Influence of Ovariectomy on Healing of Autogenous Bone Block Grafts in the Mandible: A Histomorphometric Study in an Aged Rat Model

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Purpose: *The aim of this study was to evaluate quantitatively and qualitatively the influence of estrogen deficiency on autogenous bone block grafts in aged ovariectomized rats.* ***Materials and Methods:*** *Fifty 12-month-old female Wistar rats were used in the study. They were divided into 2 groups, an ovariectomized group and a sham-operated group. After 30 days the animals received autogenous block bone grafts on the angle of the mandible, harvested from the calvaria. The animals were euthanized at 7, 14, or 28 days postoperatively.* ***Results:*** *Histologic analysis showed that at 7 days postsurgery, the interface between graft and recipient site in the sham-operated group appeared filled by a granulation tissue with angiogenic activity, whereas the ovariectomized group still exhibited a blood clot and a granulation tissue in organization. On the 14th postoperative day, the interface in the sham-operated group was partially filled by newly formed bone establishing a union between the graft and the recipient site. The interface in the ovariectomized group was typically filled by granulation tissue with discrete osteogenic activity in most specimens. On the 28th postoperative day, the graft in the sham-operated group appeared histologically integrated to the mandible. However, the interface in the ovariectomized group appeared partially filled by newly formed bone, with areas of interposed connective tissue. The statistical analysis revealed that bone neoformation was significantly greater in the sham-operated group (57.41% at 14 days and 68.35 at 28 days) in comparison with the ovariectomized group (40.82% at 14 days and 53.09 at 28 days) at the 5% level.* ***Conclusion:*** *The estrogen depletion caused by the ovariectomy hindered the healing process of autogenous block bone grafts placed in the mandibles of aged rats.* Int J Oral Maxillofac Implants 2008;23:207–214

Key words: *autograft, bone transplantation, estrogen deficiency, menopause, ovariectomy*

Surgical techniques for bone augmentation have allowed predictable reconstruction of alveolar bone and the placement of dental implants in suitable positions to facilitate functional and esthetic restorations. The ideal bone graft should be osteoinductive to stimulate osteogenesis and osteoconductive to provide a scaffold for establishing optimal conditions for ingrowth of blood vessels and cells with osteogenic potential.[1,2] These requirements are presently most adequately fulfilled by autogenous bone grafting.[3] However, local and systemic conditions may impair bone healing, including serum levels of estrogen.

Estrogen deficiency results in increased bone turnover and imbalance in favor of resorption,[4–11] causing osteoporosis.[12–15] Osteoporosis is defined as a pathologic decrease in the amount of bone and microarchitectural deterioration of bone tissue, which leads to fractures after minimal trauma.[16,17] Thus, the impact of osteoporosis on bone graft healing and its long-term outcome deserves attention.

Previous animal studies have described the relationship between osteoporosis and tooth loss[18–20] and investigated its impact on osseointegrated dental implants.[21–26] However, further animal study needs to be performed and may offer some valuable data for clinical considerations when bone grafts are used in estrogen patients.

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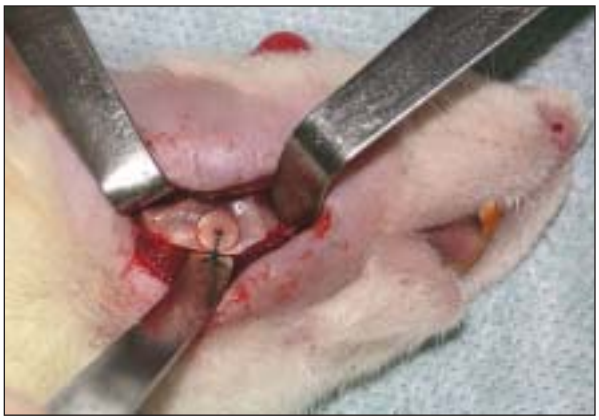

Fig 1 Bone block graft in close contact with the mandibular bone surface and stabilized by a green polyester 5-0 suture.

