Histomorphometric analysis and immunolocalization of RANKL and OPG during the alveolar healing process in female ovariectomized rats treated with oestrogen or raloxifene

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A B S T R A C T
Objective: To investigate the effects of bone-resorption inhibitors (oestrogen and raloxifene) on the RANKL/OPG balance during the chronology of the alveolar healing process in ovariectomized (OVX) rats by means of immunocolocalization and histomorphometric analysis.

Materials and methods: One hundred sixty female Wistar rats at 70 days of age were either OVX or sham-operated and divided into four groups: sham, OVX/Oil, OVX with E2 replacement (17β-estradiol, 400 μg/month), OVX with RLX treatment (1 mg/kg bw/day). The 60-day treatment started 8 days after ovariectomy. The incisors were extracted to allow analysis of 7, 14, 21, 28 and 42 days of wound healing. After obtaining the histological samples, slides were stained with hematoxylin and eosin or subjected to immunocolocalization reaction for RANKL and OPG. Results were quantitatively evaluated.

Results: Histomorphometric analysis showed that the sham group presented the highest and OVX/Oil group the lowest mean bone formation value in the post-extraction period. The immunocolocalization analysis showed a larger increase in bone turnover at 7 postoperative days in OVX/Oil and sham groups and decreasing bone turnover in the other periods. The OVX/Oil group showed a large decrease in bone turnover at 14 postoperative days, a period demonstrated by mild cellular activity. OVX/E2 and sham groups showed a decreased bone turnover at 28 postoperative days while OVX/RLX group showed a decreased bone turnover at 21 postoperative days. On the 42nd postoperative day, sham and OVX/RLX groups showed an established alveolar bone healing process.

Conclusions: Ovariectomy delays the alveolar healing process and interferes with bone turnover through the balance between RANKL and OPG. Oestrogen replacement or raloxifene treatment did not totally recover the oestrogen-deficient state. However raloxifene treatment showed more satisfactory results than oestrogen replacement.

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1. Introduction

Bone is a mineralized tissue that undergoes continuous remodelling by the combined action of osteoclasts, osteoblasts and osteocytes, which are influenced by several systemic, local and environmental factors. These factors regulate the proliferation, differentiation, function and survival of bone cells. Among systemic factors, oestrogen is a hormone well known for its inhibitory function on bone resorption by its direct effect on osteoclasts. More recently, it has been shown that oestrogen depletion promotes intense resorptive activity in the alveolar bone of rats.

Based on the protective effect of oestrogen on bone tissue, oestrogen hormone replacement currently remains among the most frequently used treatments for menopausal symptoms and prevention of osteoporosis. However the possibilities of clinical contra-indications, and risk of side-effects, have prompted the search for alternative approaches. Thus, raloxifene (selective oestrogen receptor modulators – SERM that acts as oestrogen receptor agonist in some tissues and as antagonist in others), has been proposed as promising alternative therapy for the treatment of postmenopausal osteoporosis.

In normal bone remodelling, osteoblastic bone formation follows osteoclastic bone resorption and occurs in a precise and quantitative manner, a process called coupled bone formation. The TNF family member receptor activator of nuclear factor kappa B ligand (RANKL: expressed by osteoblasts and their immature precursors) and its receptor RANK (expressed in osteoclast precursors and osteoclasts) are key regulators of bone remodelling and are essential for the development and activation of bone-resorbing osteoclasts. Bone homeostasis is achieved by a balance of bone-resorbing effects of RANKL and its natural decoy receptor osteoprotegerin (OPG; produced by osteoblasts and bone marrow stromal cells). OPG binds to RANKL thus limiting the interaction between RANKL and RANK, and inhibiting the signalling events that modulate osteoclast differentiation and lymphocyte development.

The balance between RANKL and OPG is regulated by cytokines and hormones and determines osteoclast functions. Alterations of the RANKL/OPG ratio are critical in the pathogenesis of bone diseases that result from increased bone resorption. The term osteoprotegerin has been coined for its effects on animals in which it protects against bone loss. Studies have shown that the alveolar bone healing process occurs in a dynamic manner and involves many cellular steps. The process starts with fibroblastic proliferation, mainly from the remainder of the periodontal ligament adhered to the dental socket walls. The remainder periodontal ligament originates a connective tissue that will be the foundation for calcium precipitation, leading to bone trabecular formation from the fundus and lateral walls, in order to fill the dental socket.

