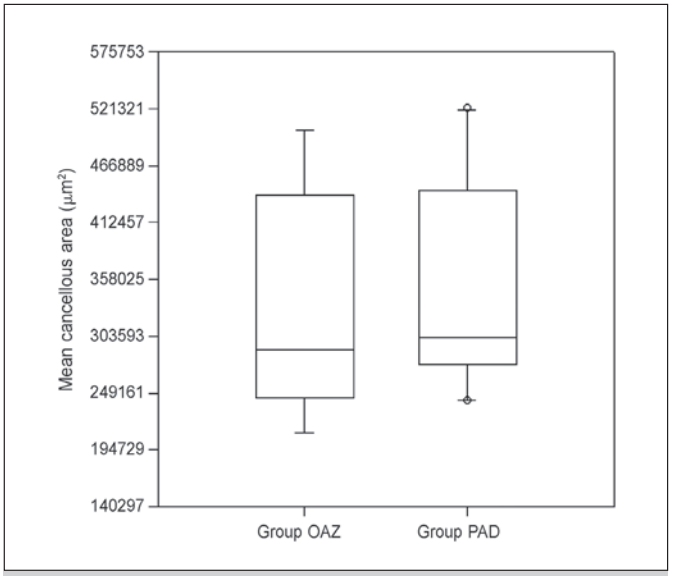

Figure 16 – *Box plot of the median of the cancellous bone area in groups OAZ and PAD (*p*=0.71).*

Linear regression analysis

Pearson's coefficient showed positive correlation between the cancellous bone area and the maximum load borne by the test specimen ($p=0.04$; $r=0.95$). (Figure 17)

DISCUSSION

The vast majority of proximal humerus fractures occurs in women (3:1) after menopause or over 60 years of age.[11] These also correspond to the fourth most frequent fracture during the osteoporosis period, responsible for 12% of all fractures.[12,13] According to Maravic *et al.*[14], in France, each humerus fracture of osteoporotic origin, generates individual annual costs around US$4,000.

Zoledronate, which acts by inhibiting the osteoclastic action and, consequently, decreasing bone resorption,[15] can be used in the prophylaxis of fractures caused by osteoporosis.

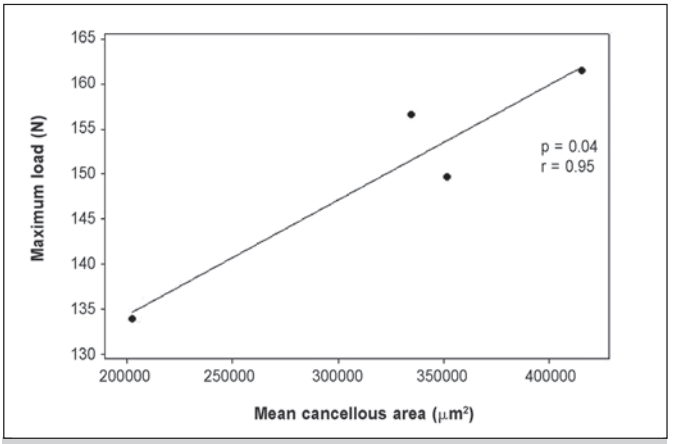

Figure 17 – *Linear regression between the cancellous bone area and the maximum load (*p*=0.04).*

For the performance of this study, the rat was chosen on account of its characteristics resembling those of human beings, as refers to the musculoskeletal and hormonal systems, besides ease of handling, availability in biotherium and low cost.[16]

In terms of body mass, the animals with hormone deprivation, in other words, ovariectomized (group O), presented greater gain of body mass than the sham group (group P) ($p=0.005$). Similar findings were obtained by several authors.[17,18] After menopause, due to the alteration not only of the distribution of fatty tissue, caused by estrogen deficiency, but also by the peripheral accumulation of fat, there is alteration of the lipid profile and increase of body mass.[19] In this study we did not verify the influence of zoledronate on the increase of the animals' body mass. Thus it is concluded that the increase of body mass was caused by removal of the ovaries (ovariectomy) and not by the substances administered.

As regards the biomechanical analysis, in comparing the maximum loan borne by the humeruses in the four groups, it was observed that the groups that received zoledronate presented significantly higher load at the time of fracture, than those that did not receive it ($p=0.02$), showing that zoledronate increased the physical resistance of the bone to fractures.[6,20] These results were repeated during the analysis of groups OAD and OAZ ($p=0.005$). The absence of significant difference between maximum loads of groups OAZ and PAD, reinforces the theory that zoledronate acted by increasing bone resistance to fracture, at a higher level than that of the non-castrated group.

Histomorphometrically, in comparing the cancellous bone area in the four groups, it could be observed that the groups that received zoledronate exhibited a significantly larger cancellous bone area ($p=0.001$) than those that did not receive it. Analyzing the cancellous bone area of groups OAD and OAZ, it was verified that the presence of zoledronate significantly increased the cancellous bone area ($p=0.01$). Pathas *et al*.[10] analyzed the effect of ovariectomy on bone diaphysis, metaphysis and epiphysis of female rats, observing the decrease of trabeculae only in the metaphyseal region. They concluded that ovariectomy exercises significant changes on the cancellous bone of long bones, especially at the extremities, and to lesser extent, in the median region. In this study, zoledronate provided the maintenance of the bone trabeculae in the humeral metaphyseal region. In the same manner as in the biomechanical study, zoledronate kept the cancellous bone area in the castrated group (OAZ) at a level statistically equal to that of the non-castrated group (PAD).

The analysis of dispersion between the cancellous bone area and the maximum load sustained by the body, showed that these are greatnesses that are positively correlated, in other words, the maximum load borne by the humerus varied, proportionally, to its cancellous area. Therefore, the quantity and quality of the spongy or trabecular bone can be considered predictive factors for fractures resulting from osteoporosis.

CONCLUSIONS

Zoledronate did not have a significant influence on the body mass of the animals. The analysis of results showed that zoledronate significantly increased the bone resistance of the proximal humerus and the cancellous bone area in the metaphyseal region of the humerus in osteoporotic rats. Other studies are necessary to evidence the effectiveness of zoledronate in other humeral regions.

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