The ovariectomized rat is considered a good animal model of estrogen deficiency–induced osteopenia.[27] There are many similarities between the aged rat model of ovarian deficiency–related bone loss and postmenopausal osteoporosis.[28] Older animals more accurately reflect the target population for any proposed therapy, as postmenopausal women present both aging and cessation of ovarian activity.

Thus, it would be appropriate to clarify the influence of estrogen deficiency on the initial healing events that occur after autogenous bone grafting. The present study was based on the experimental model described by Jardini et al[29] and reproduced by De Marco et al[30] to determine the initial healing pattern of autogenous bone block grafts and to study the revascularization of such bone blocks.

The aim of this study was to evaluate quantitatively and qualitatively the influence of estrogen deficiency on autogenous bone block grafts in aged ovariectomized rats, with an emphasis on the initial healing phase and the differences in the repair processes described in previous studies.[29,30]

MATERIALS AND METHODS

Fifty Wistar female rats (300 to 400 g and 12 months old) were included in the study. The animals were kept in plastic cages with access to food and water ad libitum. The present research protocol was approved by the São Paulo State University–UNESP Institutional Animal Care and Use Committee (no. 34/03).

Experimental Design

The animals were randomly assigned to 2 groups. The ovariectomized group received ovariectomy surgeries and autogenous bone grafts. The sham-operated group received sham surgeries and autogenous bone grafts. Autogenous bone grafting was carried out 4 weeks after the ovariectomy or sham surgical procedure. Animals were sacrificed at 7, 14, or 28 days postoperatively.

General anesthesia was obtained by intramuscular administration of xylazine (0.3 mg/100 g body weight) and ketamine (0.7 mL/100 g body weight) in all surgical procedures.

Ovariectomy and Sham Procedure

Under general anesthesia the dorsal region was trichotomized, and the skin was cleansed with 0.2% chlorhexidine digluconate. Bilateral ovariectomies were performed in 26 rats by a dorsal approach. The remaining rats were subjected to sham surgeries in which the ovaries were lifted up and returned intact to the original position. More oviarectomies than sham surgeries were carried out because of the higher risk of death in the ovariectomized animals. To confirm the success of the ovariectomy, the estrous cycle was monitored. Vaginal smear was observed for changes for 4 to 5 days of the estrous cycle in each group.

Autogenous Bone Graft Surgery

The calvaria was the autogenous bone donor area, and the angle of the mandible was the recipient area. After anesthesia, the skin of the donor and the recipient area were trichotomized, and a vigorous disinfection was carried out with 0.2% chlorhexidine digluconate. To prepare the recipient area, a linear incision of approximately 1.5 cm was made in the right mandibular angle parallel to the zygomatic process. Thus, the bone surface of the mandibular angle was surgically exposed by blunt dissection.

The bone graft was harvested from the right parietal bone using a surgical trephine bur with an internal diameter of 3.8 mm (Prudent, Lins; São Paulo, Brazil) at a controlled speed of 800 rpm under saline solution irrigation. The bone block was perforated in its center with a 0.5-mm carbide bur (KG Sorensen; Barueri, São Paulo, Brazil) at low speed. The graft was temporarily immersed in saline solution until it was positioned in the recipient area.

The recipient area was prepared by superficial cortical bone scraping and was also drilled with the same carbide bur 3 mm from the mandibular base. This allowed a green polyester 5-0 suture (Johnson & Johnson/Ethicon, Somerville, NJ) to be passed through the mandible and bone block (Fig 1). This procedure allowed the graft to be placed in close contact with the mandibular bone surface and stabilized the graft. The wound was sutured in layers

Table 1 Total Area of the Interface and Newly Formed Bone Area in the Interface at 14 and 28 Days Postoperative

	14 days				28 days			
Treatment group	n	TA Mean ± SD (mm^2)	NFBA Mean ± SD (mm^2)	NFBA Mean ± SD (%)	n	TA Mean ± SD (mm^2)	NFBA Mean ± SD (mm^2)	NFBA Mean ± SD (%)
Ovariectomized group	10	0.61 ± 0.23	0.25 ± 0.16	40.82±11.29 bB	8	0.70 ± 0.23	0.37 ± 0.17	53.09 ± 10.89 bA
Sham group	8	0.69 ± 0.24	0.40 ± 0.17	57.41±10.43 aB	8	0.73 ± 0.16	0.50 ± 0.16	68.35 ± 11.35 aA

Different lowercase and capital letters, respectively in columns and lines, indicate that they differ from each other by Tukey's Test ($P < .05$).

