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The RANK/ RANKL/ OPG interaction in the repair of autogenous bone grafts in female rats with estrogen deficiency

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Abstract: The aim of this study was to evaluate the resorption process during the repair of autogenous bone grafts with or without coverage by an expanded polytetrafluoroethylene (e-PTFE) membrane in female rats with estrogen deficiency using the immunohistochemical technique. Eighty female rats were randomly divided into two groups (OVX and SHAM). The 40 female rats in the OVX group were subjected to ovariectomy, and the 40 female rats in the SHAM group were subjected to simulated ovariectomy. The two groups were further divided in subgroup E, which was subjected to surgery for placement of autogenous bone graft (ABG), and subgroup ME, in which the ABG was covered with an e-PTFE membrane. The animals were killed at 0, 7, 21, 45 and 60 days. The specimens were analyzed using immunohistochemistry for the bone resorption markers RANK, RANK-L and Osteoprotegerin (OPG). A higher remodeling rate was observed at 7 and 21 days after the autogenous bone grafts, when the markers were more intensely expressed. At the final time point, the specimens presented similar characteristics to those observed at the initial time point. The expression of immunohistochemical markers was not altered by the estrogen deficiency. The presence of the e-PTFE membrane delayed the bone resorption process, influencing the immunohistochemical expression of markers.

Keywords: Osteoporosis; Bone Regeneration; Bone Resorption.

Introduction

Increased life expectancy has led to an increase in the demand for rehabilitation using endosseous implants and reconstructive procedures. One of the main requirements for sites receiving implants is adequate alveolar bone volume.[1,2,3] If this volume is not present, techniques for bone tissue augmentation may be used, including autogenous bone grafts and guided bone regeneration.[1,4,5] To accomplish bone regeneration, barrier membranes are often used. The use of PTFE-e membranes in GBR is based on the material's biocompatibility and its ability to maintain space for osteogenic cells and fluid migration. This event can lead to isolation of cells without osteogenic potential, protecting the wound and thereby increase bone formation.[4,5,6]

However, combined with population aging, there has been an increase in diseases that may interfere with bone repair. For example, estrogen deficiency can lead to other bone problems, such as osteoporosis, which may

interfere with bone regeneration prior to implant therapy. It is well established that estrogen deficiency increases bone turnover and alters the balance in favor of resorption, which may lead to osteoporosis. Several studies have reported the negative effects of this condition on oral bone and systemic disease.[7] However, few studies have investigated the impact of osteoporosis mediated by estrogen deficiency on bone graft repair.

To better understand the mechanism of this process, the interactions between bone repair molecular mediators must be elucidated. The RANK/RANKL/OPG interaction plays a fundamental role in the osteoclastogenesis process and is critical in the regulation of bone remodeling.[8] After menopause, bone loss occurs because RANKL activity is favored over OPG activity. The reduction of the estrogen levels decreases OPG activity and increases RANKL activity, leading to increased resorption and bone loss. The influence of osteoporosis on bone repair has been widely discussed; therefore, it is possible to study its mechanisms in animals subjected to estrogen deficiency through the immunohistochemical expression of bone resorption markers.

Consequently, this study evaluated the immunohistochemical expression of molecular markers in bone formation during the repair process of onlay autogenous bone grafts in estrogen deficient female rats.

Methodology

This study followed the Ethical Principles for Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Institutional Review Board of *Instituto de Ciência e Tecnologia* – ICT, UNESP (protocol n. 026/2008-PA/CEP).

Eighty 300-gram adult Wistar female rats aged 90 days were randomly divided into the following two groups: the OVX group, which was subjected to ovariectomy surgery, and the SHAM group, which was subjected to the same surgical procedure but without removal of the ovaries, as previously described[2]. Each group was further divided in two subgroups: subgroup E, subjected to surgery for the placement of an autogenous bone graft, and subgroup ME, in which the graft was covered with an e-PTFE membrane (WL Gore & Associates, Newark, USA).