Therefore, the purpose of the present study was to evaluate the influence of an oestrogen-deficient state, and its treatments with oestrogen or with raloxifene on the RANKL/OPG balance by means of immunocolocalization and histomorphometric analysis during different periods in the chronology of the alveolar bone healing process.

2. Material and methods

2.1. Animals

One hundred and sixty female Wistar rats, weighing between 200 and 220 g at 70 days of age at the initial time point were maintained at a temperature of 22 °C, in a 12-h light/12-h dark cycle, with free access to water and rat food. The principles of laboratory animal care (NIH publication 85-23, 1985) and national laws on animal use were complied with in the present study, which was authorized by the Animal Research Ethics Committee of the São Paulo State University, Brazil (protocol number 38/05). The estrous cycle of the rats was monitored and only animals that achieved three regular estrous cycles were selected for the experiment.

Rats were OVX or sham-operated under general anaesthesia with xylazine (0.03 ml/100 g bw/ip – Dopaser Labora-tories Calier S.A., Barcelona, Spain) and Ketamine (0.07 ml/100 g bw/ip – Fort Dodge Saúde Animal Ltda, Brazil). Animals were randomized into four groups: (1) sham; (2) OVX/Oil (subcutaneous corn oil pellets); (3) OVX/E2 (subcutaneous estradiol pellets – 400 μg/month); (4) OVX/RLX (gavage administration of raloxifene – 1 mg/kg bw/day). All the treatments were administered for a period of 60 days, starting on the eighth day after the OVX, with 40 animals in each group.

2.2. Treatment

Pellets 1.2 cm long made of silastic tubing (Dow Corning, Grand Rapids, MI, USA), were filled with 17β-estradiol (400 μg, Sigma, Saint Louis, Missouri, USA) or corn oil and were inserted subcutaneously in the rat’s back and changed after 30 days during the experimental period. For raloxifene administration, the rats were immobilized, then an adequate cannula was introduced orally and the raloxifene (Evista; Lilly, São Paulo, SP, Brazil) was released into their stomachs (1 mg/kg/day). Gavage was performed every day until the last day of the experiment (60 days).

2.3. Tooth extraction

The right maxillary incisor extractions were performed in such a way that at the end of 60 days it was possible to obtain pieces with reference to 7, 14, 21, 28 and 42 days of the alveolar healing process. Animals were anaesthetized (Coopazine; Xylazine) and after antisepsis (Polyvinylpyrrolidone iodide; Indústria Química e Farmacêutica Rioquímica Ltda, Brazil). The right maxillary incisor was luxated with the aid of a tapered instrument and extracted with a small forceps. The movement of extraction was smooth and followed the curvature of the rat incisor, so that root fracture would not occur. The atraumatic surgical technique was used, which allows tooth extraction without postoperative complications. The dental sockets were sutured with silk thread (Ethicon 4.0, Johnson and Johnson, São Paulo SP, Brazil).

2.4. Collection of materials

After the experimental periods the animals were anaesthe-tized and blood was collected and centrifuged (15 min;
2500 rpm; 2 °C). The plasma was stored at −20 °C for radioimmunoassay of estradiol (kit BioChem ImmunoSystem, Bologna, Italy). After euthanasia by anaesthetic overdose, infusion was performed with 4% formaldehyde (Acros Organics, New Jersey, USA), using a Masterflex LS perfusion pump (Cole-Parmer Instrument Company, Vermont Hills, IL, USA) to remove the right maxilla. The pieces obtained were post fixed in 4% formaldehyde, demineralized in 5% EDTA (Merck, Darmstadt, Germany) and cryoprotected in sucrose (Merck, Darmstadt, Germany). The pieces were sliced in a cryostat (Micron Zeiss, Berlin, Germany) to obtain 14 μm thick slices longitudinal to the long axis of the dental socket, which were mounted on previously gelatinized slides.

