

Evaluation of the effects of bone morphogenetic protein-2 on the healing of bone calvarial defects in ovariectomized rats

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Abstract: Bone morphogenetic proteins (BMPs) are often used with bone grafts for bone regeneration. The aim of this study was to evaluate the effect of demineralized freeze-dried bone allografts and externally applied rhBMP-2 on bone healing in experimentally generated defects in the calvarium of ovariectomized and nonovariectomized rats via histological and immunohistochemical assays. The current study was carried out on 42 female Wistar rats. After ovariectomy was performed in half of the rats, the rats were divided into three groups: control, allograft, and allograft + BMP. An experimental defect with a diameter of 4 mm was created unilaterally in the parietal bone using a trephine dental drill and a physiodispenser with saline. All animals were euthanized at the end of week 8, and bone tissues were analyzed histologically and immunohistochemically. The allografts produced better results in terms of calvarial defect healing in both the ovariectomized and nonovariectomized groups compared to the control group in enhancing bone healing, whereas BMPs combined with allografts had no positive effect on new bone formation. The allografts and BMPs applied in both groups produced fibrous tissues rather than new bone tissue. Further comprehensive studies on the effectiveness of BMPs in the formation of new bone tissue should be conducted in animals with estrogen deficiency.

Key words: Bone morphogenetic protein, allograft, ovariectomy, bone regeneration, rat

1. Introduction

Inadequate bone support for oral surgery and periodontal regeneration is a major problem for surgical applications. Additional graft materials are needed in dentistry to repair missing bone tissue. Currently, due to their osteogenic, osteoinductive, and osteoconductive effects, autogenous bone grafts are considered the gold standard under certain conditions [1]. However, other conditions, such as the presence of a second surgical site and the failure to obtain the desired amount of material, limit the use of autogenous bone grafts [2]. Because of this, many researchers have attempted to find an alternative to autografts. For instance, allografts have been used in dentistry for a long time. Allografts have osteoconductive and osteoinductive effects [3]. Regrettably, the devitalization process used to suppress the host immune response and prevent possible disease transmission in cadaveric allografts causes the loss of the mechanical, osteoinductive, and osteoconductive properties of the graft [4,5]. In addition, the maturation times of allografts are longer in defect regions than those of autografts [6,7].

Bone morphogenetic proteins (BMPs) are well known to induce osteogenic effects. Allografts typically contain BMPs that accelerate mesenchymal cell migration and attachment [3]. In both in vitro and in vivo studies of BMP-2, which is a type of BMP, the contribution of BMP-2 to ossification could be shown. BMP-2 promotes periodontal and bone regeneration in some animal models [8,9] and also induces differentiation of preosteoblast cells and thereby increases ossification [10,11]. Fundamentally, BMPs are types of growth factors and belong to the transforming growth factor (TGF- β) family [12]. There are variant BMPs that support bone regeneration, such as BMP-2, -4, -5, -6, and -7 [13]. Recombinant human bone morphogenetic protein-2 (rhBMP-2), generated by recombinant DNA technology from mammalian cells, is known to induce differentiation of osteoblasts at a cellular level and to induce osteogenesis in animal models [14]. Bone graft materials and osteoinductive agents have a synergistic effect [15]. The synergistic effect of BMPs and bone grafts on bone regeneration has been demonstrated in animal studies [11].

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In immunohistochemical studies, the type of biomarkers used is important for analyzing, imaging, and examining regeneration in the bone defect area [16]. For instance, osteocalcin (OC) is a strong marker of newly formed bone [17,18]. Osteonectin (ON) is a biological modulator and a known proliferative agent [19]. These biomarkers have been shown under various conditions to control bone cell differentiation and remodeling [20,21]. Osteopontin (OPN) is also a biomarker involved in the bone maturation and mineralization stages that occur after the accumulation and formation of a collagen network [22].

Osteoporosis is characterized by decreased bone volume and deterioration of bone cells and consequently causes bone fragility and fractures [23]. Osteoporosis and periodontitis mainly affect bone tissues [24]. Postmenopausal osteoporosis is a condition that occurs 10 to 15 years after menopause and often leads to increases in alveolar bone resorption and promotes periodontal tissue degeneration [25]. Estrogens play an important role in both inflammation and skeletal repair and remodeling by regulating important mediators of immune function. At the same time, estrogen inhibits osteoclast secretion [26]. There is a close relationship between bone metabolism and estrogen deficiency in osteoporosis [27].

The objective of the current study was to evaluate the effect of allografts and externally applied rhBMP-2 on bone healing in experimentally generated defects in the calvarium of ovariectomized and nonovariectomized rats via histological and immunohistochemical assays.

2. Materials and methods

2.1. Study design

A total of 42 female Wistar rats (250-300 g) provided by the Scientific Application and Research Center of Dicle University (Protocol No: 12-DH-53) were used. All the procedures described in the experimental protocols were approved by the Animal Ethics Committee of Dicle University (Protocol No: 2011/15, 29.03.2011). The study was performed in accordance with the Helsinki Declaration and with the permission of the Governmental Animal Protection Committee. The study was performed at the Dicle University Health Sciences and Research Center and the Faculty of Medicine Histology and Embryology Department. The weight changes in the rats were determined before sacrifice. The animals were given commercial rat chow and water ad libitum and were maintained on a 12-h light/12-h dark cycle at a temperature of 22 ± 1 °C.

The rats used in the study were divided into two groups: the control (nonovariectomized rats were assigned as the control group) and the ovariectomized group, and each group was divided into three subgroups (n = 7).

