TOMOGRAFIC AND TENSIOMETRIC ASSESSMENT ON FEMURS FROM OOPHORECTOMIZED RATS SUBJECTED TO HORMONE REPLACEMENT THERAPY

Fábio Alexandre Martynetz[1], Maria de Lourdes Pessole Biondo-Simões[2], Juliano Rodrigo Martynetz[3], Tatiana Daher Martynetz[4], Elise Zimerman[5], Heraldo Mello Neto[6]

ABSTRACT

Objective: To analyze the biomechanical and tomographic effects of hormone replacement therapy (HRT) on femurs from rats subjected to induced menopause. Methods: Forty-five adult Wistar rats were divided equally into three groups. The first and second groups consisted of rats subjected to oophorectomy, and the third was the control group, consisting of non-oophorectomized rates. After verifying that hormone failure had occurred (exfoliative cytological test), only the first group received HRT, over a two-month period. After this period, the femurs were disarticulated and subjected to biomechanical tests in a universal testing machine to evaluate their strength, and were subjected to tomographic evaluation to determine the bone mineral density. Results: The exfoliative cytological test showed that hormone failure was induced in all the oophorectomized animals. A significant difference (p = 0.030) in maximum strength measurements was observed between the groups (higher in the group with HRT). Greater bone fragility was observed in the oophorectomized animals without HRT than in those with HRT (p = 0.010), in relation to the control group (p = 0.0107). There was greater bone strength in the oophorectomized rats with HRT than in those without HRT, and these values were similar to those of the control group (p = 0.179). In the tomographic evaluation, no significant differences were found between the groups (p = 0.625). Conclusion: A significant increase in bone strength was observed with the use of HRT. However, treatment with HRT did not show any significant change in bone mineral density.

Keywords – *Menopause; Hormone replacement therapy; Tomography; Bone and bones; Osteoporosis; Femur; Rats*

INTRODUCTION

Osteoporosis is a systemic bone disease that is associated with changes to bone architecture and reduction of bone mass. This causes increased numbers of fractures, particularly among postmenopausal women[1].

Around 50% of these women may develop some type of fracture related to changes in bone quality, and 15% may have fractures of the femur[2]. Such fractures present high prevalence among women over the age of 49 years (32.7%)[3].

Fractures, and particularly femoral fractures, in patients with osteoporosis form part of orthopedists' day-to-day routine, given that their incidence has been increasing concomitantly with rising life expectancy.

These fractures are provoked or worsened by low bone quality, thus limiting the treatment methods. Only 30% of patients who suffer fractures due to osteoporosis are able to return to their previous quality of life without some functional limitation, and mortality over the first six months reaches 23.3%[4].

Prevention of the bone loss that starts soon after the menopause would help towards reducing the incidence of fractures at a later stage of women's lives[5].

1 – MSc student in the Postgraduate Clinical and Surgical Medicine Program, PUCPR.
2 – PhD in Experimental Surgery from Unifesp. Titular Professor of Research Methodology at PUCPR and Adjunct Professor IV in the Department of Surgery, Federal University of Paraná.
3 – Resident in Orthopedics and Traumatology, Cajuru University Hospital, PUCPR.
4 – Ophthalmologist from Pontifícia Universidade Católica, Paraná.
5 – Otorhinolaryngologist from Pontifícia Universidade Católica, Paraná.
6 – Neuroradiologist at X-leme. Radiologist in the Imaging Services of Cajuru University Hospital and Santa Casa de Misericórdia, Curitiba (PUCPR)

Work performed within the Clinical and Surgical Medicine Program of Pontifícia Universidade Católica, Paraná (PUCPR).
Correspondence: Rua Martim Afonso, 1168, apto. 1205, Mercês, 80430-100 Curitiba, PR – E-mail: martynetzfabio@hotmail.com

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Hormone replacement therapy (HRT), which may be oral, intradermal or parenteral, has been shown to be useful for preventing bone loss and reducing the risk of fractures during the climacteric[6,7].