2.5. Histomorphometric analysis

Two slices from each animal were stained with hematoxylin and eosin for the morphometric analysis of the bone formation in the middle thirds of the rat alveolus.16,19 The analyses were performed without the knowledge of the examiner as to sham, OVX/Oil, OVX/E2 and OVX/RLX groups. One field was analysed in each histological section and examined by light microscopy under 10× objective lenses, and images were obtained with a digital camera (JVC TK1270 Color Video Camera) mounted on the microscope and analysed with Leica Qwin Color/RGB software. The results among groups were analysed by the analysis of variance (ANOVA) followed by a post hoc Tukey’s test when the ANOVA suggested a significant difference between groups (p < 0.05).

2.6. Immunolocalization reaction

To perform the immunolocalization, the first set used was: anti-RANKL primary antibody (Goat anti-RANKL polyclonal – Santa Cruz, California, USA), and anti-OPG primary antibody (Rabbit anti-OPG polyclonal – Santa Cruz, California, USA). The second set of antibodies was: anti-rabbit conjugated to Cy3 (Jackson Immunoresearch Laboratories, West Grove, PA, USA) and anti-goat conjugated to FITC (Jackson Immunoresearch Laboratories, West Grove, PA, USA).

The sections were rinsed in 0.1 M phosphate-buffered saline (PBS, 6 × 10 min), followed by incubation in glycine and then in cold PBS containing 10% bovine serum albumin (BSA). After rinsing with PBS, the sections were reacted with anti-RANKL antibody and anti-OPG antibody (diluted 1:200 in 0.1% BSA/PBS) overnight then rinsed with PBS and the anti-rabbit conjugated to Cy3 and anti-goat conjugated to FITC were reacted for 1 h. To confirm the specificity of the immunostaining, the primary antibody was substituted with normal donkey serum at the same dilution, with 0.1% BSA/PBS used for dilution of the antibodies.

3. Results

Fig. 1 presents the plasmatic concentrations of estradiol of all experimental animals. The animals submitted to sham surgery presented the four regular stages of the estrous cycle, and the animals of group OVX/E2 presented enucleated cornified cells and higher plasmatic concentration of estradiol. The pellets were changed every 30 days because in this last period the vaginal smears were modified with the presence of large amounts of leukocytes, according to studies conducted in our laboratory (data not shown). Groups OVX/Oil and OVX/RLX presented diestrus smear, atrophied uterine horns and a lower plasmatic concentration of estradiol.

3.1. Histomorphometric analysis

Analysis of the histological events showed that in all of the evaluated groups the steps of the alveolar healing process occurred as described in the literature.16,17 Animals in the sham group showed greater bone formation in the middle third in all periods analysed whereas, animals in group OVX/Oil had lower bone formation. The alveolar bone formation of OVX animals treated with raloxifene was gradual, differing from that observed in the oestrogenized OVX group, which presented a surge in the first 14 days after extraction (Table 1). At 7 days post-extraction, the presence of a blood clot being invaded by fibroblasts, endothelial cells and macrophages, characterizing the granulation tissue were observed in all groups. In some areas, the presence of neoformed bone tissue (trabecular bone) was also observed. At 14 days post-extraction, a larger quantity of trabecular bone and osteoblasts located around the neoformed bone trabeculae was observed. At 7 and 14 days post-extraction, there was statistically significant difference between the quantity of bone formed in the sham group in comparison with the bone formation in the other groups. In OVX/E2 there was greater bone formation in comparison with OVX/Oil and OVX/RLX (Table 1). At 21 days post-extraction one could observe the presence of osteocytes inside the neoformed bone trabeculae. At 28 and 42 days post-extraction, mature bone trabeculae were observed, filling a large part of the alveolus. The treatment with oestrogen or raloxifene triggered increased bone formation after 21, 28 and 42 days of extraction, with this increase being higher in animals of the group OVX/RLX and also similar to that of the sham group (Table 1).

Fig. 1 – Plasmatic concentrations of estradiol for all groups after 60 days post-treatment. Different letter represents the statistical differences between the groups (p < 0.001).
first period analysed and the cellular activity in OVX/E2 and showed a decrease in bone turnover when compared with the other groups. The sham group at 14 postoperative days, a period demonstrated by mild cellular OVX/RLX groups.

OVX/Oil, OVX/E2 and sham groups remained the same as it was in the previous period. Sham and OVX/E2 groups differed significantly from the OVX/Oil and OVX/RLX groups.

