Original Article

Evaluation of radiographic density and proportion of trabecular bone in the femur of female rats medicated with glucocorticoid and bisphosphonate

Avaliação da densidade radiográfica e da proporção trabecular do fêmur de ratas medicadas com glicocorticóide e bisfosfonato

Abstract

Purpose: To evaluate the radiographic density and proportion of trabecular bone in the femur of female rats treated with glucocorticoid combined or not with bisphosphonate.

Methods: Thirty-six female Wistar rats were allocated to 3 groups: (1) control, (2) glucocorticoid, and (3) glucocorticoid + bisphosphonate. Group 1 was given saline solution (subcutaneous injections – 2 mL/kg), groups 2 and 3 were given methylprednisolone acetate (1 mg/kg). Group 3 also received risedronate (3 mg/kg). The animals were killed and each left femur was dissected, radiographed, and histologically processed. A single observer, blind to experimental group, measured optical density and the proportion of trabecular bone. Data were analyzed using ANOVA and Tukey test ($\alpha = 5\%$).

Results: Radiographic density was lower in group 2 than in groups 1 and 3, which were not statistically different. Group 2 had a lower mean proportion of trabecular bone than group 3; groups 2 and 3 did not differ from group 1.

Conclusions: These results suggest that administration of bisphosphonate reverses reduction in the proportion of trabecular bone resulting from glucocorticoid use, and that it is possible to detect these changes with radiography.

Key words: Osteoporosis; rats; femur; radiography; histomorphometry

Resumo

Objetivo: Avaliar a densidade radiográfica e a proporção trabecular do fêmur de ratas submetidas à administração de glicocorticóide, seguida ou não de bisfosfonato.

Metodologia: Foram utilizadas 36 ratas Wistar, alocadas a três grupos: (1) controle, (2) glico-corticóide e (3) glicocorticóide + bisfosfonato. O grupo 1 recebeu injeções subcutâneas (2 mL/kg) de solução salina, os grupos 2 e 3 receberam acetato de metilprednisolona (1 mg/kg), sendo que o grupo 3 foi tratado adicionalmente com risedronato (3 mg/kg). Os animais foram eutanasiados e cada fêmur esquerdo foi dissecado, radiografado e submetido a processamento histológico. Um observador, cego para o grupo a que pertencia a imagem, obteve a média de densidade óptica e o percentual de trabéculas ósseas por campo. Os dados foram analisados por ANOVA, complementada pelo Teste de Tukey ($\alpha = 5\%$).

Resultados: Houve médias menores de densidade radiográfica no grupo 2, diferindo significativamente do 1 e do 3, que não diferiram entre si. Quanto à proporção de trabéculas, as médias foram menores no grupo 2, diferindo significativamente do 3; os grupos 2 e 3 não diferiram significativamente do grupo 1.

Conclusões: Os resultados sugerem que a administração de bisfosfonato recupera a proporção de trabéculas ósseas diminuídas pela ação do corticóide e estas alterações são radiograficamente detectáveis.

Palavras-chave: Osteoporose; ratos; fêmur; radiografia; histomorfometria

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Introduction

Osteoporosis is defined as a systemic metabolic bone disease, characterized by reduced bone mass and microarchitecture deterioration with increased bone fragility and susceptibility to fractures (1). In the adult bone there is equilibrium between bone resorption and formation (turnover), which are related by complex biochemical interactions between osteoblast and osteoclast activity. Osteoporosis occurs when resorption predominates over bone formation (2-5).

Since few symptoms emerge, osteoporosis very often manifests as a bone fracture. Femoral fractures are the most feared complication of osteoporosis because they cause major morbidity (pain, deformity, and physical disability) and mortality, with high impact on public health. Its incidence has increased all over the world as a result of increased population survival and aging (6). According to Machado (7), osteoporosis diagnosis is made on the basis of clinical history, investigation of predisposing factors, and supplemental exams. Conventional radiographs are only able to detect bone loss above 30%. Bone densitometry is considered the gold standard for diagnosis and follow-up and should be carried out annually or at shorter intervals when necessary, for example, when the patient is undergoing corticosteroid therapy.

Glucocorticoids are widely used as pharmacological agents because of their anti-inflammatory and immunosuppressive properties. Among their adverse effects, osteoporosis stands as one of the most common sequelae, since glucocorticoids reduce osteoblast activity without a compensating reduction in bone resorption, with a high risk for fractures. Bone loss induced by glucocorticoids primarily affects trabecular bone. The mechanism of action includes a reduction in absorption of calcium in the intestine and increased renal excretion (8-10).

Bisphosphonates are pyrophosphates that inhibit osteoclast production or activity and act on osteoblasts and bone formation and mineralization. Administered orally, they are considered effective both for prevention and for treatment of glucocorticoid-induced osteoporosis, with the ability to increase bone mass in the spine and femur (8-10).

When studying bone metabolic diseases, in particular of its trabeculae, histomorphometry or quantitative histology is used. This consists of counting or measuring cell components and changes to bone microarchitecture and is the only method that allows mineralization and bone formation to be measured, especially in osteoporosis induced by glucocorticoids, which is characterized by thinning of bone trabeculae (11).