Bone graft surgery

Thirty days after the ovariectomy, surgeries were performed for placement of the autogenous bone grafts, using the calvaria as the donor site and the mandibular angle as the receptor site. A detailed description of this procedure may be found in previous studies.[2,9,10]

Initially, the graft was harvested using a trephine bur with 4.1 mm external diameter (Neodent, Curitiba, Brazil) and its center perforated with a ½ carbide bur (KGSorensen, Cotia, Brazil) at low speed, under irrigation with saline. Three perforations were performed on the mandibular angle with a ½ carbide bur under thorough irrigation, which allowed stabilization of the bone block to the receptor site using a 5.0 green braided polyester suture (Ethicon Johnson & Johnson, São José dos Campos, Brazil) and adaptation of the e-PTFE membrane in groups subjected to graft coverage with the membrane (OVX-ME and SHAM-ME). These procedures allowed graft positioning in close contact with the mandibular bone surface, stabilizing it and providing coverage of the entire grafted block and stabilization of the e-PTFE membrane in OVX-ME and SHAM-ME groups.

The muscle layer was sutured with a resorbable suture (Ethicon Johnson & Johnson, São José dos Campos, Brazil), and the skin and the donor site were sutured with a 4.0 silk suture (Ethicon, Johnson & Johnson, São José dos Campos, Brazil). After surgery, the animals received a single 1 mg/kg dose of antibiotics intramuscularly. (Pentabiótico, Fort Dodge Saúde Animal Ltda., São Paulo, Brazil.)

The animals were killed through an excessive dose of anesthetics at 0 h, 7, 21, 45 or 60 days after surgery.

Histological and immunohistochemical procedures

The specimens were demineralized with 10% EDTA solution at a pH 7.8 in a microwave processor (PELCO 3441, Ted Pella Inc., Redding, USA). The demineralized specimens were transversely sectioned at the central region of the bone graft. Each fragment was embedded in a paraffin block, following the direction of the cutting surface. The blocks were sectioned at 3 µm thickness and used for immunohistochemical analysis for the markers RANK (ab 12008, Abcam,

Inc., Cambridge, USA), RANKL (sc-7628, Santa Cruz Biotechnology, Dallas, USA) and OPG (sc-8468, Santa Cruz Biotechnology, Dallas, USA) according to the laboratory immunohistochemistry protocol of the ICT-UNESP campus de São José dos Campos.

Microscopic analysis was performed on a light microscope Axiophot 2 (Carl Zeiss, Jena, Germany) connected to a digital camera AxioCam MRc 5 (Carl Zeiss, Jena, Germany), which transfers the captured images to the AxioVision Release 4.7.2. software (Carl Zeiss, Jena, Germany).

For all periods and antibodies, the immunolabeling intensity of predefined structures and cells was classified as mild (+), moderate (++) or intense (+++).[3,11,12,13]

Results

Tables 1, 2 and 3 summarize the immunohistochemical results.

Table 1. Summary of RANK results

Group	Experimental Period	Osteoclasts
SHAM-E	7 Days	++
	21 Days	+
	45 Days	+
	60 Days	-
SHAM-ME	7 Days	+
	21 Days	+
	45 Days	+
	60 Days	-
OVX-E	7 Days	++
	21 Days	+
	45 Days	+
	60 Days	-
OVX-ME	7 Days	+
	21 Days	+
	45 Days	+
	60 Days	-

Table 2. Summary of RANK-L results

Group/Tissue Structure	Experimental Period	Osteoclasts	Osteoblasts	Granulation Tissue
SHAM-E	7 Days	+	+++	+++
	21 Days	+	++/+++	++
	45 Days	+	++	++
	60 Days	+	++	++
SHAM-ME	7 Days	++	++/+++	+/++
	21 Days	+/++	++	+/++
	45 Days	+	++	++
	60 Days	+	++	Not present
OVX-E	7 Days	+	+++	+++
	21 Days	+	++/+++	++
	45 Days	+	++	+++
	60 Days	+	++	++
OVX -ME	7 Days	++	++/+++	+/++
	21 Days	+/++	++	+/++
	45 Days	+	++	++
	60 Days	+	++	+

RANK

At the initial time (zero-hour), there was no labeling of any structure. At 7 days, there was moderate labeling of osteoclasts in the OVX-E and SHAM-E groups, compared to mild labeling in the OVX-ME and SHAM-ME groups. At 21 and 45 days, the osteoclasts exhibited mild labeling in all groups. At the final time point (60 days), the osteoclasts were not labeled (Figure 1).

