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Original Article

Ovariectomy-associated changes in interradicular septum and in tibia metaphysis in different observation periods in rats

Carina M. Esteves, Renata M. Moraes, Francine C. Gomes, Meryenn S. Marcondes, Gabriela M.G. Lima, Ana Lia Anbinder*

Department of Bioscience and Oral Diagnosis, Institute of Science and Technology, Universidade Estadual Paulista-UNESP, Av. Engenheiro Francisco José Longo, 777, Jardim São Dimas, 12245-000 São José dos Campos, Brazil

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ABSTRACT

In order to standardize an experimental model to study the effects of absence of ovarian hormones in maxillary bones compared with long bones, the aim of this research was to analyze the influence of ovariectomy (OVX) on rat alveolar bone and tibiae, in different observation periods. Thirty-six female rats were ovariectomized or sham operated. After 60, 90 or 120 days, the animals were sacrificed and their hemimandibles, maxillae and tibiae were removed and routinely prepared for hematoxylin and eosin staining. The percentage of bone matrix area in bone septum in the first molar furcation region, and in tibial metaphysis was calculated, and data were submitted to statistical analysis ($p < 0.05$). As regards the histomorphometrical analysis in jaw bones, there was no statistical difference between groups, while the effects of ovariectomy on tibiae were seen as early as 60 days. According to the methods used, there was no significant influence of absence of ovarian hormones on interradicular septum of mandibular or maxillary first molars in the periods studied, despite the reduction in bone matrix area in tibia metaphysis as early as 60 days.

Introduction

Significant attention has only recently been paid to women's health issues that have appeared in medical research conducted over the past few decades. Since then, an increasing body of sex-specific literature has emerged with regard to topics related to women [1]. Thus, many studies on post-menopausal osteoporosis have been conducted. Osteoporosis is a disease that should be considered a public health problem due to its social, physical and economic impact. It is defined worldwide as a systemic skeletal disease characterized by low bone density and microarchitectural deterioration of bone tissue, leading to increased bone fragility and risk of fracture [2].

Ovarian hormone deficiency leads to increased bone turnover and rapid loss of cancellous bone in both humans and rats [3–5], however, the rate and magnitude of such changes vary markedly between different skeletal sites [6–9]. While the effects of osteoporosis on increased fracture risk and decreased bone quality of long bones and vertebra have been well established, less is understood about the effects on the maxilla and mandible [10].

The connection between variations in estrogen levels, especially as regards deficiency and postmenopausal osteoporosis, and oral health has been a concern in several dental areas, such as periodontology, implant dentistry, endodontology, prosthodontics, orthodontics, maxillofacial surgery, and oral pathology [11]. Several studies have found effects of osteoporosis on periodontal disease and subsequent tooth loss [12,13] and on the capacity of the maxilla and mandible to integrate endosseous dental implants [14]. In orthodontics, consolidation therapy in estrogen-deficient patients probably takes longer, and relapse and therapeutic failures are more common [15]. A reduced capacity for bone tissue healing has been found to be related to estrogen deficiency [16,17]. Osteoporosis in the jaws may present a risk for accentuation of alveolar bone loss after full denture-wearing [18]. All these findings suggest that estrogen deficiency has a substantial effect on oral bone properties. Nevertheless, conflicting results have been reported as regards the influence of the absence of ovarian hormones on alveolar bone loss, in both human and animal studies [19].

Considering the limitations of cross-sectional studies, since it is difficult to establish and control variables, and the challenges that disturb prospective studies in humans, experimental animal

* Corresponding author. Tel.: +55 12 39479058; fax: +55 12 39479010.
E-mail addresses: anaanbinder@hotmail.com, ana.anbinder@fosjc.unesp.br (A.L. Anbinder).

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models have been extensively used to study the relationship between osteoporosis/osteopenia and the periodontium. The experimental model for osteopenia induced by ovariectomy in female rats is the most commonly used animal model for evaluating problems related to bone loss in postmenopausal women. However, there is no standard ovariectomy (OVX) model [8]. Throughout the literature, it is possible to find osteoporosis studies that have used ovariectomized rats in the age-range from 3 [19,20] to 11 months old [8]. Certain combinations of age, skeletal site, and time post-OVX [8] and methods used to analyze bone disease can lead to varying levels of bone deterioration in response to estrogen deficiency. Such data may be useful for understanding the discrepancies between different studies, in addition to improving study design and interpretation. Bone deterioration can be determined by measuring bone mineral levels using dual energy X-ray absorptiometry (DEXA) and assessing the trabecular microarchitecture using micro-computed tomography (micro-CT), among other cutting edge technologies. In addition, there are simpler methods such as histomorphometry, an affordable method for most laboratories, which has been used in many osteoporosis studies [4,21].

Based on the foregoing, with a view to standardizing an experimental model to study the effects of absence of ovarian hormones in maxillary bones compared with long bones, the aim of this research was to analyze the influence of ovariectomy (OVX) on rat alveolar bone and tibiae, in different observation periods.

Material and methods

Thirty six adult female Wistar rats (*Rattus norvegicus*) aged approximately 90 days were used. The study was approved by the Institutional Ethics Committee.