Therefore, the objective of this study was to evaluate the radiographic density and proportion of trabecular bone in the femurs of rats subjected to glucocorticoid administration, followed or not by bisphosphonate use.

Methodology

This study was approved by the Research Ethics Committee of the School of Dentistry, Universidade Federal do Rio Grande do Sul, protocol 42/06. All procedures were in accordance with international recommendations for research with animals.

Thirty-six female *Rattus Norvegicus* rats, Wistar strain, were obtained from the animal house at the Universidade Luterana do Brasil (ULBRA) at 13 weeks' age and weighing 150 to 200 g. The rats were identified by colored marks on their tails and kept in separate cages, with six rates per cage, in an environment with temperature controlled at 23±3ºC, relative air humidity of 55±15%, and a 12 h light-dark cycle. They were fed on standard laboratory chow and water *ad libitum*. These rats were allocated to three groups, each containing 12 animals: 1 - control, 2 - glucocorticoid, and 3 - glucocorticoid + bisphosphonate. The animals in all three groups were kept for 12 weeks, during which time group 1 was given subcutaneous injections (2 mL/kg) of saline solution three times per week. Group 2 were given subcutaneous injections of methylprednisolone acetate (1 mg/kg) in suspension and diluted in saline solution, three times per week. Group 3 were given the same treatment as group 2, followed by risedronate (3 mg/kg) diluted in distilled water at a volume of 5 mL/kg for 12 weeks, six times a week, orally.

The animals in groups 1 and 2 were killed after 12 weeks and those in group 3 after 24 weeks. The left femur was removed from each animal. The anatomic specimens were conditioned individually in labeled plastic pots, containing neutral buffered formalin solution at 10%. The pieces of bones were radiographed from the front, with a Dabi Atlante (Ribeirão Preto, São Paulo, SP, Brazil) X-ray machine, using 50 kV, 8 mA, and no. 2 periapical films (Ektaspeed Plus, Kodak, Rochester, USA). The locater cylinder of the X-ray machine was fitted to a support so that the vertical angle was 90° and focal distance was 30 cm, with an exposure time of 0.3 s. Films were processed in new solutions, in a standardized manner, using the temperature-time method (Kodak, Rochester, USA).

All conventional X-rays were digitized at 300 dpi and saved in BMP format (Bitmap for Windows) files, which were imported into the Adobe Photoshop™ CS software (version 7.0, Adobe Systems Inc., San Jose, CA, USA). A standardized method was used to select the region of interest of each image for optical density analysis. A single observer, who was blind to the experimental groups, used the histogram function to obtain the mean density of the selected area, in pixels (Fig. 1).
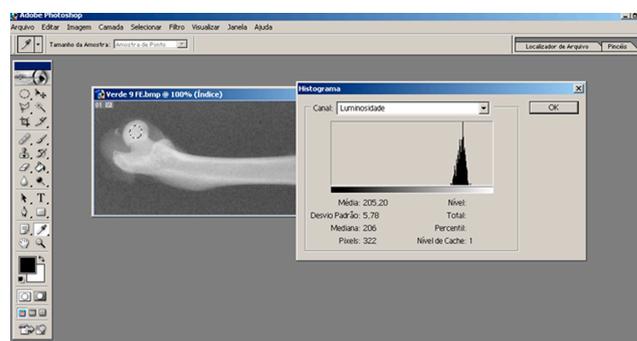

Fig. 1. Obtaining values for radiographic density (RD) using the software Adobe Photoshop™. The mean and standard deviation for the RD of the area selected in the image are shown in the histogram window.

The samples were decalcified in a 1:1 solution of formic acid 50% and sodium citrate 20%, at the Oral Pathology Laboratory of ULBRA. They were kept immersed in the solution, with the substance being replaced daily, throughout the decalcification period. The time taken for decalcification was controlled by means of attempting to transfix the specimen with a histological needle. The decalcified specimens were processed for setting in paraffin. Three semi-sequential sections were taken in the axial direction, with a thickness of 4 μm and 1mm apart from each other, measuring from the extremity of the previous section, using a Leica 2155 rotary microtome. The sections were collected on histological slides. The sections were then stained with Hematoxylin and Eosin (HE) for observation using a transmitted light microscope.

The center of each histological section was selected, which was captured using a microscope at 4X magnification and then photographed using a digital camera. The captured images were then imported into Adobe Photoshop™. The area of trabecular bone observed in each field was quantified, with the aim of determining the proportion between the area of trabecular bone and marrow space (Fig. 2). The specimens were evaluated in stages: A maximum of ten images were evaluated per day and, after a 7-day interval, a second evaluation was performed.

Data on density values of the radiographic images and proportion of trabecular bone per field were analyzed by Analysis of Variance and Tukey's Multiple Comparisons Test, at the significance level of 5%, using the statistical software SPSS, version 10.0.

