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Raloxifene therapy inhibits osteoclastogenesis during the alveolar healing process in rats

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ABSTRACT

Objective: To investigate the expression of OPG, RANKL and TRAP during alveolar healing process (7, 14, 21, 28 and 42 postoperative days) in ovariectomized rats treated with raloxifene or with oestrogen replacement therapy, using immunohistochemistry reaction approach.

Materials and methods: Wistar female rats (10 weeks age) were submitted to ovariectomy surgery (OVX) or sham surgery. The female rats were divided in four groups: (1) sham; (2) OVX/O (ovariectomy and oil); (3) OVX/E2 (ovariectomy and oestrogen replacement); (4) OVX/RLX (ovariectomy and raloxifene therapy).

Results: It was observed high amount of OPG immunolabelling with predominance at 14 and 21 postoperative days on sham and OVX/RLX groups, respectively. At 7 postoperative days, there was no difference between the groups for TRAP protein. Otherwise, to the other periods, it was observed greater expression of TRAP and RANKL protein on OVX/O group compared to sham, OVX/E2 and OVX/RLX groups. It was also observed a discrete TRAP immunolabelling at 28 and 42 postoperative days on OVX/RLX group.

Conclusions: Oestrogen deficiency induces osteoclastogenesis in the alveolar healing process. Quantitative changes in the osteoclastic activity could be prevented through the raloxifene therapy.

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1. Introduction

Raloxifene (benzotiofen analogue) is a selective modulator of oestrogen receptors (SERMs) that prevents bone loss. The medicament is used in the treatment and prevention of osteoporosis in the United States and in many other countries, due to its selective activity to the oestrogen receptors of the bone tissue. According to the literature, raloxifene reduces the expression of bone turnover markers, increases bone mineral density, reduces vertebral fractures risk from 50% to 30% in precocious menopause women,[1] decreases the breast cancer incidence[2] and changes the lipids concentration in the bloodstream.[3] However, the mechanisms whereby this compound modulates bone cells activities are less known.

Selective markers of bone turnover as osteoprotegerin (OPG), Kappa B factor ligand of the tumoural necrosis factor (RANKL) and tartrate resistant acid phosphatase (TRAP) are used for analysis of the effects of pharmacological agents and pathogenesis of bone diseases in ovariectomized rat model (OVX). These markers have been considered relatively specific for osteoblasts (OPG and RANKL)[4,5] and osteoclasts (TRAP).[6,7]

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Therefore, the aim of this study is to compare the effect of raloxifene therapy with oestrogen replacement therapy in ovariectomized rats during the chronology of the alveolar healing process. To better understand the potential of raloxifene in improving bone quality a semi-quantitative evaluation of the osteoclastogenesis during the alveolar healing process were proceeded by means of immunohistochemistry reactions of OPG, RANKL and TRAP protein.

2. Material and methods

2.1. Animals

Laboratory principals of animal care[8] and the national laws of the animal use were followed in the present study that was authorized by the Ethics Committee in animal experimentation of the São Paulo State University, Brazil. One hundred and sixty female Wistar rats (10 weeks age) were kept in the adequate place (22 ± 2 °C, 12 h light/12 h dark), with food and water *ad libitum*.

Rats with regular estrous cycle were submitted to ovariectomy (OVX)[9] or sham surgery under xilazine (0.03 ml/100 g bw/ip-Dopaser Laboratories Calier S.A., Barcelona, Spain) and ketamin (0.07 ml/100 g bw/ip-Fort Dodge Saúde Animal Ltd., Brazil). The animals were randomly separated in 4 groups with 40 animals each one: (1) sham, (2) OVX/O, (3) OVX/E2 and (4) OVX/RLX. Every treatment started at the 8th day after ovariectomy and lasted for 60 days.

