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# Calvarial bone defects in ovariectomized rats treated with mesenchymal stem cells and demineralized freeze-dried bone allografts

Healing of calvarial bone defects with stem cells

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## Abstract

The aim of the study was to investigate the ability of a combination of bone marrow mesenchymal stem cells (BM-MSCs) with and without demineralized freeze-dried bone allografts (DFDBAs) to induce bone regeneration in calvarial defects in ovariectomized rats. Critical size defects were filled with a combination of demineralized freeze-dried bone allografts and bone marrow mesenchymal stem cells (BM-MSCs) or BM-MSCs alone. Eight weeks after calvarial surgery, the rats were sacrificed. The samples were analyzed histologically and immunohistochemically. No difference was observed in vascularization between groups C1 (animals with cranial defect only, control group) and O1 (animals with cranial defect only, ovariectomy group). Intramembranous ossification was observed at a limited level in groups C2 (animals with cranial defect with MSCs, control group) and O2 (animals with cranial defect with MSCs, ovariectomy group) compared to C1 and O1. In group C3 (animals with demineralized freeze-dried bone allografts with MSCs, control group), the fibrous structures of the matrix became compact as a result of a bone graft having

been placed in the cavity, but in group O3 (animals with demineralized freeze-dried bone allografts with MSCs, ovariectomy group), the fibrous tissue was poorly distributed between the bone grafts for the most parts. We conclude that the insertion of BM-MSCs enhances bone healing; however, the DFDBA/BM-MSC combination has little effect on overcoming impaired bone formation in ovariectomized rats.

**Key words: bone healing, bone marrow mesenchymal stem cells (BM-MSCs), demineralized freeze-dried bone allografts (DFDBAs), ovariectomy, calvarial defect**

## **INTRODUCTION**

Estrogen deficiency is an important cause of postmenopausal bone loss. It leads to an imbalance in osteoblast and osteoclast number. The effect of estrogen on bone metabolism is mediated by proinflammatory cytokines. In estrogen deficiency conditions, monocytes and macrophages produce large amounts of the cytokines IL-1, IL-6, TNF- $\alpha$ , granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, and prostaglandin-E<sub>2</sub> that stimulate mature osteoclasts, and consequently induce bone resorption (1).

Several studies have suggested that there is a relationship between systemic low bone density and the onset of periodontitis, which is characterized by the loss of connective tissue and alveolar bone, and risk factors, such as genetics, environmental factors, hormone levels, smoking and diabetes, associated with osteoporosis. Authors have also remarked that osteoporosis may have an effect on the progression of periodontitis via the loss of bone mineral density in the maxilla and mandible in postmenopausal women (2,3).

Diminished bone density enhances the destruction of alveolar bone, which complicates bone regenerative procedures (4).

Thus, immune cells directly contribute to bone remodeling, and the bone healing process is known to be negatively affected by estrogen deficiency in elderly women, as estrogen promotes osteoclastic activity. Many experimental studies have also shown delayed wound healing (impaired bone healing) in ovariectomized rats (5-8).

The treatment of bone defects is particularly controversial in the case of osteoporosis. The bone grafting procedure is thought to be the most widely used method to enhance bone regeneration and repair bone defects, but it has certain drawbacks, including risks during collection, hemorrhage, infection, chronic pain, sterilization, storage, especially foreign body reactions, and disease transmission (9,10). Therefore, a more effective treatment is needed to

improve bony defects in osteoporosis. Mesenchymal stem cells (MSCs) have the ability to differentiate into osteoblasts and are available from a wide variety of sources. Tissue regeneration using autologous stem cells to form a suitable scaffold is an alternative to using autografts and allografts (11). The bone-regeneration potential of mesenchymal stem cells has been evaluated in bone defects in animals with or without scaffolds (12,13). Currently, different methods for efficient tissue regeneration are being developed with various combinations of stem cells and scaffolds (14,15).

In light of this information, we hypothesized that treatment with BM-MSCs combined with bone grafts would facilitate bone repair in osteoporotic bone damage conditions. There is little information available about the healing capacity of BM-MSCs used in combination with demineralized freeze-dried bone allografts (DFDBAs) for the treatment of calvarial bone defects. For this reason, we aimed to evaluate the effects of BM-MSCs and allografts on bone healing in ovariectomized rats via the expression of immunohistochemical markers.

## **MATERIALS AND METHODS**

### ***Animals***

A total of 48 female Wistar rats (250-300 g) provided by Scientific Application and Research Centre of Dicle University (Protocol No: 12-DH-53) were used. All of the procedures involved in the experimental protocols were approved by the Animal Ethics Committee of Dicle University (Protocol No. 2011/15). The study was performed in accordance with the Helsinki Declaration and with the permission of the Governmental Animal Protection Committee. Because six animals died, the study was conducted with 42 rats. Whole animals were provided with commercial rat chow and water ad libitum and were maintained on a 12 h light/12 h dark cycle at a temperature of  $22 \pm 1$  °C.

The animals were anesthetized by the intraperitoneal administration of xylazine and ketamine and then subjected to ovariectomy. Ovariectomy was preceded by a 3 cm long midline dorsal skin incision, approximately halfway between the middle of the back and the base of the tail, according to the method described by Pires-Oliveira et al. (16). The animals were monitored for infection.

Thirty days following ovariectomy, all animals were anesthetized for the introduction of calvarial bone defects. After the head hair was shaved, a longitudinal midsagittal skin incision was made to expose the parietal bones, and flaps were retracted in a subperiosteal plane, exposing the parietal bones. A 4-mm-diameter full-thickness round-sized cranial defect

was made unilaterally in the parietal bone using trephine dental drills with saline water irrigation. Care was taken to avoid injury to the dura in all animals (17).