These comprised the following: the control group: C1, cranial empty defect; C2, defect with demineralized freeze-dried bone allografts (DFDBAs); and C3, defect with allograft and bone morphogenetic protein (BMP-2); and the ovariectomized group: O1, cranial empty defect; O2, defect with allograft; and O3, defect with allograft and BMP-2.

2.2. Surgical procedure

The 21 rats subjected to ovariectomy were intraperitoneally administered xylazine (3 mg/kg) and ketamine (35 mg/kg) and then ovariectomy was performed. According to the procedure recommended by Pires-Oliveira et al., between the middle of the dorsum and the tail base, a 3-cm-long incision was made in the midline dorsal skin for ovariectomy [28]. The animals were observed for infection. At the end of day 30, all animals were anesthetized prior to the generation of the bone defects in the calvarial zone. After shaving the head hair, a longitudinal midsagittal incision was made in the skin to expose the parietal bones, and the flaps were retracted in the subperiosteal plane to expose the parietal bones. A 4-mm diameter full-thickness critical-size cranial defect was made unilaterally in the parietal bone using a trephine dental drill and a physiodispenser with saline [29,30].

After the cranial defect was generated in the nonovariectomized group, the defects were left empty in group C1. DFDBAs (Raptos, Citagenix, Laval, QC, Canada) were placed in the defects in group C2, and the defects in group C3 were treated with allografts and rhBMP-2 (produced in *E. coli*, Gibco, Frederick, MD, USA). The same experimental plan was used in the ovariectomized rats.

After placement of the grafts, the flaps were sutured with 3/0 silk sutures. No infection or side effects were observed during the recovery period. Eight weeks after the calvarial surgery, all of the rats were sacrificed using an overdose of intraperitoneal ketamine hydrochloride prior to histological and immunohistochemical evaluation.

2.3. Preparation of the biomaterial

Considering the manufacturer's recommendations and the dosage used in other studies, 10 µg of BMP was diluted to 0.2 mg/mL [31,32]. An equal amount of allograft was soaked with 12.5 µL of rh-BMP-2 solution before being placed in the defect site.

2.4. Sample and histological process

The operation area was extracted using a saw, remaining 2 mm around each defect area. The samples were fixed in 10% neutral buffered formaldehyde solution for 48 h, and decalcified in 10% ethylenediaminetetraacetic acid (EDTA) prepared in 0.1 M Tris-HCl buffer (pH 7.4) for 14 days. Subsequently, the specimens were dehydrated, cleared, and embedded in paraffin. Then 5-µm-thick serial sections were cut from the center of the bone defect. Four

slides were prepared from each sample and each slide contained a minimum of 3 sections that were at least 50- μ m apart. Three slides were used for immunohistochemical detection, and the other, stained by Gomori's method (to determine ossification), was used for histological analysis.

2.5. Immunohistochemical staining

To perform the immunohistochemical analyses, the streptavidin–biotin–peroxidase method [33] was performed using the Zymed Histostain Plus Bulk Kit (code: 85-9043, Histostain Plus Bulk Kit, Zymed, South San Francisco, CA, USA). Sections were treated with 3% H₂O₂ prepared in methanol for 15 min to block endogenous peroxidase activity after deparaffinization and rehydration and were washed with phosphate-buffered saline (PBS). Antigen retrieval was performed in citrate buffer (0.01 M, pH 6.0) at 95 °C for 30 min, and the sections were allowed to cool before immunostaining. After they were washed twice in PBS for 5 min, the sections were treated with protein blocking solution (Ultra V Block) at room temperature for 5–10 min to block nonspecific staining. Then the sections were incubated with a mouse monoclonal antibody against OPN (Santa Cruz Biotechnology, sc-21742) and rabbit polyclonal antibodies against OC (Santa Cruz Biotechnology, sc-30044) and ON (Santa Cruz Biotechnology, sc-25574) at a dilution of 1:200 for 20 h at 4 °C. After incubation, the sections were washed three times in PBS for 5 min, treated with biotinylated secondary antibody at room temperature for 20 min, and then washed three times in PBS for 5 min. The sections were incubated in streptavidin peroxidase solution for 20 min and washed three times in PBS for 5 min. To visualize the reaction, the sections were treated with 3',3'-diaminobenzidine (DAB) chromogen for 5–10 min and counterstained with Mayer's hematoxylin for 2–3 min. Negative controls were used to ensure the accuracy of

staining, and distilled water was used instead of primary antibodies in these controls.

The intensity of the positive staining was defined as + weak, ++ medium, +++ strong, +/++ weak to moderate, or ++/+++ moderate to strong. The slides were examined and photographed using a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) equipped with a digital camera (Nikon Coolpix-4500) (Table 1).

2.6. Statistical evaluation

Kruskal–Wallis and Mann–Whitney U tests, which are nonparametric test methods, were used, and statistical analyses were considered significant for P < 0.05.

3. Results

3.1. Histological findings

At the fornix of the defect cavity in the C1 group, formations, such as spicules, that were associated with intramembranous ossification were observed. Fibrous tissue was also commonly observed in the cavity space and the presence of vascularization was noted (10 \times). For the ovariectomized group (O1), no intramembranous ossification was observed in the cavity and fibrous tissue was formed only at the fornix of the cavity and did not show a homogeneous distribution. In addition, vascularization was not intense in this group (10 \times).

In the C2 group, the presence of regular and common fibrous tissue in the defect area was noteworthy. It was also determined that vascularization showed a more regular structure (10 \times). The fibrous tissue around the bone graft was poorly formed and gaps between bone grafts were common in the ovariectomized group (O2). In addition, while the high abundance of cellular elements in the defect area was remarkable, vascularization was irregular (10 \times).