The OVX/Oil group showed a large decrease in bone turnover at 14 postoperative days, a period demonstrated by mild cellular activity when compared with the other groups. The sham group showed a decrease in bone turnover when compared with the first period analysed and the cellular activity in OVX/E2 and OVX/RLX remained the same as it was in the previous period.

On the 21st postoperative day, the bone turnover in the OVX/Oil, OVX/E2 and sham groups remained the same as it was in the previous period, and only in OVX/RLX was there a large decrease in the OPG/RANKL ratio in comparison with the previous period. Sham and OVX/E2 groups differed significantly from the OVX/Oil and OVX/RLX groups (Fig. 2).

The OVX/Oil group showed a large decrease in bone turnover at 14 postoperative days, a period demonstrated by mild cellular activity when compared with the other groups. The sham group showed a decrease in bone turnover when compared with the first period analysed and the cellular activity in OVX/E2 and OVX/RLX remained the same as it was in the previous period.

On the 21st postoperative day, the bone turnover in the OVX/Oil, OVX/E2 and sham groups remained the same as it was in the previous period, and only in OVX/RLX was there a large decrease in the OPG/RANKL ratio in comparison with the previous period. Sham and OVX/E2 groups differed significantly from the OVX/Oil and OVX/RLX groups (Fig. 3).

On the 28th postoperative day, sham, OVX/Oil and OVX/RLX groups showed similar bone turnover, however it was increased in comparison with that of the OVX/E2 group. OVX/Oil group analysis showed statistical difference when compared with that of the other groups. OVX/E2 group analysis showed statistical difference when compared with that of the OVX/RLX group.

On the 42nd postoperative day, sham and OVX/RLX groups showed a decreased bone turnover in comparison with that of the other periods in the same groups, which indicated an established alveolar bone healing process, which had not yet occurred in the other groups, indicating a delay in the alveolar healing process. Sham and OVX/E2 groups differed significantly from the OVX/Oil and OVX/RLX groups.

### 3.2 Immunolocation of RANKL/OPG analysis

The overlap of the slices showed the presence of OPG and RANKL in the studied groups. The yellow colour represents the overlap of the areas captured with both wavelength filters, for visualization of FITC (green) and CY3 (red) fluorochromes, respectively. The double labeling of RANKL and OPG in osteoblasts around the neoformed bone trabeculae suggests the bone turnover step, as well as synthesis of both proteins by osteoblasts during the alveolar healing process (Fig. 2).

On the seventh postoperative day, the OVX/Oil and sham groups showed intense cellular activity demonstrated by an increased bone turnover (represented by colocalization of RANKL/OPG) followed by OVX/E2 and OVX/RLX groups. Sham and OVX/Oil groups differed significantly from the OVX/E2 and OVX/RLX groups.

The OVX/Oil group showed a large decrease in bone turnover at 14 postoperative days, a period demonstrated by mild cellular activity when compared with the other groups. The sham group showed a decrease in bone turnover when compared with the first period analysed and the cellular activity in OVX/E2 and OVX/RLX remained the same as it was in the previous period.

On the 21st postoperative day, the bone turnover in the OVX/Oil, OVX/E2 and sham groups remained the same as it was in the previous period, and only in OVX/RLX was there a large decrease in the OPG/RANKL ratio in comparison with the previous period. Sham and OVX/E2 groups differed significantly from the OVX/Oil and OVX/RLX groups (Fig. 3).

On the 28th postoperative day, sham, OVX/Oil and OVX/RLX groups showed similar bone turnover, however it was increased in comparison with that of the OVX/E2 group. OVX/Oil group analysis showed statistical difference when compared with that of the other groups. OVX/E2 group analysis showed statistical difference when compared with that of the OVX/RLX group.

On the 42nd postoperative day, sham and OVX/RLX groups showed a decreased bone turnover in comparison with that of the other periods in the same groups, which indicated an established alveolar bone healing process, which had not yet occurred in the other groups, indicating a delay in the alveolar healing process. Sham and OVX/E2 groups differed significantly from the OVX/Oil and OVX/RLX groups.

### 4. Discussion

Alveolar healing process provides a suitable model for the study of bone formation in rats and can be considered a sensitive indicator of bone damage under different experimental conditions. Ovariectomy has been considered a particularly useful model to investigate the loss of bone trabeculae in response to oestrogen deficiency, since the loss of cortical bone is minimal.