(ie, the muscle was sutured with 5-0 polyglactin 910 [Vicryl; Johnson & Johnson/Ethicon], and the skin in the donor and recipient areas was sutured with 4-0 black silk [Johnson & Johnson/Ethicon]). Postoperatively, the animals received soft food and water ad libitum for 5 days.

Ten ovariectomized rats were sacrificed at 14 days; 8 rats per group were sacrificed at all other time points. After sacrifice (7, 14, or 28 days), the right hemimandible was removed and fixed in 10% formalin for 48 hours, decalcified in 18% EDTA for approximately 40 days, and embedded in paraffin. Semi-serial sections 6 µm thick were obtained in a transversal direction, as demonstrated in Fig 2. The sections were stained with either hematoxylin-eosin or Masson trichrome for histologic and histometric analysis.

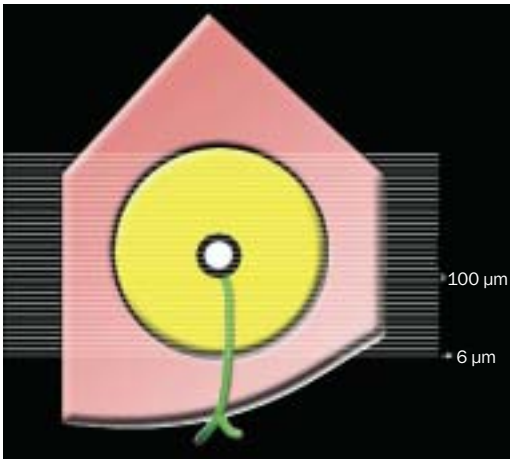

Fig 2 Schematic illustration of transversal direction of the semi-serial sections obtained for histologic and histometric analysis.

Histologic and Histometric Analysis

Histologic analysis was performed by investigating all the semi-serial stained sections of each specimen in both groups. The histologic sections were analyzed by a single blinded calibrated observer with an Olympus CH2 binocular microscope (Olympus Optical, Tokyo, Japan) between the bone block graft and the receptor bed for the following: presence of granulation tissue, presence of blood clot, osteogenic activity, occurrence of resorption, and newly formed bone areas. Moreover, the general characteristics of the bone graft, receptor bed and graft periphery; the occurrence of resorption, revascularization of the bone graft, and the filling of medullar spaces were noted.

Jandel Sigma Scan Pro (Jandel Corporation, Version 2.0, San Rafael, CA) software was used for quantitative data analysis at 14 and 28 days. There was no newly formed bone in the interface at observation period of 7 days in either group; thus, histometric analysis was not performed for these groups.

The 2 most central sections of each specimen block were selected. Sections from the center perforated area were excluded. Each section was photographed with a digital camera (Olympus DP 10, Olympus Optical) connected to a light microscope (Olympus BX 50 F4, Olympus Optical) and then saved as a digital image in a computer. It was not possible to capture the entire interface in a single image at the level of magnification that was used. Thus, a composite digital image was created by combining 3 smaller images.

After calibration, histometric measurements of the total area of the interface and the newly formed bone area in the interface were obtained in pixels and converted into mm^2 by measurement calibration using a histologic slide rule. The following criteria were used to standardize the histometric analysis:

- The total area of the interface was delineated corresponding to the space between the bone block graft and the receptor bed. This was considered 100% of the area to be analyzed.
- The newly formed bone area within the total was calculated by delineating each bone unit separately and then summing the areas.

Percentages were calculated (ratio of newly formed bone/interface area) and submitted to statistical analysis (Table 1).