The findings obtained in the C3 group (4 \times) were similar to those in the C2 group described above. Likewise,

Table 1. Staining intensity scores of OPN, OC, and ON immunoreactivity in the rat calvarium.

	Extracellular matrix OPN, OC, ON	Osteoprogenitor cells OPN, OC, ON	Osteoblasts OPN, OC, ON	Osteocytes OPN, OC, ON
C1	+ / ++, +, +	-, -, -	+, +, -	-, -, -
C2	++, ++ / ++++, ++ / ++++	-, -, -	++, ++, ++	-, -, -
C3	++, +++, +, ++	-, -, -	++, ++, ++	-, -, -
O1	-, +, +	-, -, -	-, +, -	-, -, -
O2	+ / ++, + / ++, ++	-, -, -	++, ++, ++	-, -, -
O3	+ / ++, ++, ++	-, -, -	++, ++, ++	-, -, -

OPN: Osteopontin, OC: Osteocalcin, ON: Osteonectin, C: Control group, C1: (Empty defect), C2: (Empty defect + graft), C3: (Empty defect + graft + BMP), O: Ovariectomized group, O1: (Empty defect), O2: (Empty defect + graft), O3: (Empty defect + graft + BMP), Staining intensity: +++ strong, ++ moderate, + weak, - negative.

the findings in the ovariectomized group (O3) and the O2 group were parallel. Additionally, addition of BMP to allografts had no effect in the C3 or O3 groups (Figure 1).

3.2. Immunohistochemical evaluation

3.2.1. Osteopontin involvement

In the C1 group, osteoblast cells in the defect area showed low involvement of OPN, and the extracellular matrix also showed only low- to medium-level involvement of OPN. In addition, blood vessels in the defect area were stained (20×). OPN immunoreactivity was observed in normal bone tissue in the O1 group, whereas OPN immunoreactivity was observed only in blood vessels in defect areas (4×).

In the C2 group, OPN immunoreactivity was not observed in osteoprogenitor cells at the fornix of the defect cavity, but moderate OPN staining was observed in osteoblasts that formed around the bone graft. The extracellular matrix and blood vessels showed positive staining for OPN (4× and cropped image 40×). The findings in the O2 group were similar to those in the C2 group, and the extracellular matrix around the bone graft showed remarkably strong OPN staining (4×).

Osteoprogenitor cells at the fornix of the defect area showed no OPN involvement in the C3 group. It was determined that the osteoblast cells that formed around the bone graft material had moderate OPN immunoreactivity and that fibrous tissue in the defect area was abundant and homogeneously distributed (10×). While the osteoblast cells that formed around the bone graft showed moderate staining, the extracellular matrix around the graft showed minimal staining (10×) (Figure 2). The comparisons of the parameters within the groups for OPN staining are shown in Table 2.

3.2.2. Osteocalcin involvement

In the C1 group, OC involvement in osteoprogenitor cells was not observed, and OC showed weak staining in osteoblasts at the fornix of the defect cavity. It was also determined that the extracellular matrix was dense at the fornix of the defect cavity and that the vessels were positively stained with OC (20×). Similar findings were shown in the O1 group compared to the C1 group, and the only difference was that the staining in the extracellular matrix was not homogeneously distributed (20×).

In the C2 group, negative staining was observed in osteoprogenitor cells at the fornix of the defect area, but unlike the findings in the C1 group, osteoblasts that formed in the defect area showed moderate involvement. In addition, it was observed that the extracellular matrix and blood vessels were intensely stained both at the fornix of the defect area and within the defect (10×). In the O2 group, in terms of OC, the extracellular matrix showed strong involvement at the fornix of the defect cavity, but

the involvement in the defect area was weak and localized. While osteoprogenitor cells showed no reaction with OC, moderate OC immunoreactivity was detected in osteoblasts. In addition, blood vessels showed positive staining for OC (10×).

In the C3 group, no immunoreactivity was observed in osteoprogenitor cells except for those at the fornix of the cavity, but moderate involvement was observed in osteoblasts. In this group, it was observed that the extracellular matrix was stained more intensely in the defect area and especially around the bone graft, and blood vessels also positively stained for OC (10×). A lack of staining was observed in osteoprogenitor cells in the O3 group, and moderate involvement was observed in osteoblasts. There was staining in the extracellular matrix, both at the fornix of the defect cavity and within the defect area, but less staining was detected around the bone graft compared to that detected in the C3 group. In addition, apparent OC expression was observed in the blood vessels (10×) (Figure 3). The comparisons of the parameters within the groups for OC staining are shown in Table 3.

3.2.3. Osteonectin involvement

In the C1 group, ON involvement in osteoprogenitor cells at the fornix of the defect and in osteoblasts in the defect cavity was not found. In addition, the staining of the extracellular matrix was weak, and blood vessels showed moderate ON immunoreactivity (10×). The O1 group showed results similar to those of the C1 group.

In the C2 group, a lack of ON staining was observed in osteoprogenitor cells at the fornix of the defect area; unlike those in the C1 group, osteoblasts that formed in the defect area in the C2 group showed moderate ON staining. The extracellular matrix in the fornix of the defect area was intensely but not homogeneously stained in the cavity, and ON expression was also detected in the blood vessels (4× and cropped image 20×). The O2 group showed results similar to those of the C2 group, but the extracellular matrix was not intensely stained.

In the C3 group, osteoprogenitor cells in the fornix of the defect area did not show ON expression, but moderate staining of osteoblasts that formed around the bone graft was observed. The extracellular matrix and blood vessels showed intense ON expression (10×). In the O3 group, while osteoprogenitor cells had no staining, which was similar to the results in the C3 group, osteoblast cells around the bone graft showed moderate ON staining. The extracellular matrix in the fornix of the defect area showed intense staining, but the staining was decreased inside the defect area. ON expression in the blood vessels was also observed (4×) (Figure 4). The comparisons of the parameters within the groups for ON staining are shown in Table 4.