The effect of raloxifene on bone tissue without affecting the endometrium was observed when comparing the uterine weight of female rats. Studies in literature have shown that raloxifene may control bone loss and reduce cholesterol without affecting uterine hypertrophy.

Both studied proteins were immunodetected in osteoblasts, osteocytes and/or in bone lining cells, which are the predominant cells at the studied stages of alveolar bone healing. Bone formation is the main activity of cells that repair the dental socket. Osteoblasts expressed both OPG and RANKL, playing a main role in maintaining balanced bone dynamics by secreting both proteins at early stages of alveolar bone healing.

The OVX/Oil group bone turnover decreased drastically after the first period analysed. This observation is probably the result of apoptosis of the osteoblast-lineage cells via up-regulation of toll-like receptors which is induced by the ovariectomy, evoking a decrease in the release of OPG and RANKL by these cells. In relation to the histomorphometric evaluation, it was observed that at 7 and 14 days post-extraction, statistical analysis showed significance decrease in the quantity of bone formed in the other groups, comparing with the sham group.

Ovariectomy increases the bone loss in ligature induced periodontitis and negatively affects the sustentation bone tissue, decreases bone repair and its density around titanium implants. Different studies have observed that the decrease in bone density due to ovariectomy affects bone turnover after tooth extraction with an increase in bone resorption and decrease in bone formation. This is in agreement with the results of the present study, in which less than 50% of the middle third of dental sockets were observed to be filled by bone, also affecting bone turnover which was represented by RANKL/OPG colocalization.

The procedures of oestrogen removal and replacement were important to enable a complete evaluation of the physiological role of this hormone in bone metabolism. Other than that the ovariectomy procedure led to the removal of oestrogen and progesterone, but only oestrogen was replaced. This is believed to be one of the reasons why even after the oestrogen or raloxifene replacement therapy, the quantity of neoformed bone in both groups was lower than that of the sham group.

### Table 1 - Mean values ± standard error of the mean (S.E.M.) of histometric results from neoformed bone area in middle thirds of rat alveolus (% of total area) for all groups after 7, 14, 21, 28 and 42 days post-extraction. Statistical analysis was performed between groups of the same period after tooth extraction. Different letters represent the statistical differences between the groups (p < 0.05).

<table>
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<th></th>
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<th>28 d</th>
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<td>23.64b</td>
<td>1.6</td>
<td>29.5b</td>
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<td>2.62</td>
<td>49.81b</td>
<td>2.5</td>
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<tr>
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<td>1.0</td>
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<td>0.82</td>
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3.2. Immunolocation of RANKL/OPG analysis

The procedures of oestrogen removal and replacement were important to enable a complete evaluation of the physiological role of this hormone in bone metabolism. Other than that the ovariectomy procedure led to the removal of oestrogen and progesterone, but only oestrogen was replaced. This is believed to be one of the reasons why even after the oestrogen or raloxifene replacement therapy, the quantity of neoformed bone in both groups was lower than that of the sham group.
Fig. 2 – (A–T) Histological sections of the middle third of the alveolus during the periods in the chronology of the alveolar healing process at 7, 14, 21, 28 and 42 postoperative days from sham (first and third column) and OVX/Oil (second and forth column) groups. The first and second columns are representing the histological section stained with hematoxylin and eosin (10×). A stepwise increasing in bone formation and maturation was observed during all analysed periods in both groups. However, sham group showed greater bone formation in all analysed periods compared to OVX/Oil group. This difference between both groups is well illustrated comparing the first and second column of the figure. The third and forth columns are representing the histological section from OPG/RANKL immunocolocalization. A stepwise decreasing of OPG/RANKL ratio was observed at sham group (third column) during all analysed periods. OVX/Oil group (forth column) showed an intense bone turnover at the seventh postoperative day and a large decreasing during the other periods. The white arrows show the double labeling of RANKL and OPG in osteoblasts around the neoformed bone trabeculae.
The association of histomorphometry and RANKL and OPG colocalization brings relevant data to the questions raised in this study. The colocalization technique labels the synthesized protein simultaneously in the same cell, allowing a special analysis of the tissue to be made. It is important to observe that the gene expression of mRNA of a protein does not always mean that this same protein will be synthesized. Moreover, serological evaluation techniques do not always show whether the proteins are in activity or not, because it is possible for the OPG in serum to be linked to another plasma protein and it may be inactive32; RANKL is also produced by a variety of cell types and its expression is regulated by many physiologic and pathologic factors33 therefore, the quantity of RANKL serum does not refer to bone tissue only.