Women who start to use estrogen before the age of 60 years and continue with HRT maintain their bone mineral density, lose less bone and have a risk of fracture that is 37% lower[8].

Computed tomography is a very important tool within medical practice. One of its properties is its capacity to qualify and quantify bone architecture, thus enabling better morphological evaluations[9].

HRT may increase bone mass, reduce its loss and reduced the risk of fractures and their sequelae[3,4,10,11].

METHODS

Forty-five rats (*Rattus norvegicus albinus, Rodentia, Mammalia*) of the PUCPR Wistar lineage were used. Their ages were between 90 and 100 days, they weighed between 150 and 200 grams and they were supplied by the Central Vivarium of Pontifícia Universidade Católica, Paraná. The rats were kept under constant environmental conditions, with day/night cycling of 12 hours and a controlled ambient temperature of 20 ± 2 °C. The relative air humidity and noise levels were appropriate for the environment. The animals had free access to water and feed that was appropriate for this species. Five rats were housed in each cage, and all cages were positioned at the same distance from the light source.

The sample was divided into three groups with 15 animals each. The first and second groups consisted of oophorectomized rats, while the third (the control group) consisted of healthy rats that were not subjected to this procedure. The rats in group 1 received HRT.

The animals were firstly subjected to median laparotomy, under anesthesia. Bilateral oophorectomy was performed on the rats in groups 1 and 2. The animals in group 3 underwent simulated laparotomy.

Material for vaginal smears was collected using the Papanicolaou technique at the start of the study, in order to determine the hormone patterns of each animal in the sample.

Twenty-eight days after the surgical procedure, a second vaginal smear was collected from the animals in all three groups, in order to confirm the lack of hormones.

After detection of an induced “menopause”, daily HRT was started, in the following manner: the animals in group 1 received 50 µg of conjugated estrogen, in association with 2 mg medroxyprogesterone per day. The medication was administered by means of an orogastric probe. The rats in groups 2 and 3 received an equal volume of 0.9% NaCl, through the same route.

Fifteen days later, vaginal smears were again collected from the animals in all three groups, to confirm the hormonal status.

After two months of therapy, the animals were sacrificed using a lethal dose of sodium thiopental, intraperitoneally. After death had been confirmed, the right femur was removed and was subjected to tomographic and strength evaluations.

The imaging examination was performed using the Siemens® Somatom tomography apparatus, Esprit model (made in Erlanger, Germany). Tomographic slices through the femur were produced to evaluate three regions (two slices of 1.5 mm in thickness per region): 1) proximal region, going from the subcapital area to the lesser trochanter; 2) diaphyseal region of the isthmus, as far as one centimeter distally; and 3) distal metaphysis (Figure 1).

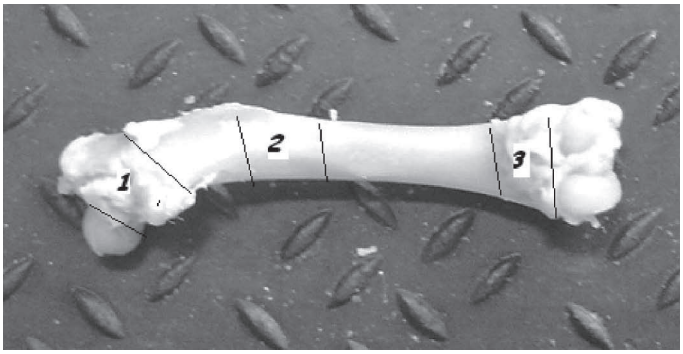

Figure 1 – Demonstration of the areas of the femur that were examined using computed tomography

The bone density was analyzed in each area, using the relationship between the cortical and medullary bone structure (Figure 2).