The animals were divided into two groups, with one half being submitted to bilateral ovariectomy (OVX group) and the other half, to sham surgery for surgical stress simulation (SHAM group).

After anesthesia with a mixture of 13 mg/kg of 2% xylazine hydrochloride (Rompun – Bayer – São Paulo, SP, Brazil) and 33 mg/kg of ketamine base (Francotar – Virbac – Roseira, SP, Brazil), the lateral abdominal region was shaved, the skin and musculature were incised longitudinally below the last rib, and the ovary was identified and exposed. In the OVX group, hemostasis was performed by ligation of the upper part of the Fallopian tube with no. 4.0 silk suture, and the ovary was excised together with the surrounding fat, oviduct and a small portion of the uterus. The muscle layer was then closed with absorbable no. 4.0 catgut and the skin sutured with no. 4.0 silk. In the SHAM group, after exposure of the ovary, the organs were returned to the abdominal cavity, which was sutured.

Six animals of each group (OVX and SHAM) were anesthetized with anesthetic overdose and euthanized by decapitation, after the observation periods of 60, 90 or 120 days. The hemimandibles, maxillae and tibiae were removed and fixed in 10% formalin.

All animals were weighed on the day of ovariectomy or sham surgery (initial weight), and on the day of sacrifice, and the percentage weight gain during the experiment was calculated.

Hemimandibles and maxillae were decalcified in a 6% aqueous solution of (ethylenedinitrilo) tetraacetic acid solution, and then embedded in paraffin. We used mesiodistal histological sections cut at a fixed distance of 20 μm between them, in order to evaluate the entire volume of the first molar furcation. From all the slides obtained, five sections equidistant from each other, in which the medial roots could not be seen, were chosen from each hemimaxilla and hemimandible. Sections with the presence of first molar medial root were excluded, because its presence diminished the amount of alveolar bone available for evaluation in the furcation. Proximal tibiae were decalcified with 5% trichloroacetic acid, hemisectioned in the longitudinal direction and embedded in paraffin parallel to the sectioned surface. Semi-serial sections, with a 20 μm distance between them, similar to those from the jaw bones were then obtained, and four equidistant sections were selected for analysis.

The image of the interradicular septum between mesial and distal roots in the furcation region was digitized (50× magnification) using a Axioplan2 microscope (Zeiss, Oberkochen, Baden-Württemberg, Germany) and the percentage of bone matrix in an elliptical area of 8.495 mm^2 in the mandible and of 8.732 mm^2 in the maxilla were histomorphometrically evaluated. In summary, the image of the bone matrix area, including the osteocyte lacunae was selected with the aid of the Adobe Photoshop 7.0.1 (Adobe Systems Incorporated, San Jose, CA, USA) software program. The image was then transferred to the ImageJ 1.31p software (National Institutes of Health, Bethesda, MD, USA), in which it was binarized, and the area was calculated [22]. To analyze the percentage of bone matrix area in the tibial metaphysis, planimetry by point counting was used. In this method, a grid of equidistant parallel test lines with 96 cross-points was placed on a digital microscope image (100× magnification) immediately under the growth plate. The percentage of all points along the bone matrix to the total number of points of the grid was considered.

The five sections selected from each hemimaxilla and hemimandible and the 4 sections of each tibia, were analyzed per animal. The mean percentage of bone matrix of all sections per animal was taken as the value used for statistical analyses.

For statistical analysis, the independent variables were “time” (60, 90, 120 days) and “ovarian hormones” (SHAM, OVX), whereas the dependent variables were “weight”, “percentage of bone area in the furcation region of the first molar in the maxilla, mandible” and “percentage of bone area in tibial metaphysis”. All data were submitted to two-way analysis of variance (ANOVA) and the Sidak post hoc test, when necessary. The level of significance was set at 5% for all tests performed, for which the GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA) software program was used.

Results

Completeness of ovariectomy was confirmed at death in all OVX rats by the absence of ovarian tissues and atrophied uterine horns. Due to technical difficulties, four animals from each group were used ($n = 4$) for the analyses of maxillary and mandibular areas, and one animal from OVX-120 group was excluded from the tibial analysis.

Body weight

The body weight values of the animals at day 0 and sacrifice day (60, 90 or 120 days) were transformed into percentage of weight gain and submitted to two-way ANOVA. An interaction effect between “ovarian hormones” and “time” was found ($F_{df(2;30)} = 5.44$; p-value = 0.01 < 0.05) as well as statistically significant difference for main effect “ovarian hormone” ($F_{df(1;30)} = 70.02$; p-value = 0.001 < 0.05). The Sidak test indicated statistical difference with regard to the variable “ovarian hormones” after 60 and 90 days (Fig. 1a). In other words, the percentage of body weight gain was lower in the SHAM than the OVX group after 60 days (SHAM 60 d = 9.87 ± 4.35%; OVX 60 d = 35.30 ± 6.03%) and 90 days (SHAM 90 d = 14.37 ± 2.21%; OVX 90 d = 31.58 ± 2.28%). No significant difference was found for the main effect “time” ($F_{df(2;30)} = 0.46$; p-value = 0.638 > 0.05).