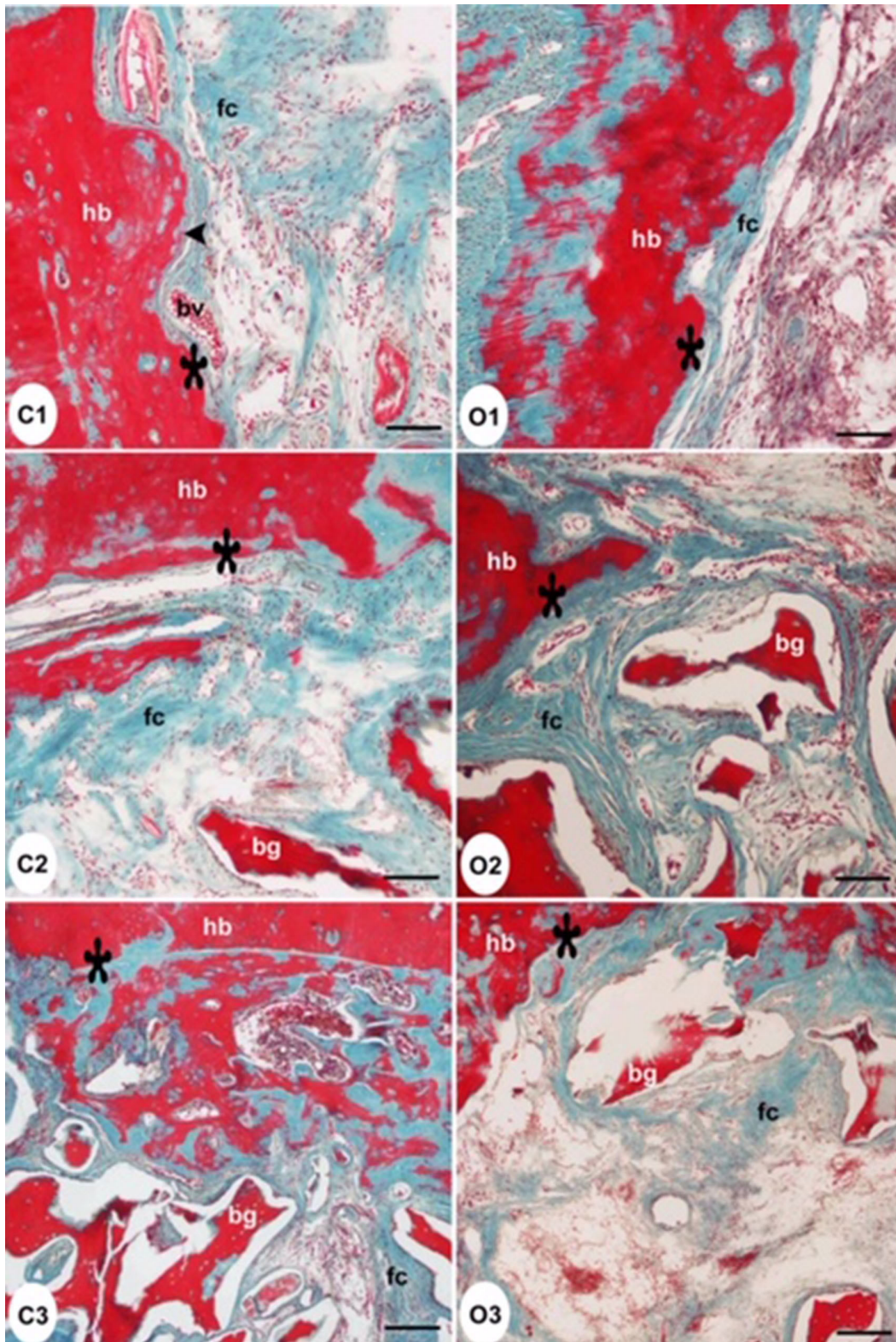


Figure 1. Histological analysis of defect areas in rat calvarial bone in the control (C1, C2, and C3) and ovariectomy (O1, O2, and O3) groups. The defects were empty as shown in C1 and O1 and defect areas were treated with bone grafts (C2, O2) and bone grafts + bmp (C3, O3). hb: host bone, fc: fibrous connective tissue, bg: bone graft, (*): interface between host bone and the defect, (▼): bone spicule. Scale bars: 100 μ m (C1, C2, O1, and O2); 250 μ m (C3 and O3). Gomori's staining method.