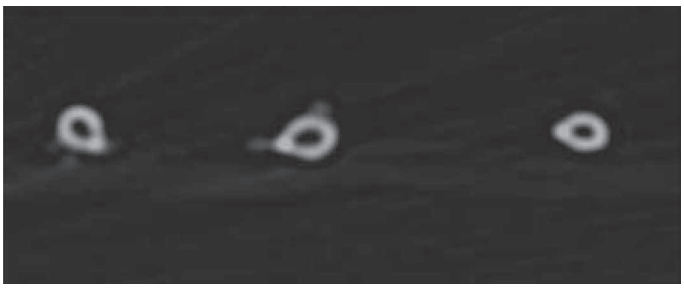

Figure 2 – Characteristics of the axial tomographic slices from the middle segment of the three groups

Hounsfield units (HU) were used to measure bone density. This density measurement unit is used in computed tomography, with an empirical scale that identifies the diagnostic radiation penetrability according to the tissue type.

Bone strength was evaluated in a destructive test carried out in the Destructive Test Laboratory of the Department of Mechanical Engineering, in the Technology Park of Pontifícia Universidade Católica, Paraná.

The femurs were subjected to destructive testing in a three-point flexion strength test apparatus (tension/compression): Emic®, model DL-500, made in Brazil (Figure 3). This device was coupled to a microcomputer running the M-test mechanical test software, by means of an Rs-232 standard serial channel. This software made it possible to convert the absolute numerical values into newton units, thereby assessing the force required to fracture a given material.

To make measurements on the mechanical test apparatus, a steel structure had to be constructed to support the diaphysis of the femur. Each support, of one centimeter in size, was positioned on the metaphyseal region of the femur, and the flexion force was applied by means of a third force in the opposing direction, which was also adapted through a steel tip of 0.5 cm in size. The Lateral supports were measured empirically, given that they would not influence the test. The position of the 0.5 cm tip was determined as the average diameter measurement of the cross-section through the femurs examined[12]. The force was applied until fracturing was achieved (Figure 3).

The results obtained were subjected to statistical analysis. To compare the groups in relation to the study variables that presented normal distribution, one-way analysis of variance was used.

Multiple comparisons were performed using the LSD test. For variables that presented asymmetrical distribution, comparisons between the groups were made using the nonparametric Kruskal-Wallis test.

To investigate whether variables presented normal distribution, the Shapiro-Wilks test was used, and to assess the homogeneity of the variance, the Levene test was used.

Associations between quantitative variables were evaluated by estimating Spearman's correlation coefficient. P-values < 0.05 were taken to indicate statistical significance.

RESULTS

On the 15th day after "menopause" had been induced, it was observed on vaginal smears that all the oophorectomized rats were in the diestrus phase, thus characterizing the presence of a hormone status compatible with hypoestrogenism.

During the study period, 16 animals died. Group 1 presented the greatest number of deaths, such that only six animals remained; in group 2, 12 animals remained and in group 3, 11 animals remained.

The remaining 29 samples underwent tomographic analysis and the strength test, in accordance with their respective groups.

There was a significant difference ($p = 0.030$) between the groups regarding the maximum strength measurements in the strength test: it was greater in the group with HRT than in the group without HRT (Table 1). It was observed that group 2 presented greater fragility in the strength test than did group 1 ($p = 0.010$) or group 3 ($p = 0.0107$).

Analysis on the breaking strength of the experimental specimens showed that there was a significant difference between the groups ($p = 0.049$) (Table 2), most clearly between groups 1 and 2 ($p = 0.016$). The breaking strength was similar between the animals with HRT

Figure 3 – Demonstration of specimen positioning in the test apparatus for producing fractures (left) and fracture pattern obtained (right)

Table 1 – Maximum strength values in newtons for each group

GROUP	N	MEAN	MEDIAN	MINIMUM	MAXIMUM	STANDARD DEVIATION
1	6	47.22	48.61	36.48	57.61	7.49
2	12	40.72	40.83	32.93	48.63	4.24
3	11	43.98	43.86	39.52	49.80	3.03

LSD test: p = 0.030

and the controls (p = 0.179). The femurs of the animals in the control group presented greater strength than did those in group 2, but without statistical significance (p = 0.171).