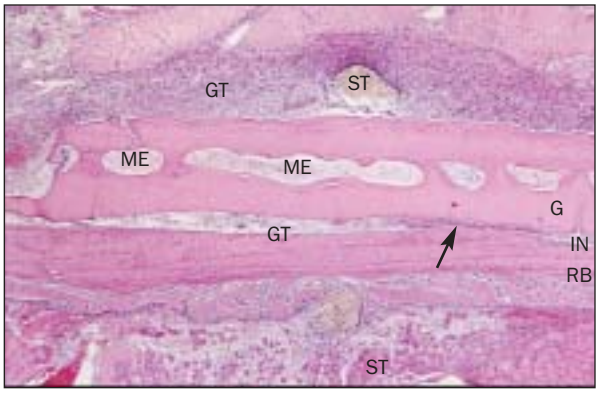

Fig 3 Sham-operated group, 7 days. Organized granulation tissue involving the graft periphery may be observed. Such tissues is also present in the graft-bed interface (IN). Vestiges of newly formed immature bone matrix can be seen in the central area of the interface *(arrow)*. G = graft, GT = granulation tissue, RB = receptor bed, ME = medullary space, ST = suture (H&E; original magnification ×10).

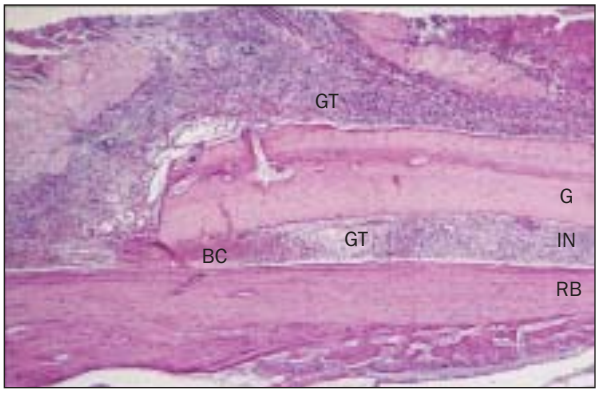

Fig 4 Ovariectomized group, 7 days. Remnants of blood clot may be observed at the interface and graft borders. Immature granulation tissue (GT) involving the graft periphery as well as the graft-bed interface can also be observed. IN = interface, G = graft, RB = receptor bed, BC = blood clot. (H&E; original magnification ×10).

The significance of differences between groups in relation to percentage of newly formed bone in the interface was determined by analysis of variance (ANOVA), followed by a post-hoc Tukey test. Results were considered significant where *P* was less than .05.

RESULTS

Histologic Analysis, 7 Days

In the sham-operated group, the amount of granulation tissue observed was typical, with a significant amount of fibroblast-like cells, many blood vessels, and an organized net of fibrin around the graft periphery as well as in its interface and lateral edges. Discrete osteogenic activity was noted in the most central area of the bed-graft interface, characterized by bits of newly formed immature bone matrix surrounded by numerous large osteoblast-like cells (Fig 3). In the ovariectomized group, the graft periphery and the interface presented an immature granulation tissue containing some blood vessels and fibroblast-like cells as well as remnants of blood clot (Fig 4). Resorption could also be seen along the graft surface in both the sham-operated and ovariectomized groups, although revascularization channels through preexisting haversian channels with vascular infiltration were only noted in the sham-operated group.

Histologic Analysis, 14 Days

In the sham-operated group, woven bone with intertrabecular spaces was observed in the interface, establishing bridges that connected the receptor bed and the internal surface of the graft. The trabecular bone contained numerous osteocytes and was surrounded by large osteoblasts. Woven bone trabeculae partially filled the medullary spaces in most specimens. Many osteoblasts were observed adjacent to the borders of the newly formed bone tissue. In most specimens, remnants of granulation tissue in the interface and around the graft periphery were well revascularized and rich in fibroblasts (Fig 5). In the ovariectomized group, granulation tissue was typically densely vascularized along the bed-graft interface. However, discrete osteogenic activity was observed along the bed-graft interface with small amounts of newly formed bone tissue. In most specimens, new bone formation was very minimal and restricted to the central area within the interface. Most medullary spaces contained degenerating cells and also presented fibroblast-like cells and osteoprogenitor cells. Few medullary spaces presented partially filled by a thin woven bone (Fig 6).