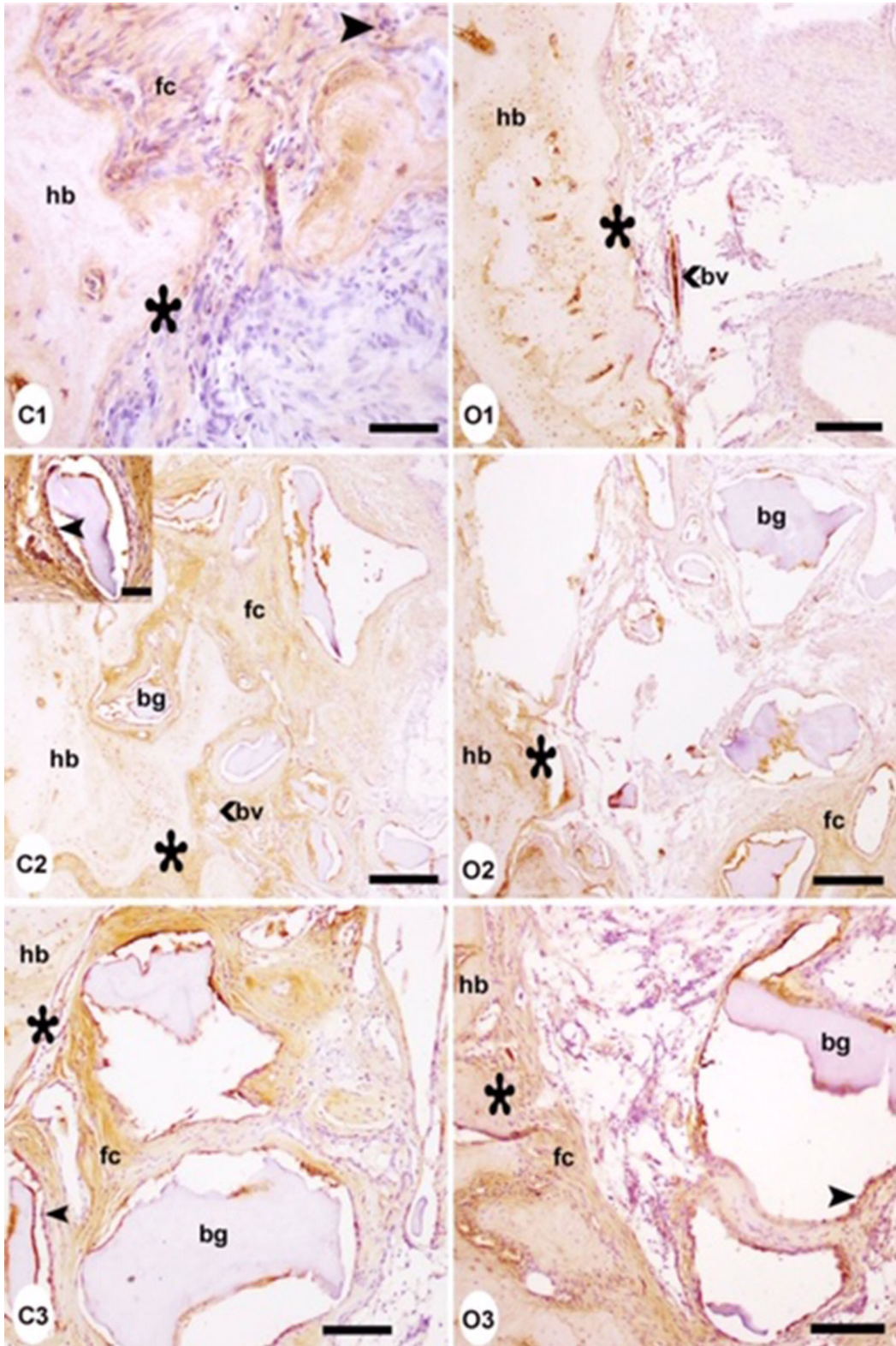


Figure 2. OPN expression in defect areas in rat calvarial bone in the control (C1, C2, and C3) and ovariectomy (O1, O2, and O3) groups. The formations in the groups are shown in the histochemical figure. hb: host bone, fc: fibrous connective tissue, bv: blood vessel, bg: bone graft, (*) : interface between host bone and the defect, (▼): osteoblast. Scale bars: 50 μm (C1), 100 μm (C3 and O3), 250 μm (O1, O2, and C2; the magnification within the image is 25 μm).

Table 2. Semiquantitative analysis of the parameters for osteopontin in calvaria. $P < 0.05$.

Groups	P	Parameters	n	Average rank	Different ($P < 0.05$) from groups
(1) C1	0.0269	1	7	7.57	(2)
(2) C2			7	15.14	(1)
(3) C3			7	10.29	ns
(1) C1	0.0064	3	7	5.43	(2)(3)
(2) C2			7	14.14	(1)
(3) C3			7	13.43	(1)
Groups	P	Parameters	n	Average rank	Different ($P < 0.05$) from groups
(1) O1	0.0004	1	7	5.00	(2)(3)
(2) O2			7	17.29	(1)(3)
(3) O3			7	10.71	(1)(2)
(1) O1	0.0004	3	7	4.00	(2)(3)
(2) O2			7	14.00	(1)
(3) O3			7	15.00	(1)

Parameters: 1, extracellular matrix; 2, osteoprogenitor cells; 3, osteoblasts; 4, osteocytes.

ns: nonsignificant ($P > 0.05$) Since average ranks of parameters 2 and 4 were equal, no significant difference could be established (average rank = 11.00).

4. Discussion

The results suggest that the use of rhBMP-2 with allografts may increase bone regeneration in estrogen-deficient rats. Autografts are still acknowledged as the gold standard due to their osteogenic, osteoconductive, and osteoinductive properties. However, they have some disadvantages such as limited supply and donor site morbidity [34]. Allografts, which are produced as an alternative to autografts, are considered the gold standard for bone regeneration and are obtained from cadavers. Although allografts contain BMPs, they lose some of their effectiveness in preventing disease as a result of the sterilization procedure [4]. Bone graft materials such as allografts are used in bone regeneration in oral surgery and periodontology [35,36]. Many factors affect the effectiveness of the graft material, such as the type of graft and the growth factors, and living cells and vascularization in the graft material and defect area [37,38]. Additionally, the existence of osteoprogenitor cells in the defect area directly influences bone regeneration. Moreover, when these cells are triggered by growth factors and have adequate vascularization, bone regeneration occurs robustly [7,39]. rhBMP is a kind of TGF- β and was used to enhance new bone formation in the present study.