Table 2 – Breaking strength values in newtons in the three groups

GROUP	N	MEAN	MEDIAN	MINIMUM	MAXIMUM	STANDARD DEVIATION
1	6	46.30	47.70	35.55	57.15	7.72
2	12	40.02	39.88	31.18	47.38	4.19
3	11	42.88	43.16	36.91	49.72	3.55

LSD test: p = 0.049

From evaluating the mean bone mineral density of the femur, using tomography, a clear tendency towards similarity of values was observed, but without statistical significance (p = 0.625) (Table 3).

Table 3 – Mean bone mineral density values (Hounsfield units)

GROUP	N	MEAN	MEDIAN	MINIMUM	MAXIMUM	STANDARD DEVIATION
1	6	1.53	1.53	1.37	1.66	0.10
2	12	1.63	1.65	1.26	1.97	0.21
3	11	1.66	1.58	1.29	2.39	0.28

LSD test: p = 0.625

The animals that received HRT presented lower bone mineral density in the proximal segment, on tomography, than did the other two groups, but without statistical significance (p = 0.073).

The animals in the control group presented higher bone mineral density in the middle and distal segments, on tomography, than did the experimental groups (p = 0.0437) (Table 4).

In group 1, the breaking strength did not show any statistically significant correlation with tomographic bone mineral density. This was also seen in relation to the femurs of animals without HRT and in the control group (Table 5).

Table 4 – Bone mineral density values in the middle segment of the femur (Hounsfield unit)

GROUP	N	MEAN	MEDIAN	MINIMUM	MAXIMUM	STANDARD DEVIATION
1	6	1.51	1.46	1.39	1.68	0.12
2	12	1.62	1.62	1.21	2.20	0.33
3	11	1.75	1.66	1.17	2.39	0.41

LSD test: p = 0.0437

Table 5 – Correlation between bone mineral density and breaking strength, using Spearman's correlation coefficient

GROUP	N	SPEARMAN'S CORRELATION COEFFICIENT	p VALUE
1	6	-0.257	0.623
2	12	0.137	0.672
3	11	-0.118	0.729

Maximum strength did not present any statistically significant correlation with tomographic bone density in the femurs of groups 1 and 2, or in the control group (Table 6).

Table 6 – Demonstration of the correlation between bone mineral density and maximum strength, using Spearman's correlation coefficient

MAXIMUM STRENGTH			
Group	N	Spearman's correlation coefficient	p value
1	6	-0.086	0.872
2	12	0.294	0.353
3	11	-0.145	0.670

DISCUSSION

Humans are bipedal, with femurs that have become adapted to support greater loads, relative to rats. Structurally, in the morphological and spatial layout, divergence between the mechanical and anatomical axes of the lower limbs can be seen, which provides greater resistance to external forces.

On aging, the quantity of trabeculated bone diminished and an area of weakness is observed in the proximal region of the femur. This, together with the layout of the traction-compression force lines in the neck and pertrochanteric regions, makes the femur more susceptible to fractures. In turn, these regions have been described and classified radiologically according to the degree of disappearance of the trabecular lines[13].

In 1941, *apud* Nordin and Polley[14], Albright correlated the development of osteoporosis among menopausal women with hypoestrogenism. This author believed that this state of reduction of serum estrogen levels would reduce bone formation. Today, it is known that absence of this hormone increases bone turnover, thereby increasing both bone formation and bone reabsorption.

Over the last ten years, the average life expectancy among Brazilians has increased from 68.1 years to 72.3 years, especially among women, for which the estimated is between 72 and 76.1 years[15]. The increase in the geriatric population has led to increased incidence of osteoporosis[2] and, consequently, to increased risk of fracture among this population.

HRT may increase bone mass by decreasing the losses, through inhibiting bone reabsorption. Theoretically, it makes bones more resistant. Estrogen acts on this system by inhibiting bone reabsorption, through inducing osteoclast apoptosis by means of alpha receptors in bone cells, thereby influencing the formation, maintenance and reabsorption of bone tissue. It thus prevents bone loss and may diminish the risk of fractures[16].