RANK- L

At the initial time point, moderate labeling was observed at the osteocyte lacunae in the central region of the graft. At 7 days, the OVX-E and SHAM-E groups exhibited intense labeling of connective tissue at the bed-graft interface, while in the OVX-ME and SHAM-ME groups, this labeling was mild or moderate. The OVX-E and SHAM-E groups exhibited intense labeling of osteoblasts, while in the OVX-ME

Table 3. Summary of OPG results

Group/Tissue Structure	Experimental Period	Osteoclasts	Osteoblasts	Granulation Tissue	Osteocytes in newly formed bone
SHAM-E	7 Days	-	++	+/++	++/+++
	21 Days	-	++	+	++/+++
	45 Days	-	+/++	+	++
	60 Days	-	+	+	+
SHAM-ME	7 Days	-	++	++	++/+++
	21 Days	-	+++	++	++/+++
	45 Days	-	+/++	+	++
	60 Days	-	+	+	+
OVX-E	7 Days	-	++	+/++	++/+++
	21 Days	-	++	+	++/+++
	45 Days	-	+/++	+	++
	60 Days	-	+	+	+
OVX-ME	7 Days	-	+++	++	++/+++
	21 Days	-	+++	++	++/+++
	45 Days	-	+/++	+	++
	60 Days	-	+	+	+

and SHAM-ME groups this labeling ranged from moderate to intense. The osteocyte lacunae in the newly formed bone exhibited intense labeling in all groups. Conversely, the osteoclasts presented moderate labeling in groups covered with the membrane and mild labeling when the membrane was not used. At 21 days, the connective tissue at the bed-graft interface exhibited moderate labeling in the OVX-E