2.2. Treatment

The OVX animals received pellets (1.2 cm silastic tubing; Dow Corning, Grand Rapids, MI, USA) with 17β-estradiol (400 μg; Sigma, Saint Louis, MO, USA) – OVX/E2 group or pellets with corn oil – group OVX/O. The pellets were subcutaneously inserted in the back of the rats and changed each 30 days during the experimental period because in this last period there was modification in the vaginal smears with the presence of large amounts of leukocytes, according to previous studies conducted in our laboratory (date not shown). Raloxifene (1 mg/kg/day; Evista; Lilly, São Paulo, SP, Brazil) was directly liberated in the stomach of the experimental animals, through gavage. The treatments were performed for 60 days.

2.3. Dental extraction

The animals were anesthetized with xylazine (0.03 ml/100 g body weight [bw]/intraperitoneal [ip]; Dopaser® Laboratories Calier SA, Barcelona, Spain) and ketamine (7 μl/kg bw/ip; Fort Dodge Saúde Animal Ltd., Brazil), and after the antisepsis (polyvinylpyrrolidone iodide; Indústria Química e Farmacêutica Rioquímica Ltd., Brazil), the right upper incisive was extracted with appropriate instrumental.[10] The dental sockets were sutured with silk thread (Ethicon 4.0, Johnson and Johnson, São Paulo, SP, Brazil). The extractions were realized in 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 alveolar wound healing.

2.4. Collection of materials

After 60 days, the animals were sacrificed by intracardic perfusion (Cole Parmer Instrument Company, Vernon Hills, IL, USA) with a 4% paraformaldehyde solution (Acros organics, NJ, USA) then, the right maxilla was removed. The obtained pieces were postfixed in 4% paraformaldehyde solution, demineralized with 1% EDTA (Merck, Darmstadt, Germany) and crioprotected with sucrose (Merck, Darmstadt, Germany). The pieces were longitudinally sectioned through the long axis of the alveolar process with a cryostat (Micron Zeiss, Berlin, Germany) in order to obtain slices with 14 μm thickness, that were mounted in previously gelatinized slides.

2.5. Immunohistochemistry reaction

For the immunohistochemistry reactions, primary antibodies anti TRAP, anti OPG and anti RANKL (Goat anti trap polyclonal, Goat anti opg polyclonal; Goat anti rankl polyclonal – Santa Cruz, CA, USA) and the biotinylated donkey anti goat antibodies (Biotin-SP-AffiniPure donkey anti goat IgG-Jackson Immunoresearch Laboratories, West Grove, PA, USA) was the secondary antibody; the immunohistochemistry reaction signal was amplified with the Avidin–Biotin system (Kit ABC Vectastain Elite ABC, Vector Laboratories, Burlingame, CA, USA) and the reaction was revealed using diaminobenzidine (DAB – Sigma, Saint Louis, MO, USA) as the cromogen.

The analysis of the data was realized in a semi-quantitative manner, the scores presented a variation from "–" for no labelling to "+, ++ and +++" to less, moderate and intense labellings, respectively.

3. Results

As described in previous studies from our lab,[11,12] estrous cycle was monitored and OVX/O and OVX/RLX group presented diestrus smear, atrophied uterine horns and lower plasmatic concentration of estradiol. In contrast, 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.

For all experimental groups, positive immunolabelling for OPG and RANKL protein were visualized in cells of connective tissue, osteoblasts around the trabeculae bone and in osteocytes aprisioned in the bone tissue formed during the alveolar healing process. TRAP protein was observed in osteoclasts present around the alveolar walls and close to the neoformed trabeculae bone.

At 7 postoperative days, besides the great amount of haemosiderin, it was observed discrete RANKL immunolabelling in osteoblasts around trabeculae bone and osteocytes of the middle third (Fig. 1). Fibroblasts of the connective tissue presented moderate immunolabelling of OPG protein (Fig. 2). OVX/O group presented the highest immunolabelling for OPG and RANKL protein than the other groups. TRAP immunolabelling were not visualized in the middle third, only a discrete labelling in the borders of the dental socket with no significant difference between the groups (Fig. 3).