In the present study, oophorectomy promoted hormone deficiency and led to reduced bone mineral density in the middle segment, along with decreased bone strength in the destructive test. This model was efficient for promoting a state of hypoestrogenism. Cardoso Netto *et al*[17] previously demonstrated that oophorectomy induced the menopause and reduced the bone deformation limit, thus making the femur less resistant.

It was also noted in the present study that the maximum strength and breaking strength of the femurs of the animals with HRT were significantly greater than among those without this treatment. Thus, this study proved that HRT had protective action on bones.

The destructive testing method with force applied at three points made it possible to evaluate bone strength reliably. This method had already been used by Probst *et al*[18].

Nowadays, diagnoses of osteoporosis are confirmed by means of bone densitometry. Bone density reflects the quantity of mineral within a given area of the skeleton, thus representing bone mineral density. It is expressed as grams per measured unit of area or volume. Dual energy X-ray densitometry (DEXA) is an effective technique that today is considered to be the gold standard for determining bone density[19].

According to Marshall *et al*[20], bone mineral densitometry is a method that can predict the risk of fracture. However, it is unable to identify individuals with fractures, and systematic osteoporosis screening programs for menopausal women are not recommended[20].

In the literature, tomography has been established as a method for evaluating bone density, although densitometry is today the gold standard examination for determining bone density in humans. It was decided for the present study to use tomography because its accuracy is similar to that of DEXA and its use on small animals is well defined in the literature[21].

Bone analysis using computed tomography is of prime importance for proving the effects of bone reabsorption and formation[22-24], and for faithfully characterizing bone architecture, thus providing better comprehension of bone morphology in rats[25].

Tomography has the capacity to establish a correlation between biomechanical properties and bone morphology in rat femurs[26,27]. This examination may also clearly reflect the parameters of the bone morphological parameters, and it may be used for *in vivo* experiments[28].

Lima *et al*[9] demonstrated the efficacy of three-dimensional computed tomography for morphological evaluations on the bone structure of femurs from Wistar rats. However, the high cost of this apparatus and our limited access to such technology only made it possible to use conventional axial computed tomography.

In the present study, it was observed that HRT in rats led to a significant increase in bone strength. However, no significant increase in bone mineral density was observed in the femurs of the animals that were administered HRT. It is possible that the method used for this evaluation may not have presented sufficient sensitivity to detect possible changes within this sample.

It was decided to carry out the bone strength test in the diaphyseal middle third of the femur because of the impossibility of reproducing the trauma and fracture mechanism in the proximal segment, as occurs in humans. The middle segment was chosen for the evaluation because this was the area subjected to the direct action of the force during the three-point test.

Rats are quadruped animals and the layout of their strength lines, mechanical axis and anatomical axis are not the same as those of humans. This made it impossible to conduct a torsion test that would reproduce a fracture in the proximal test, with a mechanism similar to what often affects the geriatric population.

The aim of the three-point flexion test was to establish the relative strength of the bone when forces were applied, such as deforming flexion on an axis. The bone first broke under tension, and the fracture propagated to the side under compression, thereby opening up a butterfly wing[12,26].

The three-point destructive testing model is reliable and capable of measuring numerical values in newtons that enable comparisons and statistical studies with safe specificity[12].

In the present study, although the femurs of the control group animals presented tomographic density that was greater than the densities in the other two groups, no statistically significant correlation was observed when comparing the breaking strength and the maximum strength in the three groups.

Studies have proven that HRT produces beneficial changes in animals that are subjected to such therapy. A change in bone mineral composition is seen, particularly in areas that are critical regarding fractures and especially in long bones[29-31].

Knowledge of fracture prevention mechanisms and fracture treatment may contribute towards better quality of life for elderly populations.

CONCLUSION

The present study made it possible to conclude that HRT administered to rats led to significantly greater bone strength. The tomographic examination used did not demonstrate any significant changes in bone mineral density.

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