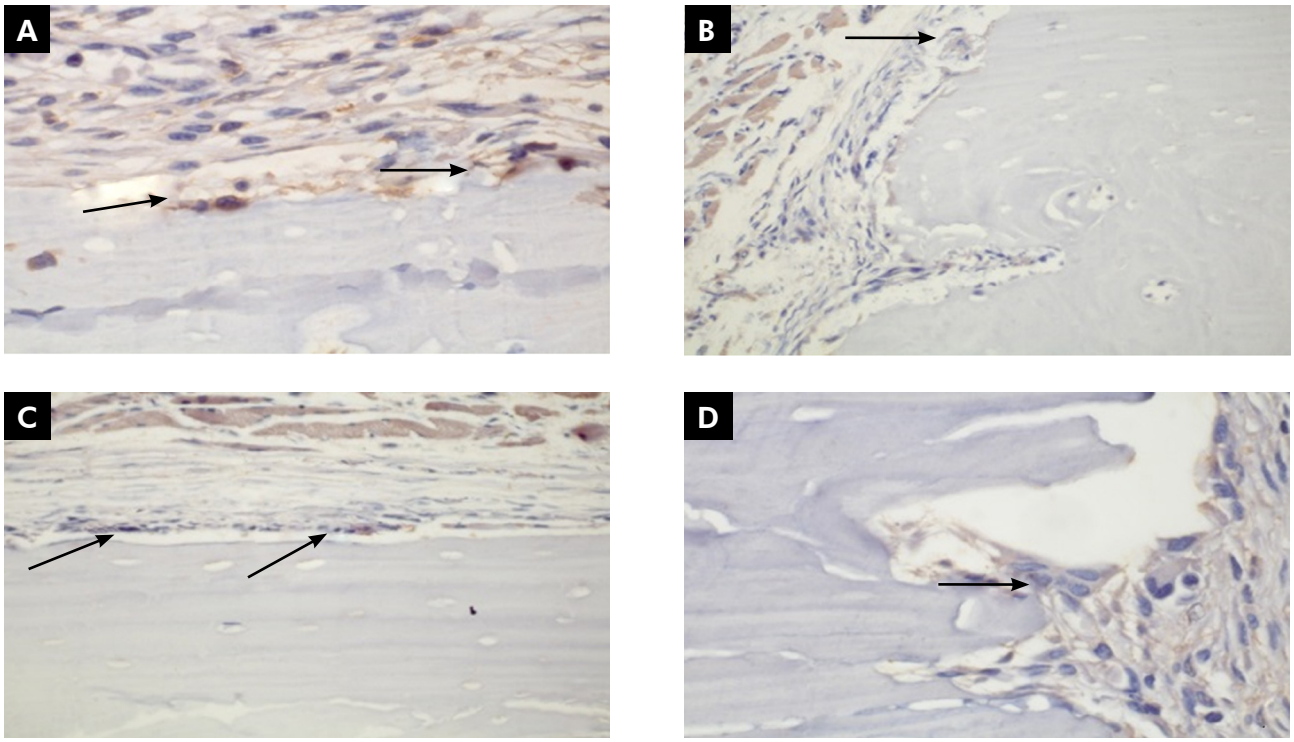

Figure 1. Photomicrographs showing the Rank staining a) Day 7 Group OVX-E: moderate labeling of osteoclast (→) x400. b) Day 7 Group OVX-ME: mild labeling of osteoclasts (→) around bone graft. x400. c) Day 21 Group SHAM-E: mild labeling of osteoclasts (→) x200. d) Day 21 Group SHAM-ME: mild labeling of osteoclasts (→) around bone graft x1000.

and SHAM-E groups, while labeling varied from mild to moderate in the OVX-ME and SHAM-ME groups. The osteoblast labeling was positive, ranging from moderate to intense in the OVX-E and SHAM-E groups, while moderate labeling was observed in the OVX-ME and SHAM-ME groups. The osteocytes in the newly formed bone were moderately labeled, while the osteoclasts presented alternately mild or moderate labeling in groups with membranes and mild labeling in groups without membranes. At 45 days, the OVX-ME, SHAM-E and SHAM-ME groups presented moderate labeling of connective tissue at the bed-graft interface, while only the OVX-E group presented intense labeling. The osteoblast and osteocyte labeling was moderately positive. The osteoclasts exhibited mild labeling. At the final time point, there was moderate labeling of connective tissue at the bed-graft interface in the OVX-E and SHAM-E groups, compared to mild labeling in the OVX-ME group, while the SHAM-ME group presented only bone tissue at the bed-graft interface. The osteoblasts exhibited moderate labeling, while the osteocytes exhibited lacunae with mild to moderate labeling. The osteoclasts exhibited mild labeling (Figure 2).

OPG

At the initial time point, specimens in the four groups exhibited similar characteristics. At the graft region, the lacunae of osteocytes in the central region presented moderate labeling. At 7 days, the OVX-E and SHAM-E groups presented a considerable quantity of connective tissue with mild to moderate labeling at the bed-graft interface, compared to moderate labeling in the OVX-ME and SHAM-ME groups. The osteoblasts presented intense labeling in the OVX-ME and SHAM-ME groups, and moderate labeling in the OVX-E and SHAM-E groups, while osteocytes in the newly formed bone presented moderate to intense labeling. The osteoclasts were not labeled. At 21 days, the connective tissue at the bed-graft interface exhibited mild labeling in the OVX-E and SHAM-E groups, while in the OVX-ME and SHAM-