Histologic Analysis, 28 Days

In the sham-operated group, the bed-graft interface already showed integration between the lower face

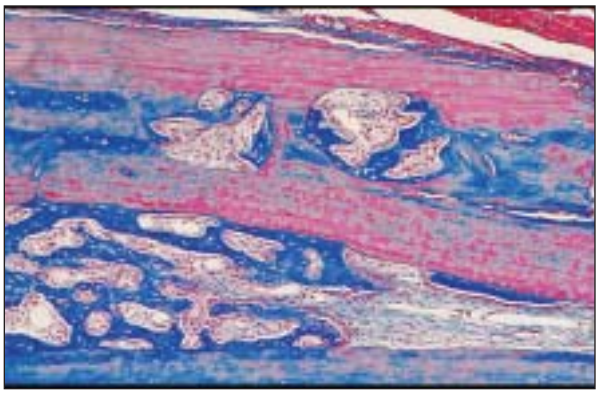

Fig 5 Sham-operated group, 14 days. Note the newly formed bone (NFB) in the interface (IN), connecting the recipient bed (RB) and the lower surface of the graft (G). Medullary spaces (ME) were partially filled by woven bone trabeculae (Masson trichrome; original magnification ×25).

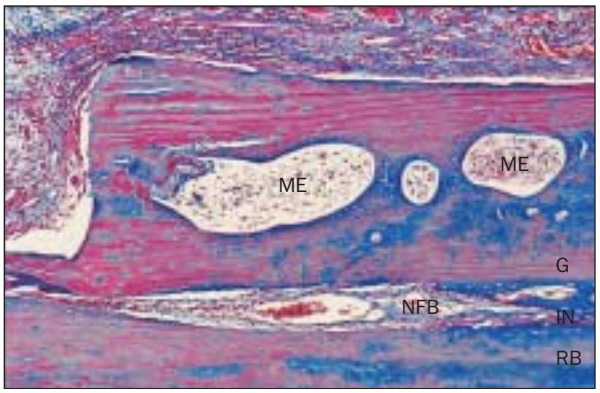

Fig 6 Ovariectomized group, 14 days. Newly formed bone (NFB) restricted to the central area within the interface (IN). Note the connective tissue interposed between the graft (G) and the recipient bed (RB). Medullary spaces (ME) contained degenerating cells. (Masson trichrome; original magnification ×25).

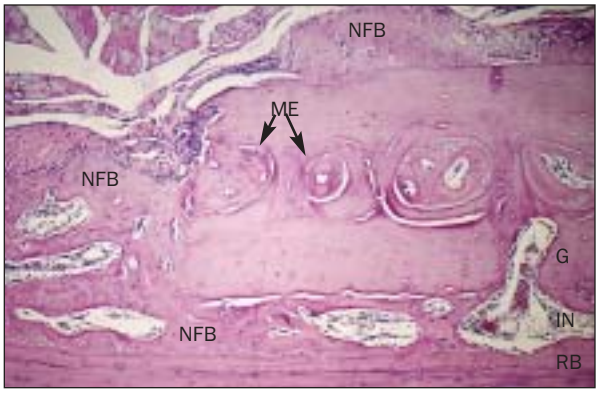

Fig 7 Sham-operated group, 28 days. Newly formed bone (NFB) can be observed filling the graft-bed interface (IN), reaching the graft's lateral borders and beyond the external surface of the graft (G). The medullary spaces (ME) were completely filled by newly formed bone. RB = receptor bed. (hematoxylin-eosin; original magnification ×25).

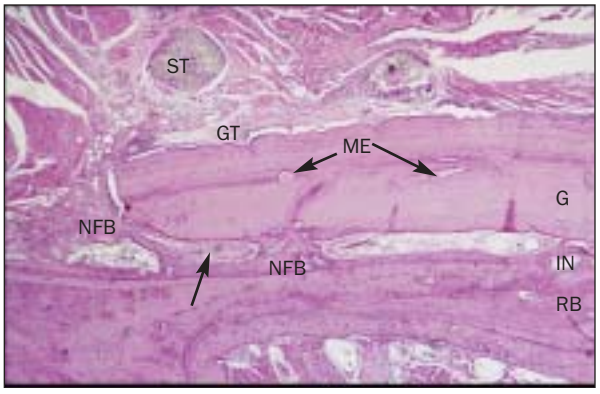

Fig 8 Ovariectomized group, 28 days. Woven bone bridges may be observed between the bed and the graft. Connective tissue interposed *(arrow)*. Note irregular contour along the graft (G). IN = interface, NFB = newly formed bone, GT = granulation tissue, RB = recipient bed, ME = medullary space, ST = suture thread (H&E; original magnification ×10).