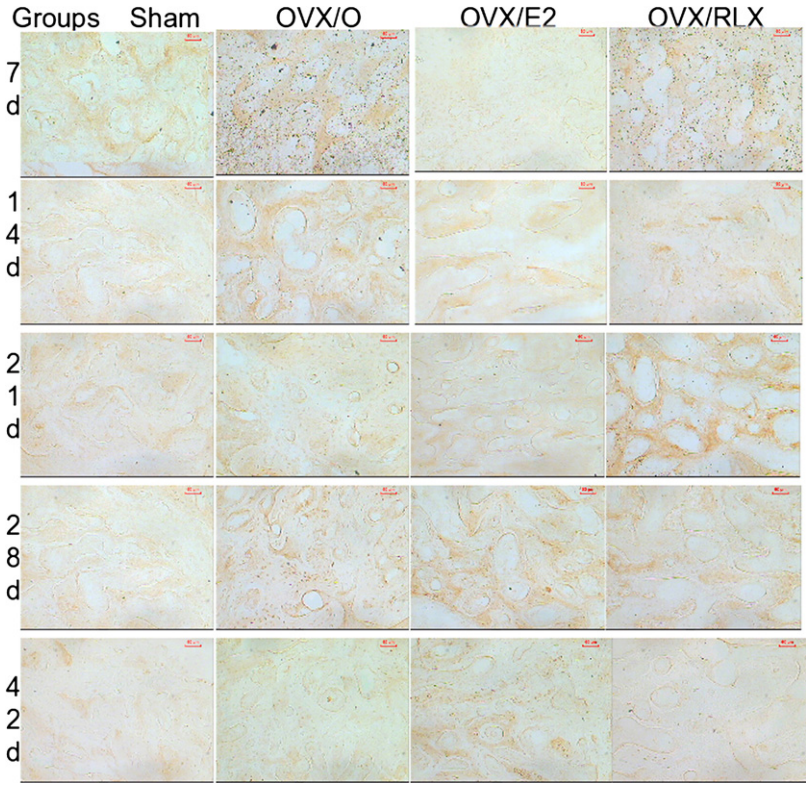

Fig. 1 – RANKL immunolabelling at 7, 14, 21, 28 and 42 days post-extraction in animals of sham, OVX/O, OVX/E2 and OVX/RLX groups.

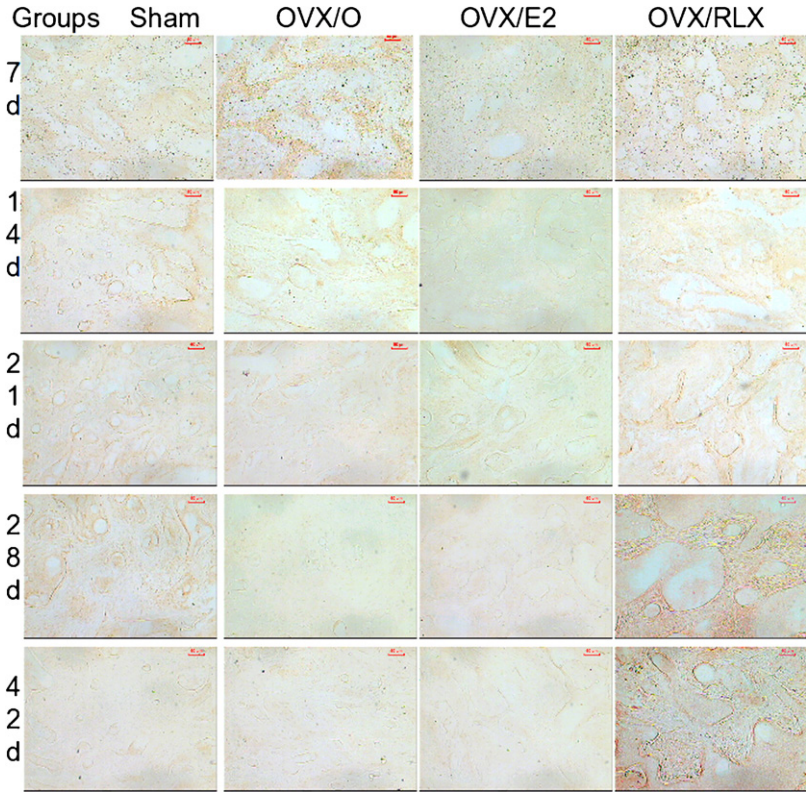

Fig. 2 – Osteoprotegerin immunolabelling at 7, 14, 21, 28 and 42 days post-extraction in animals of sham, OVX/O, OVX/E2 and OVX/RLX groups.