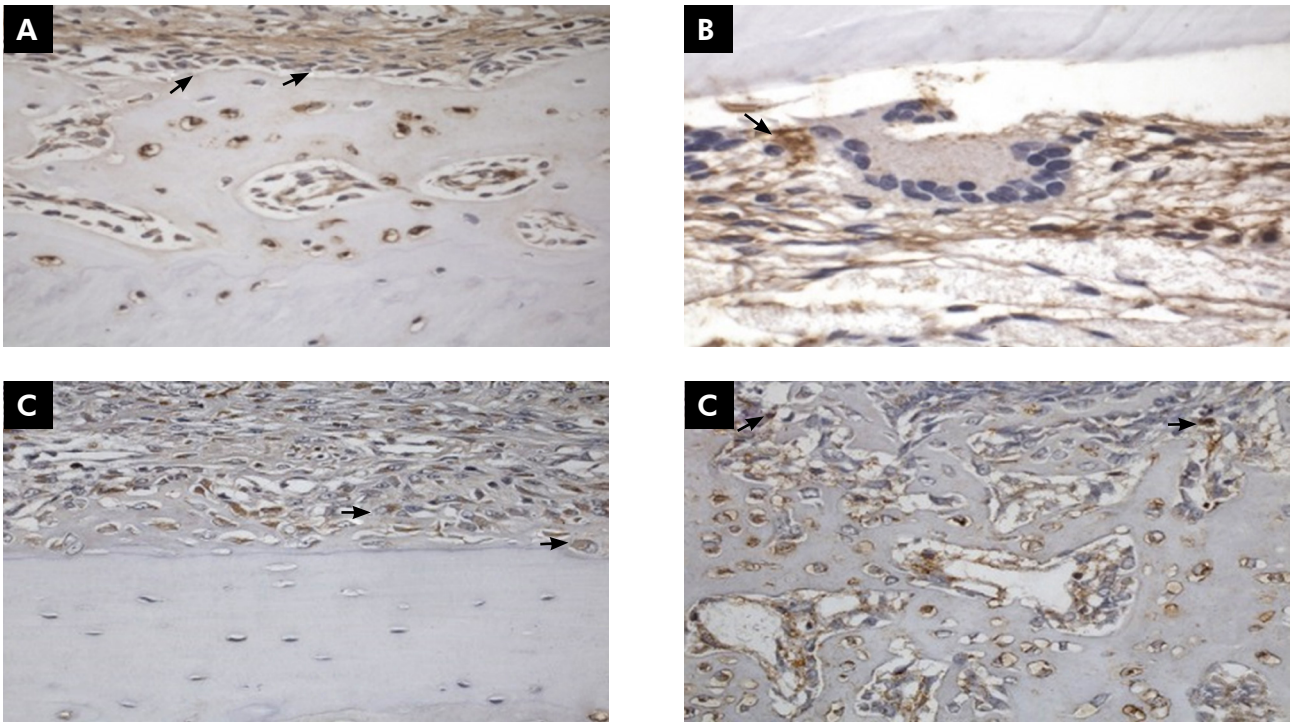

Figure 2. Photomicrographs showing the Rank-L staining a) Day 7 Group OVX-ME: moderate/intense labeling of osteoblasts (→) of newly formed bone. x400. b) Day 7 Group OVX-ME: moderate labeling of osteoclast next to the graft. (→) x1000. c) Day 7 Group SHAM-ME: moderate/intense labeling of osteoblasts(→) of newly formed bone. x200 d) Day 7 Group SHAM-ME: moderate/intense labeling of osteoblasts (→) of newly formed bone x400.