A critical-size bone defect is defined as an intraosseous wound that will not heal itself completely without intervention [40,41]. In the present study, as in previous studies, the size of the critical-size defect was selected as 4 mm [7,29]. Although it is difficult to compare human biology and animal biology, rats are frequently used to ensure the reproducibility of experiments and for studies that seek to attain a 'proof of principle' [18,42]. The rat

calvarial site defect model is an excellent model for bone regeneration in the craniofacial region. As the calvarial site is stable, defects in this site are useful for the examination of histological and immunohistochemical parameters [18]. In the present study, the experiment period was determined as 8 weeks. This period was selected based on previous studies [43–45]. Akita et al. [29] and Dazai et al. [46] stated that endogenous bone healing was completed after 8 weeks.

Recently, the appropriate dosage of rhBMP has become controversial. A high BMP dosage could have negative consequences, such as osteolysis, hematoma, and ectopic bone formation, and these could be caused by disproportionate growth of osteoblasts [47–49]. Moreover, Kimelman Bleich et al. claimed that even using sequestered BMPs can cause osteolysis [50]. Additionally, the results of a study carried out by Abdala et al. with different doses of rhBMP (1, 3, 5, and 7 μg) showed that there were no significant differences in new bone formation in rat calvarial defects [51]. Pelaez et al. added different amounts of rhBMP-2 to graft materials and examined the effect on the amount of new bone. It was found that a dose greater than 2.5 μg had no effect on new bone formation [43]. By considering the presence of BMP in the allograft and based on the results of the studies mentioned above, the dosage of rhBMP was selected as 0.2 mg/mL in the present study.

According to the immunohistochemical results of our study, the OC, OPN, and ON staining results in osteoblasts, osteocytes, the extracellular matrix, and osteoprogenitor cells were similar among the groups, and rhBMP-2 had no effect on ossification or immunological staining in either the nonovariectomized or ovariectomized groups.

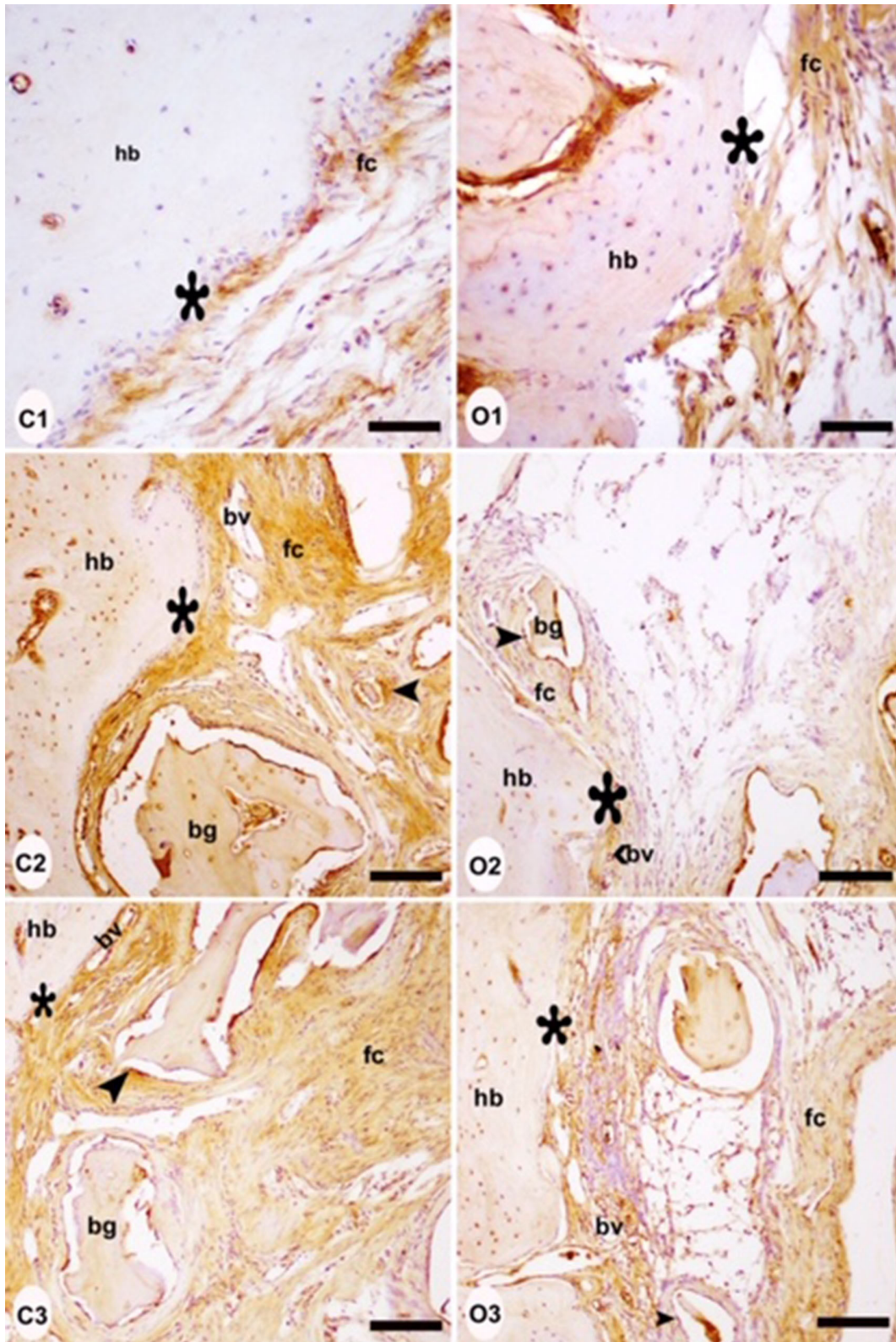


Figure 3. OC expression in defect areas in rat calvarial bone in the control (C1, C2, and C3) and ovariectomy (O1, O2, and O3) groups. The formations in the groups are shown in the histochemical figure. hb: host bone, fc: fibrous connective tissue, bv: blood vessel, bg: bone graft, (*): interface between host bone and defect, (▼): osteoblast. Scale bars: 50 μ m (C1 and O1), 100 μ m (C2, C3, O2, and O3).