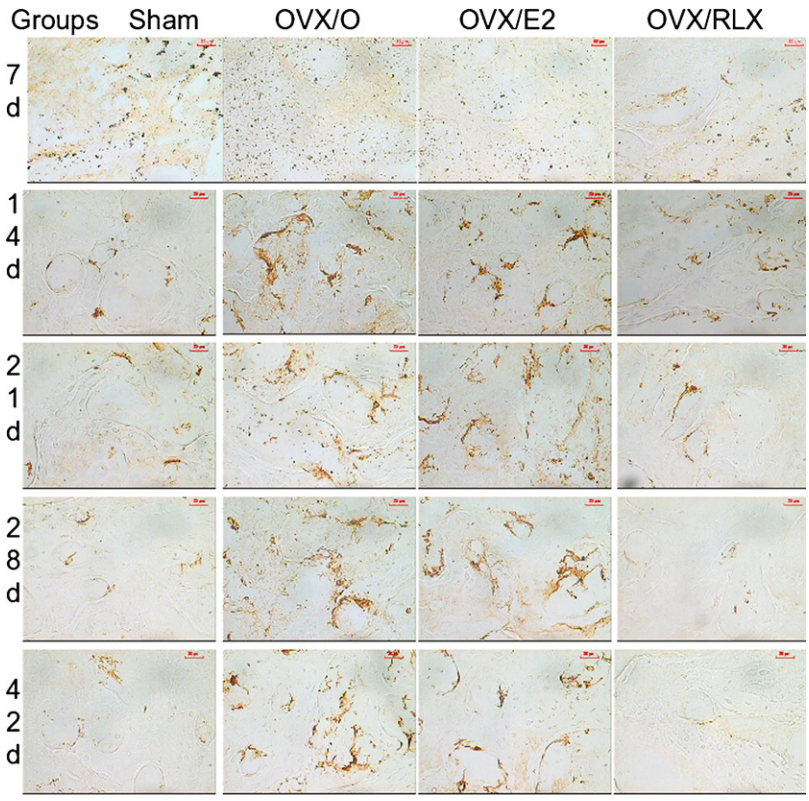

Fig. 3 – TRAP immunolabelling at 7, 14, 21, 28 and 42 days post-extraction in animals of sham, OVX/O, OVX/E2 and OVX/RLX groups.

At 14 postoperative days, it was observed RANKL immunolabelling (Fig. 1) similar to the previous period of all groups. Sham and OVX/RLX groups showed similar OPG immunolabelling (Fig. 2) compared to the previous analysed period, whilst OVX/O and OVX/E2 showed a decreasing of OPG immunolabelling. No background labelling with haemosiderin was observed which facilitates the visualization of the area. OVX/O group showed intense TRAP immunolabelling, moderate for OVX/E2 group and discrete for sham and OVX/RLX groups (Fig. 3).

At 21 postoperative days, OVX/O group showed a decreasing OPG immunolabelling whilst it was increased for OVX/RLX group compared to the previous period (Fig. 2). Additionally, an increasing of RANKL immunolabelling was observed for all experimental groups (Fig. 1). These findings suggest an increasing in the cellular activity of bone remodelling process in order to form bone tissue in the presence of raloxifene. Considering TRAP immunolabelling, OVX/O group showed an intense expression, OVX/E2 group showed a moderate expression whilst sham and OVX/RLX showed a discrete expression, similar to previous analysed period (Fig. 3).