According to the results obtained, it is possible to describe the alveolar repair process associated with the RANKL and OPG pattern of expression in the control (sham) group as being inversely proportional. As there was an increase in bone formation during the course of the analysed periods, there was a reduction in the expression of these proteins, indicating complete filling of the alveolar process associated with a balance in bone turnover.

Implant of biomaterials into the post-extraction socket of osteoporotic female rats promotes an increase in the trabecular bone formed, when compared with the connective tissue volume and blood clot remnants.31 These observations show that there are materials available that can act locally helping to solve adverse situations that occur in the presence of systemic changes. The advances of biological engineering have researched bone morphogenetic proteins as an alternative.34,35

The importance of RANKL and OPG-regulated processes has been demonstrated by the phenotype of genetically modified mice. Severe osteoporosis develops in mice with OPG gene deletion36 because of the increased numbers and activity of osteoclasts, whereas overexpression of OPG results in osteopetrosis due to a reduction in number and activity of bone-resorbing osteoclasts.15

The OPG/RANKL/RANK system plays an important role in the regulation of bone turnover.37,38 Anomalies of this system have been implicated in the pathogenesis of postmenopausal osteoporosis.39 Conceptually, an elevated RANKL/OPG ratio within the skeleton promotes bone loss,13 while restoring a balanced RANKL/OPG ratio or blunting RANK responsiveness prevents osteoclast activation and bone resorption. Different strategies can be considered for therapeutic purposes. In vitro and in vivo studies suggest that RANKL expression is suppressed by 17β-estradiol,40 and RANKL effects can be blocked by synthetic OPG fusion proteins,41–43 soluble RANK fusion protein14 or RANKL antibodies. RANK activation can be suppressed by peptidomimetics that prevent RANK binding to RANK44 or by blocking post-receptor signaling.45 In vitro experiments show that OPG production can be stimulated by 17β-estradiol,46 raloxifene,21 bisphosphonates47, otherwise, in vivo experiments show that OPG production is stimulated by mechanical strain48 and selective small molecule stimulators49.

This study is the first to demonstrate the influence of an oestrogen-deficient state, and its treatments with oestrogen or with raloxifene on the RANKL/OPG balance during the periods in the chronology of the alveolar bone healing process. The present study demonstration of colocalized OPG with RANKL confirms the specific cellular synthesis. In addition the present study was able to show oestrogen and raloxifene modulated OPG and RANKL expression in the osteoblastic cell lineage in the alveolar healing process. The results of this study suggest that the oestrogen and raloxifene treatment increased the osteoblastic cell lineage synthesis of OPG, and the decreased RANKL synthesis could provide such contribution.

Fig. 3 – Mean values ± S.E.M. of immunolocation of RANKL/OPG analysis in middle thirds of rat alveolus (% of total number of stained cells) for all groups after 7, 14, 21, 28 and 42 days post-extraction. Statistical analysis was performed between groups of the same period after tooth extraction Different letter represents the statistical differences between the groups (p < 0.05).

This study showed that cells involved in the alveolar healing process presented both OPG and RANKL expression, which changed during its development. Both expressions represent the bone turnover and osteoblastic activity, reflected in the quantity of neoformed bone in the evaluated periods of alveolar healing process. In the sham group, direct
inhibitory effects were shown to be exerted on RANKL-induced osteoclast differentiation. The increase in trabecular quantity was associated with a decrease in the bone turnover (OPG/RANKL ratio).

5. Conclusion

Ovariectomy delays the alveolar healing process and interferes with bone turnover. The oestrogen replacement or raloxifene treatment did not totally recover the oestrogen-deficient state. However raloxifene treatment showed more satisfactory results than oestrogen replacement.

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Conflict of interests: No conflict of interest.

Ethical approval: Animal Research Ethics Committee of the São Paulo State University, Brazil (protocol number 38/05).

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