Table 3. Semiquantitative analysis of the parameters for osteocalcin in rat calvaria. $P < 0.05$.

Groups	P	Parameters	n	Average rank	Different ($P < 0.05$) from groups
(1) C1	0.0848	1	7	7.93	ns
(2) C2			7	13.21	ns
(3) C3			7	11.86	ns
(1) C1	0.0007	3	7	4.36	(2)(3)
(2) C2			7	13.71	(1)
(3) C3			7	14.93	(1)
Groups	P	Parameters	n	Average rank	Different ($P < 0.05$) from groups
(1) O1	0.5134	1	7	11.00	ns
(2) O2			7	9.50	ns
(3) O3			7	12.50	ns
(1) O1	0.0026	3	7	5.07	(2)(3)
(2) O2			7	15.00	(1)
(3) O3			7	12.93	(1)

Parameters: 1, extracellular matrix; 2, osteoprogenitor cells; 3, osteoblasts; 4, osteocytes.

ns: nonsignificant ($P > 0.05$) Since average ranks of parameters 2 and 4 were equal, no significant difference could be established (average rank = 11.00).

Ishigaki et al. revealed that OC staining of osteoblasts was weak in the graft group [52]. Ivanovski et al. observed that OC staining of osteoblasts and osteocytes was strong in the graft group [53]. Another study showed that osteocyte staining was moderate in both the ovariectomized and nonovariectomized groups [54]. In the present study, OC staining of osteoblasts was moderate, and no staining of osteocytes was observed in either the ovariectomized or nonovariectomized groups. ON staining of osteocytes was not observed in the ovariectomized or nonovariectomized groups in our study, and the ON staining of osteoblasts was moderate. The results of studies by Tera et al. [52] and Ishigaki et al. [54] were similar to those of our study. In the literature, many immunohistochemical and histological studies have shown that rhBMP-2 is effective in increasing new bone formation [18,31,40,55–57]. Cheng et al. showed that BMP-2 did not produce a statistically significant difference in bone volume in an experimental study in rats. They also argued that the use of allografts in combination with BMP-2 did not achieve the full repair of defects [58]. Koerner et al. showed that some adverse side effects of rhBMP-2 were increased inflammatory reactions and the expression of inflammatory cytokines [59]. When histological and immunohistochemical studies of BMP in the literature were examined, it was found that BMP was used with different growth factors and agents to prevent the side effects mentioned above. For instance, Lee et al. reported that the use of BMP and FGF-2 together with biphasic calcium phosphate (BCP) in an animal model increased periodontal tissue regeneration and was more

successful than BCP used with BMP-2 alone. The BMP-2 + BCP results at the end of 4 weeks were better than the BMP-2 + FGF-2 + BCP results, whereas the BMP-2 + FGF-2 + BCP group results were better at the end of 8 weeks [11]. Similarly, Sharmin et al. reported that the combination of vascular endothelial growth factor and BMP was superior to BMP-2 alone [60].

Although estrogen is the primary sex hormone in women, it plays a crucial role in bone regeneration and homeostasis in both women and men [26]. Estrogen deficiency leads to both early and late osteoporosis in postmenopausal women and increases the occurrence of osteoporosis in older men [61]. Ovariectomy is considered the most common method to induce osteopenia [54]. Similar to the results of the study by Tera et al. [54], the immunohistochemical markers did not show any alterations due to osteopenia. Several animal studies have shown delayed bone repair effects similar to those observed in the present study [62–64]. Fuegl et al. stated that the enhancement of bone formation was minimal in ovariectomized rats [65]. In another study, it was shown that the amount of newly formed bone was small and that bone formation was slow [66]. Durão et al. correspondingly indicated that the rate of new bone formation was slow [67]. Song et al. reported that bone resorption in the ovariectomized rat cortical tibial area started at the end of the eighth week, and bone formation was not observed at the end of the fourth week [68]. In a study conducted by Yang et al., bone formation occurred at the twelfth week in ovariectomized rats [69].