At 28 and 42 postoperative days, OVX/O group showed a decreasing in the OPG immunolabelling (Fig. 2), as well as a significant increase in RANKL immunolabelling (Fig. 1). OVX/E2 group started to show a decreasing in OPG immunolabelling for osteoblasts and osteocytes. OVX/O group presented expressive labelling against RANKL. Raloxifene administration caused a reduction in RANKL immunolabelling at 28 days and absence immunolabelling at 42 days. TRAP immunolabelling was kept

Table 1 – Scores from the semi-quantitative manner, the scores presented a variation from "–" for no labelling to "+, ++ and +++" to less, moderate and intense labellings, respectively.

	OPG	RANKL	TRAP
7 Days			
SHAM	++	+	+
OVX/O	+++	++	+
OVX/E2	++	+	+
OVX/RLX	++	+	+
14 Days			
SHAM	++	+	+
OVX/O	++	++	+++
OVX/E2	+	+	++
OVX/RLX	++	+	+
21 Days			
SHAM	++	++	+
OVX/O	+	+++	+++
OVX/E2	++	++	++
OVX/RLX	+++	++	+
28 Days			
SHAM	++	++	+
OVX/O	+	+++	+++
OVX/E2	+	++	++
OVX/RLX	++	+	–
42 Days			
SHAM	–	–	+
OVX/O	–	+++	++
OVX/E2	–	++	+
OVX/RLX	++	–	–

intense to moderate for OVX/O and OVX/E2 groups respectively and reduced for the other groups, primarily for the OVX/RLX group (Fig. 3) (Table 1).

4. Discussion

Oestrogen deficiency systemically affects bone remodelling through OPG/RANKL signalisation during the events that modulates osteoclasts cellular differentiation and lymphocytes development. In the experiments realized in our laboratory, the osteoprotective effect of oestrogen in inhibits bone resorption is confirmed after treating OVX rats with 17β-estradiol. Which increased bone mass in the middle third of the alveolar bone, however the action of raloxifene was not as pronounced as E2.[11,12]

The intense immunolabelling for RANKL and TRAP observed in OVX animals showed the signalling action of the members of the tumour necrosis factor (RANKL) on osteoclastic responses (TRAP). The oestrogen deficiency following ovariectomy leds to a high bone turnover during the alveolar healing process after tooth extraction whilst, oestrogen and raloxifene treatments led to bone formation. However TRAP expression at 28 and 42 days post-extraction in OVX animals treated with raloxifene was very low, whilst this expression was more expressive in OVX animals treated with oestrogen. Our results suggest that raloxifene treatment may compensate the changes induced by ovariectomy reducing the number of pre-osteoclasts and mature osteoclasts.

Studies have shown that oestrogen deficiency leads to an increase of osteocytes apoptosis in human beings[13] and in female rats[14] and the osteocytes apoptosis can be reverted through oestrogen replacement therapy[14,15] or through raloxifene therapy.[16] Studies have suggested an autocrine mechanism, through a Fas ligand (FasL), in which oestrogen-induced osteoclast apoptosis[17] and a paracrine mechanism in which oestrogen affects osteoclast survival through FasL upregulation in osteoblast cells leading to pre-osteoclasts apoptosis,[18] this may explain the osteoprotective function of oestrogen as well as of SERMs. However, Kawamoto et al.[19] evaluated the effects of oestrogen deficiency state in osteoclastogenesis of the periodontal tissue at 7 postoperative days and did not find any difference in the number of osteoclasts between oestrogen replacement therapy and sham groups. The authors also observed a significant increase of TRAP expression at 14 postoperative days on OVX group compared to the others, these finding are in agreement to our findings.

The increasing of OPG expression at 7 postoperative days observed on OVX/O group suggests a transient increase in the osteoblastogenesis during the initial step of the alveolar wound healing process. This finding was also observed by Miyazaki et al.[20] Changes in bone formation marker (OPG) were transient whilst changes in bone resorption markers (RANKL and TRAP) were constant. These results were confirmed by the immunohistochemistry of OPG protein, where the increase in the osteoblast cells was only transient during the initial step of the alveolar wound healing in OVX rats (7 postoperative days), whilst the increase in the osteoclastic differentiation was constant throughout the experiment.