Results

Significant differences of radiographic density and proportion of trabecular bone were found between groups (Table 1). Groups 1 (control) and 3 (glucocorticoid+bisphosphonate) did not differ but exhibited higher means of radiographic density than group 2 (glucocorticoid) (F=17.20; $P<0.01$).

For proportion of trabecular bone, Group 3 (glucocorticoid+bisphosphonate) showed a significantly higher mean value than group 2 (glucocorticoid). Group 1 (control) did not differ significantly from either of the other groups (F=4.27; $P=0.02$).
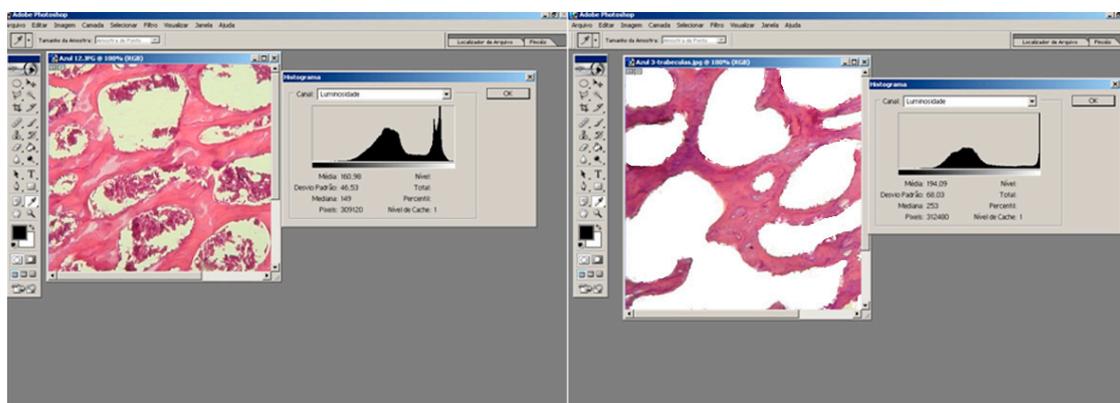

Fig. 2. Selecting trabeculae images and obtaining the trabecular area using the histogram function.

Table 1. Comparison between study groups (n=12/group) in terms of radiographic density (in pixels) and proportion of trabecular bone (%).

Group	Mean*	SD	95%CI	F	P
Radiographic density					
Control	197.24 A	12.39	[189.37 to 205.11]	17.20	<0.01
Glucocorticoid	179.37 B	6.15	[175.45 to 183.27]		
Glucocorticoid + Bisphosphonate	199.33 A	7.79	[194.38 to 204.28]		
Proportion of trabecular bone					
Control	54.96 AB	9.00	[49.24 to 60.67]	4.27	0.02
Glucocorticoid	47.98 A	6.88	[43.61 to 52.35]		
Glucocorticoid + Bisphosphonate	58.26 B	10.20	[51.78 to 64.74]		

* Means followed by the same letter are not statistically different (ANOVA and Tukey test, α=5%).

Discussion

This study analyzed the radiographic density and proportion of trabecular bone in the femur of female rats medicated with glucocorticoid and bisphosphonate, detecting significant differences between groups. Administration of high dose of glucocorticoids for prolonged periods is associated with loss of bone mass, which is more evident and occurs more rapidly in trabecular bone (12-16). The dose used in this study has previously been employed by Takahashi et al. (9) to induce bone loss in female rats, which loss was of the order of 6% compared with a control group and measured by bone densitometry of vertebrae.

The present study showed that the administration of bisphosphonate was capable of reversing the loss of radiographic density resulting from the administration of glucocorticoid, leading to a slightly higher proportion of trabecular bone than in the control group, confirming previous studies (9,15). These results for radiographic density are comparable with the findings by Pinto et al. (17), which described reduced bone density as a typical radiographic feature of osteoporosis induced by dexamethasone and increased density in response to administration of sodium alendronate.

The area of trabeculae in each field, measured using an image analysis software, was determined from the proportion between the area of the trabeculae and the area of the marrow space. In relation to group 1 (control), this proportion was reduced as a result of the administration of glucocorticoid and its increase was related to administration of bisphosphonate, but these differences did not attain statistical significance. Statistically significant differences were only observed when group 2 (glucocorticoid) was compared with group 3 (glucocorticoid+bisphosphonate). Pinto et al. (18) used histomorphometric analysis to confirm the possibility of inducing osteoporosis by administration of glucocorticoid and of recovering bone mass by administration of bisphosphonate. However, these authors used a graticule to quantify trabecular bone during histological analysis. Histomorphometry by counting points was also employed by Piai et al. (19). These authors analyzed the quantities of bone matrix formed in response to risedronate, in bone repair of the tibia of rats with osteopenia. The quantity of neoformed bone tissue, in percentage of area, was determined from the ratio between the number of dots superimposed on the bone matrix and the total number of dots on the grid, indicating that treatment with risedronate was ineffective for bone repair and confirming that the effect of bisphosphonates is dose-dependent.

Conclusions

These results suggest that the administration of bisphosphonate reverses the reduction in the proportion of trabecular bone caused by the effect of glucocorticoids. These changes to the bone tissues can be detect with radiography.

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