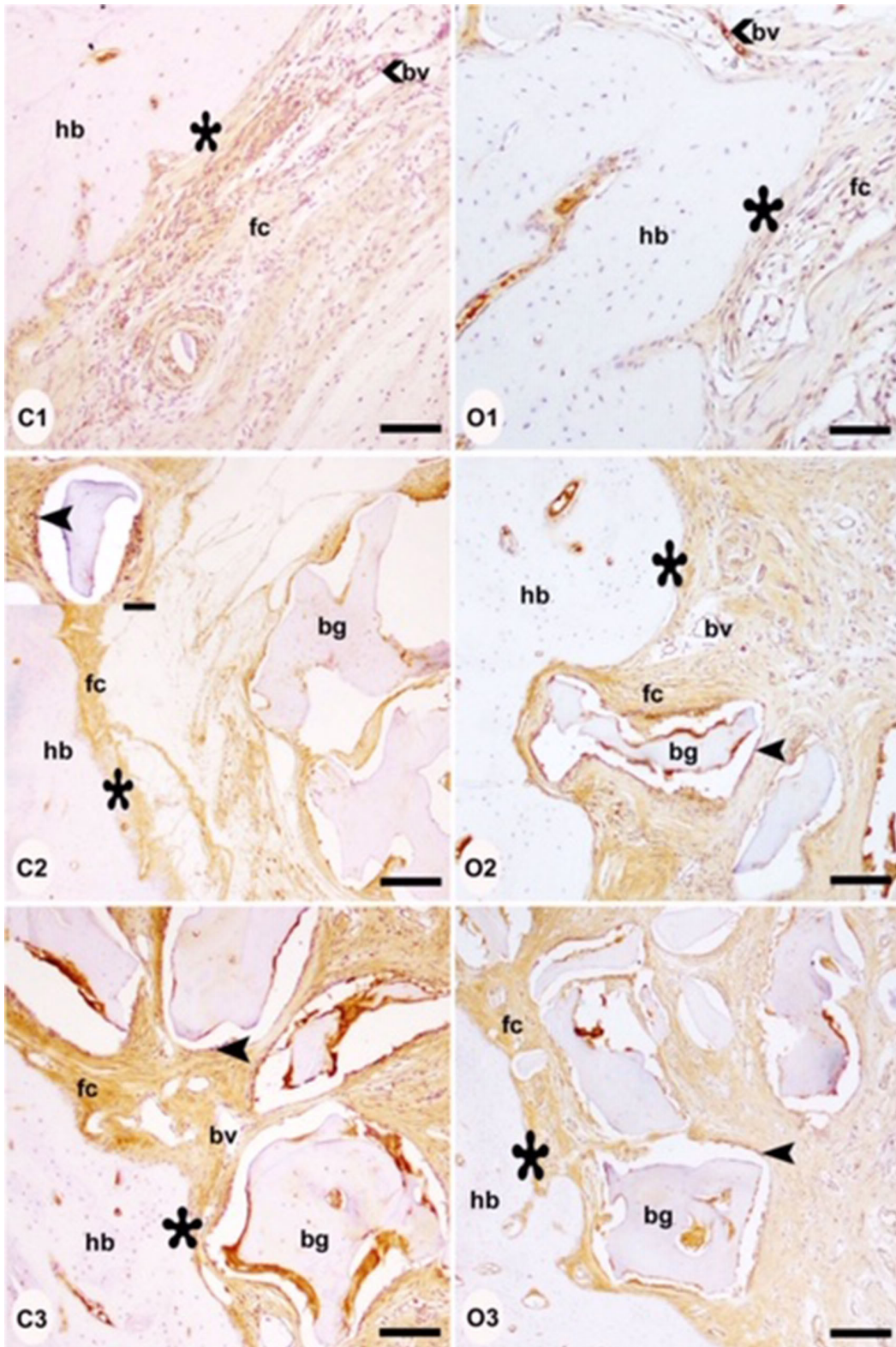


Figure 4. ON expression in defect areas in rat calvarial bone in the control (C1, C2, and C3) and ovariectomy (O1, O2, and O3) groups. The formations in the groups are shown in the histochemical figure. hb: host bone, fc: fibrous connective tissue, bv: blood vessel, bg: bone graft, (*) : interface between host bone and defect, (▼) : osteoblast. Scale bars: 100 μ m (C1, C3, O1, and O2), 250 μ m (O3 and C2; the magnification within the image is 25 μ m).

Table 4. Semiquantitative analysis of the parameters for osteonectin in rat calvaria. $P < 0.05$.

Groups	P	Parameters	n	Average rank	Different ($P < 0.05$) from groups
(1) C1	0.0004	1	7	4.00	(2)(3)
(2) C2			7	13.50	(1)
(3) C3			7	15.50	(1)
(1) C1	0.0002	3	7	4.00	(2)(3)
(2) C2			7	14.50	(1)
(3) C3			7	14.50	(1)
Groups	P	Parameters	n	Average rank	Different ($P < 0.05$) from groups
(1) O1	0.0013	1	7	4.71	(2)(3)
(2) O2			7	13.71	(1)
(3) O3			7	14.57	(1)
(1) O1	0.0004	3	7	4.00	(2)(3)
(2) O2			7	14.50	(1)
(3) O3			7	14.50	(1)

Parameters: 1, extracellular matrix; 2, osteoprogenitor cells; 3, osteoblasts; 4, osteocytes.

Since average ranks of parameters 2 and 4 were equal, no significant difference could be established (average rank = 11.00).

It is known that regeneration in the calvarial region can be more difficult to induce than regeneration in the tibial region. In a previous study, it was shown that experimentally created defects in the calvarial region may lead to insufficient supply of blood vessels and a lack of osteoprogenitor cells. Thus, it has been claimed that calvarial defects result in a poor osteogenic response and a challenging recovery environment during the evaluation of therapeutic agents [65]. In the present study, considering the weak effect of BMPs and above all the lack of estrogen, the poor conditions in the calvarial region and the loss of periosteum were considered to have a negative effect on bone healing.

In conclusion, in both the control and ovariectomized groups, although there was an improvement in terms of the production of fibrous tissue in the allograft groups, no ossification areas were observed in the defects treated with allografts/allografts + BMPs. In addition, the control group

showed a more positive result for fibrous tissue compared to the ovariectomy group. In addition, OPN, OC, and ON were expressed in the matrix and osteoblasts in areas where ossification was expected, but bone formation was not sufficient. Further studies are necessary to investigate the use of bone regeneration biomaterials in estrogen deficiency conditions.

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Conflict of Interest

The authors have no potential conflict of interest regarding any relationships with other people or organizations that could inappropriately influence this study.

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