of the graft and the receptor bed. Intense osteogenic activity was observed in this period. A greater amount of woven bone formation with large blood vessels was observed. The graft contour was more irregular along its lateral borders, suggesting resorption. Newly formed bone could also be seen reaching beyond the limits of the graft's lateral borders and above the external surface of the graft. The medullary spaces were completely filled by newly formed bone in most specimens (Fig 7). In the ovariectomized group, woven bone formed a connection between the bed and the graft, particularly in the central area of the bed-graft interface. However, areas of connective tissue poor in collagen fibers and infiltrated by blood vessels were still noted interposed between the bone graft and the receptor bed. Few medullary spaces were filled by thin woven bone, and osteogenic activity was not observed in any of the specimens. The lateral borders and external surface of the graft exhibited resorption areas and lamellar bone apposition, resulting in an irregular contour along the graft (Fig 8).

Statistical Analysis

ANOVA showed significant differences between groups ($P < .001$) and between healing periods ($P = .0055$), but there was no significant interaction between groups and periods ($P = .8644$).

The means were compared by the Tukey test. This comparison revealed that bone neoformation was significantly greater in the sham-operated group (57.41% at 14 days; 68.35% at 28 days) compared with the ovariectomized group (40.82% at 14 days; 53.09 at 28 days) in both observation periods (Table 1).

DISCUSSION

The rat model used in the present study was previously used to analyze the initial repair pattern[29] and the revascularization[30] of autogenous bone block grafts. The main goal of the present study was to use this method to investigate, in 12-month-old female rats, the influence of ovariectomy on the healing of autogenous bone block grafts in mandible by histometric analysis, emphasizing the histologic events that occur during the initial healing phase.

Based on the bone growth pattern of female Wistar rats Kalu et al[28] believed that from 6 months of age, changes in femur density and calcium were minimal, and that by 12 months, all the bone parameters measured had reached plateau levels. On the basis of these findings, the use of 12-month-old animals ensured that the skeletal changes observed following ovariectomy in the aged rat model were mainly due to ovarian hormone deficiency. However, its influence is less evident in young animals because of continued bone growth and in old animals because of age-related bone loss and osseous disease. Regarding all that, 12-month-old female rats were chosen for the present study, taking into account that the pattern of human postmenopausal bone loss and that of bone loss induced by ovariectomy in aged rats are similar.

Other studies have investigated the impact of osteoporosis on osseointegrated implant outcomes. Some of them have demonstrated that estrogen deficiency may influence bone healing around titanium implants.[21–26] However, the influence of the estrogen deficiency on the healing of bone grafts has still not been elucidated.

At the time points that were histometrically analyzed (14 and 28 days), the results indicated that the newly formed bone area obtained in the interface was significantly greater in bone grafts done in sham-operated rats (ie, non–estrogen-deficient rats) than in ovariectomized rats, which were characterized by estrogen deficiency. This result can be explained by the action of gonadal hormones, particularly estrogen, on growth factor production and cytokine expression in osteoblasts and osteoclasts in vitro, which may be important for regulating bone remodeling. Some of these growth factors are essential to stimulate angiogenesis.[31] The osteoblastic cells under estrogenic stimulation produce transforming growth factor-β and the insulin-like growth factors (IGFs). Transforming growth factor-β, which is a potent mitogen in osteoblastic cells, has been reported to inhibit osteoclast recruitment and resorption.[31,32]

Histologic findings seemed to be in accordance with the quantitative results, which also showed a delay on the healing of autogenous bone block grafts in the mandibles of ovariectomized rats in comparison to sham-operated rats.