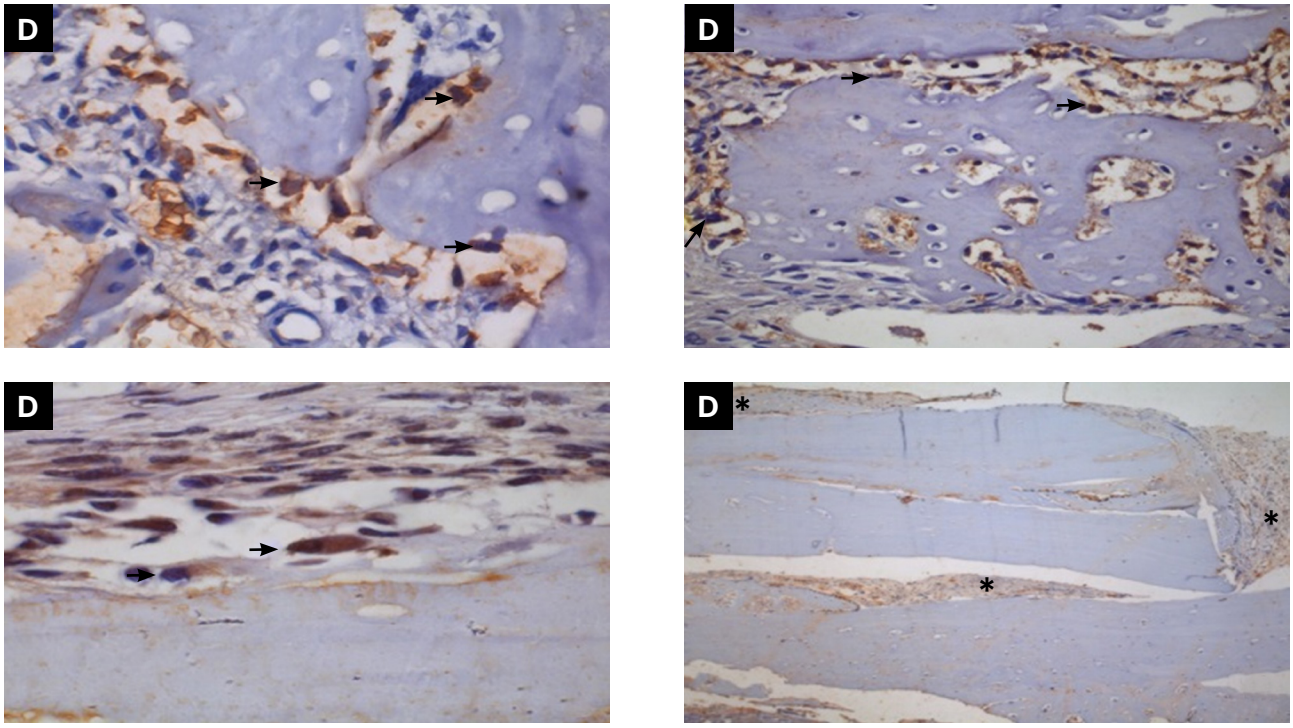

Figure 3. Photomicrographs showing the OPG staining a) Day 7 Group OVX-E: moderate labeling of osteoblasts (→) of newly formed bone x400. b) Day 7 Group SHAM-E: moderate labeling of osteoblasts (→) of newly formed bone x400. c) Day 21 Group OVX-ME: intense labeling of osteoblasts (→) of newly formed bone x1000. d) Day 21 Group SHAM-ME: moderate labeling of connective tissue (*) x100.

ME groups there was moderate labeling. The osteoblast labeling was moderately positive in the OVX-E and SHAM-E groups, while the same structures were strongly marked in the OVX-ME and SHAM-ME groups. The osteocytes in the newly formed bone exhibited moderate to intense labeling. The osteoclasts were not labeled. At 45 days, all groups presented mild labeling of connective tissue at the bed-graft interface. The osteoblasts were labeled mildly to moderately. Conversely, the osteocytes presented moderate labeling. The osteoclasts were not labeled. In all groups, the labeling of connective tissue at the bed-graft interface was mildly positivity at 60 days. The osteoblasts and osteocytes were mildly positive. The osteoclasts were not labeled (Figure 3).

Discussion

This study evaluated the immunohistochemical expression of bone resorption markers during the repair process of autogenous block bone grafts with or without e-PTFE membrane coverage in rats with estrogen deficiency. As the population ages, oral health quality becomes increasingly relevant. It is important to understand the exact process of autogenous graft healing because of the increasing demand for periodontal and implant surgical procedures using this technique.

Here, we used a well-established and extensively documented animal model.[14,15] Thirty days after the ovariectomy, the animals already exhibited changes in bone metabolism due to estrogen deficiency. As a consequence, bone alterations were detected, including bone loss, especially in the trabecular bone pieces. Additionally, low rates of bone formation were observed in bone wounds.[10,12,16]

The OPG/RANK/RANK-L interaction plays a fundamental role in osteoclastogenesis and is critical for the regulation of bone remodeling. *In vitro* studies of human osteoblasts have revealed that estrogen induces OPG production.[17,18,19]

After menopause, bone loss is related to the favoring of RANKL activity over that of OPG. RANKL is the main stimulating factor for the differentiation, development, maturation, activation, and survival

of osteoclasts. It stimulates osteoclast differentiation through its physiological receptor, RANK, which is expressed in osteoclast-lineage cells. An increase in RANK-L secretion by osteoblasts accelerates bone resorption and leads to the loss of bone mass. The action of RANK-L is antagonized by osteoblast-produced OPG, which prevents RANK activation via binding to RANK-L, thereby interrupting osteoclastogenesis and limiting bone resorption.[20]

A reduction in the estrogen level decreases the OPG activity and increases the RANKL activity, leading to increased resorption and bone loss. However, the literature presents conflicting results concerning the period after menopause, when bone loss is related to favoring RANKL activity over that of OPG. In a cohort study, Mezquita-Raya *et al*.[21] and Chiba *et al*.[22] found no correlation between OPG and bone resorption markers in women, regardless of their menopause status. According to the results, this study also demonstrated that the expression of bone markers during repair of autogenous bone grafts was not altered by estrogen deficiency.