Our findings suggest that raloxifene therapy reduces osteoblastic cells apoptosis and, probably, acts blocking the formation of osteoclasts brush borders more efficient than estradiol therapy. As the literature shows controversial findings,[21–28] this findings are less discussed maybe due to the limited number of scientific papers that compare both therapies.

Studies has shown that raloxifene therapy, in a dose dependent manner, protects bone tissue blocking osteoclastogenesis, mature osteoclasts activation and their survival.[27,29] Our findings indicate that raloxifene therapy compensates OVX statement by reducing the number of pre-osteoclasts and mature osteoclasts. As showed in this study in which OVX/RLX group presented a minor TRAP labelling at 28 postoperative days and an absence of TRAP labelling at 42 postoperative days compared to sham and OVX/E2 groups. Also we observed a minor RANKL expression on OVX/RLX group at 28 and 42 postoperative days compared to OVX/E2 group.

The intense RANKL immunolabelling was more significant at 28 and 42 postoperative days on OVX group. This finding is in agreement to our previous studies in which we observed the least amount of bone formation at the same period and same group.[11,12] An important observation is the intense expression of RANKL and TRAP protein observed in some experimental groups emphasizing previous evidences[4,5,19,27–29] that suggest the signalling role of the tumoural necrosis factor members (RANKL) on the osteoclastic responses (TRAP). Considering the signal cellular responses, raloxifene therapy decreased RANKL immunolabelling and increased OPG immunolabelling, consequently decreasing TRAP. This finding is confirmed by previous studies[4,5,19,27–29] that show the role of raloxifene therapy in protecting bone tissue that brings an important therapeutic option to keep bone tissue homeostasis.

Studies of Cheung et al.[30] in bone marrow cloned cells cultures (HCC1) with osteoblastic characteristics and primary human osteoblasts (HOB) showed a significant reduction in RANKL expression in cells treated with raloxifene whilst oestrogen treatment did not show significant changes. As RANKL/OPG balance showed a reduction on OVX/RLX group compared to OVX/E2 group. Another important finding of our study in which raloxifene acts is increasing OPG expression. A result also observed by Viereck et al.,[31] Messalli et al.[32] and Michael et al.[33] These findings suggest that the raloxifene and oestrogen present different mechanisms of action in the expression of OPG, RANKL and TRAP. Furthermore, oestrogen and SERMs present different clinical profile, differently modulating ERα and Erβ transcription activities.[23,34–36] In recent study realized by Yan et al.,[37] with OPG knockout female rats, the authors observed an increase in bone trabecular area, bone mineral density and bone resistance after raloxifene therapy as well as a reduction in osteoclasts number and RANKL transcription, suggesting that raloxifene mechanism of action do not depend on OPG protein.

SERMs preserve the positive effects of oestrogen on bone tissue without adverse effects in uterine and breast tissues.[38] Whilst raloxifene has shown protective action of osteocytes apoptosis induction caused by OVX,[24,29,39] the molecular mechanism of this protection remains unknown. Structurally different from oestrogen, raloxifene retain a cyclohexane

hydroxyl group C3 which may potentially facilitate its antioxidant action.

More studies are necessary to better evaluate the biological mechanisms in which raloxifene acts. Even though, our experiments have shown an important participation of tumoural necrosis factor in signalising osteoclastic activity inhibition. RANKL immunolabelling reduction and OPG immunolabelling increasing and its consequent reduction of TRAP immunolabelling observed on OVX/RLX group shows the role of raloxifene therapy in protecting bone tissue that brings an important therapeutic option to keep bone tissue homeostasis.

5. Conclusion

Oestrogen deficiency induces osteoclastogenesis in the alveolar healing process. Quantitative changes in the osteoclastic activity could be prevented through the raloxifene therapy.

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