Graft surface resorption and the presence of revascularization channels along the extension on the graft was observed in both groups at the seventh day of the period, which suggested that graft revascularization was already under way. In 1975, Enneking et al[33] reported that cortical graft vascular penetration is primarily a result of peripheral osteoclastic resorption and then vascular infiltration through Volkmann and haversian canals. However, the infiltration of capillaries, fibroblast-like cells, and osteoprogenitor cells through Volkmann channels was only evident in the sham-operated group. Newly formed bone was observed in association with some of these channels in some specimens of this group.

After 7 days, vestiges of newly formed immature bone matrix in the central area of the bed-graft interface were observed in the sham-operated group, whereas in the ovariectomized group the graft-bed interface presented an immature granulation tissue. Although Jardini et al[29] observed the formation of immature trabecular bone 7 days postoperatively, they used male rats younger than those used in the present study.

The 14-day results suggest that woven bone formation within medullary spaces is intimately related to the revascularization process. De Marco et al, who used India ink perfusion to evaluate revascularization of block grafts, noted revascularization from the recipient bed and also from the surrounding connective tissue by the seventh day.[30] Once more, it was not possible to establish comparison to the same period of the present study because they used younger male rats. Otherwise, the delayed repair in the ovariectomized group could be explained by the deficiency of estrogen, which induces the production of growth factors. These growth factors induce organic matrix apposition and epithelial proliferation, resulting in effective local angiogenesis in the wound area.[34,35] In this manner, the discrete angiogenesis and the delayed bed-graft integration in ovariectomized rats noted in this study could be related to lower estrogen levels induced by the ovary removal. Furthermore, early vascular penetration and nutrition of the graft are key factors for its future integration with the receptor bed, which explains the advanced repair of graft-bed interface in the sham-operated group.

The results of the study developed by Jardini et al[29] showed signs of graft incorporation after 21 days, starting at the periphery of the graft. According to the authors cited, this could be explained by the

revascularization process, as well as by the fact that incorporation begins in areas of bed-graft contact. However, in the present study, initial signs of incorporation of the graft to the receptor bed were observed in the sham-operated group after 28 days, most evidently in the central area of the wound and adjacent to the inferior border of the graft. It is thus suggested that the inferior border of the graft was in close contact with the receptor bed, leading to faster incorporation, and that the incorporation in the central area may have been faster because the revascularization process originated from the perforated bone area.

Complete immobilization of the graft and the bed preparation by perforation and/or decorticalization becomes necessary to provide adequate incorporation of the graft, as demonstrated in the study of de Ċarvalho et al.[36] To encourage revascularization of the graft, Buser et al[37] and Misch and Misch[38] proposed perforations in the bone bed. In the present study, perforations were not performed in the receptor bed, as previously described, to avoid fractures due to the thinness of the cortical bone of the mandible. A superficial cortical bone scrape was performed, supporting an adequate bone graft adaptation and incorporation.

The Jardini et al[29] results and the results of the present study showed that the materials and methods used provided adequate incorporation of the graft. Although fixation of the bone graft can reportedly be accomplished with a compressive screw,[39] the present authors have tried this without success. Most of the graft was encapsulated by connective tissue, probably due to the thinner cortical bone of the mandible angle. Donos et al[39] observed a zone of fibrous connective tissue that frequently formed between the bone graft and recipient bone (lateral aspect of the mandible) when it was fixed with microimplants. The authors speculated that the microimplant may have resulted in insufficient fixation of the graft.

Despite the delayed healing process in the ovariectomized group, the observation of a continuous bone gain after 45 days by Jardini et al[29] suggests that this bone repair process would have continued over a longer period. The significant difference in newly formed bone area between the control and experimental groups suggests that the estrogen deficiency delays the healing process of autogenous bone block graft. Further studies are necessary, involving other animal species and humans, other graft materials, longer periods, and hormonal reposition and histochemical techniques to clarify the influence of the estrogen deficiency on the healing process of bone grafts. Long-term results could strongly support the use of bone grafts in postmenopausal women.

CONCLUSION

The estrogen depletion caused by the ovariectomy hindered the healing process of autogenous block bone grafts placed in the mandibles of aged rats. Although the repair process appeared delayed in the ovariectomized group, there was evidence that it would have been completed after a longer period of time.

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