In contrast, Fahrleitner-Pammer *et al*.[23] and Indridason *et al*.[24] found a positive relationship between bone turnover markers and OPG in postmenopausal women.

Pedrosa Júnior *et al*.[25] performed bone graft surgeries on rabbit mandibles with or without perforations and observed the expression of both RANK-L and OPG 7 days postoperatively. However, no difference was observed between the two markers, suggesting that the procedure did not influence the expression of these markers. Similar to the study by Pedrosa Júnior *et al*.,[25] this study also included perforations in the receptor bed and similarly revealed that OPG, OC and RANK-L were not altered and remained stable during all stages.

Comparatively, Luvizuto *et al*.[26] studied RANK-L and OPG during the alveolar healing process in female ovariectomized rats treated with estrogen or raloxifene. They found that 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 at 7, 14, 21, 28 and 42 days. In this study, osteoblasts were intensely labeled for OPG during the early stages of bone regeneration (7-45 days), and this intensity of labeling decreased at 60 days in all groups (OVX-E, Sham-E, OVX-ME and Sham-ME). The staining for OPG in osteocytes localized in newly formed bone followed the same pattern as that observed in osteoblasts. For RANK-L, intense labeling was noted in osteoclasts at 21 days in the SHAM-ME and OVX-ME groups, in osteoblasts at 7 days in the SHAM-ME and OVX-ME groups and at 21 days in groups SHAM-E and OVX-E. The author affirms that osteoblasts expressed both OPG and RANKL, playing a major role in maintaining balanced bone dynamics by secreting both proteins during the early stages of alveolar bone healing.

The present study demonstrated that the expression of bone markers during the repair of autogenous bone grafts was not altered by estrogen deficiency; however, it was influenced by the presence of the e-PTFE membrane. Without the membrane, the groups showed a higher expression of RANK in osteoclasts at 7 days, confirming its importance during the resorptive process of graft healing.[10] Using a similar experimental model, Jardini *et al*.[2] observed that graft specimens covered with an e-PTFE membrane had a lower rate of bone resorption, while significant bone resorption was observed when the e-PTFE membrane was not used, which is in agreement with the results reported by Alberius *et al*.[27] and Jensen *et al*.[28]

The results of Nascimento *et al*.[10] showed that the bone healing process is not impaired by osteoporosis because all groups showed graft substitution and adequate integration into the receptor bed by the end of the experimental period. However, a significant difference was observed in the quality of the regenerated bone and the receptor bed of OVX animals. Osteoporotic rats showed larger quantities of medullary spaces in both regenerated bone and the receptor bed when compared to sham-operated rats. Regarding the amount of regenerated bone, this study compares favorably with others that have shown larger amounts of bone augmentation for membrane-protected bone grafts,[2,4,27] and osteoporotic rats responded as well as sham-operated rats. Furthermore, Luize *et al*.[16] showed that ovariectomy impairs bone healing after implantation of autogenous block graft. On postoperative day 28, the sham-operated group

showed graft RANK-L integration to the mandible, while the ovariectomized group had not completed the healing process with areas of connective tissue and newly formed bone.

According to our results, the mechanism that leads to impaired healing after autogenous block graft placement in ovariectomized rats did not seem to be related to the bone resorptive signaling pathways involving RANK, RANK-L, OPG. However, caution must be exercised here. In the present study, the sample size of each group was small. Although some trends could be observed, the differences between groups were not statistically significant, most likely due to the small group size. Studies using greater sample sizes are recommended. Other molecules involved in resorption have been studied and have been shown to be linked to the absence of estrogen, such as melatonin,[29] PTH[30] and others.[31] Furthermore, estrogen deficiency is also related to the altered expression of bone formation markers.[32]

Conclusions

We concluded that the expression of immunohistochemical markers was not altered by the estrogen deficiency. The presence of the e-PTFE membrane delayed the bone resorption process, influencing the immunohistochemical expression of markers.

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