The animals were randomized. The two groups were separated into three subgroups (n=7). Group 1 (control group) was divided into **C1**; animals with cranial defect only, **C2**; animals with cranial defect with MSCs, and **C3**; animals with demineralized freeze-dried bone allografts with MSCs. Group 2 (ovariectomy group) was divided into **O1**; animals with cranial defect only, **O2**; animals with cranial defect with MSCs, and **O3**; animals with demineralized freeze-dried bone allografts with MSCs. Eight weeks after the calvarial surgery, all of the rats were euthanized with an intraperitoneal overdose of ketamine hydrochloride for histological evaluation.

### ***Isolation and culture of rBM-MSCs***

The isolation and culturing of rBM-MSCs (rat bone marrow mesenchymal stem cells) were performed in vitro according to a published protocol (18). MSCs were isolated from the bone marrow of rats. Under sterile conditions, femurs and tibias were excised from each rat, bone marrow cells were isolated by flushing the bone marrow cavity with complete medium (L-DMEM supplemented with 10 % fetal bovine serum [FBS, Gibco/Life Technologies] and 1% penicillin/streptomycin) delivered through a 21 gauge needle. After washing, the isolated bone marrow cells were cultured in complete medium at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for three days. The unattached cells were removed, and the adhered cells were continually cultured until reaching 70-80% confluence. The cells were trypsinized and passaged at a ratio of 1:2 or 1:3. The third-passage rBM-MSCs were pooled and used for characterization and treatment.

### ***Characterization of rBM-MSCs***

Undifferentiated rBM-MSCs were subjected to flow cytometry analysis (FACS Calibur [BD Biosciences, San Jose, CA]). The cell suspension was spun at 1000 RPM for 5 minutes and the supernatant was decanted. The pellet was resuspended in 1X PBS. The cells were counted with a hemocytometer. The desired total number of cells was added to a flow tube (0.5-1 x 10<sup>6</sup> per sample). Wash the cells by adding ~ 1 ml 1X PBS to the flow tube. The cell suspension was spun at 1000 RPM for 5 minutes and the supernatant was decanted. Gently tap the tube to loosen the cell pellet. An appropriate amount of staining buffer (50 ul per 1 x 10<sup>6</sup> cells) was added and Add 1 x 10<sup>6</sup> cells (50 ul) was added to the desired number

of flow tubes. Finally, immunophenotyping analysis was performed for the antigens CD29, CD45, CD54, CD90, CD106, MHC class-I and MHC class-II (BD Biosciences).

### ***In vitro differentiation of rBM-MSCs***

Adipogenic and osteogenic differentiation was performed in vitro according to a published protocol (19). Adipogenic differentiation was performed by incubating rBM-MSCs with L-DMEM supplemented with 0.5 mM isobutyl-methylxanthine, 10<sup>-6</sup>M dexamethasone, 10 µg/ml insulin and 200 µM indomethacin for two weeks. The medium was refreshed every 3–4 days. The formation of intracellular lipid droplets, which indicates adipogenic differentiation, was confirmed by staining with 0.5% oil red O (Sigma-Aldrich, St. Louis, MO). For osteogenic differentiation, the cells were cultured with L-DMEM supplemented with 100 nM dexamethasone, 0.05 µM ascorbate-2-phosphate, and 10 mM β-glycerophosphate for four weeks. After four weeks, osteogenic differentiation was assessed via staining with 2% alizarin red S (pH 4.1–4.3; Fluka, Buchs, Switzerland).

### ***Green fluorescent protein (GFP) labeling of rBM-MSCs***

Mesenchymal stem cells were transfected with pGFP-N (Clontech, Palo Alto, CA) by electroporation (Neon Transfection System, Invitrogen, Carlsbad, CA) following the instructions provided by the manufacturer. After transfection, the cells were cultured with L-DMEM (supplemented with 10% FBS), and the transformed cells were selected with G418 (Gibco/Life Sciences; 200 µg/ml) under standard culture conditions for 48 h. GFP-positive cells were maintained in the same medium supplemented with G418 (200 µg/ml) for three passages. The number of GFP+ cells was monitored by flow cytometry; > 90% of the cells used in the treatment were GFP positive.

### ***Immunohistochemical analysis of GFP+rBM-MSCs in the tissue***

Consecutive sections, each 4 µm thick, were taken from each paraffin-embedded tissue. To detect GFP+rBM-MSCs, an immunofluorescence staining protocol was performed. Slides were deparaffinized with xylene for 5 min twice and rehydrated in a series of graded alcohol solutions (70 to 100%). Endogenous peroxidases were inhibited by incubation with 3% H<sub>2</sub>O<sub>2</sub> in PBS buffer. For antigen retrieval, the samples were heated to 98–99 °C in antigen retrieval buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) and incubated for 30 min in a pressurized vessel. Nonspecific staining was blocked with a mixture of 1.5% serum in PBS for 30 min at room temperature, and the sections were incubated with a mouse

monoclonal anti-GFP antibody (SC-9996) at a 1:50 dilution for 1 h at room temperature. After incubation with appropriate fluorophore-conjugated secondary antibodies, the sections were covered with mounting medium containing DAPI (Santa Cruz, Heidelberg, Germany). The cells were investigated under a fluorescence microscope (Leica DMI 4000B, Wetzlar, Germany).

### ***Light microscopy***

Calvarial tissues were removed and fixed in 10% neutral buffered formaldehyde solution for 48 h and then decalcified in 10% ethylene diaminetetraacetic acid (EDTA) prepared in 0.1 M Tris-HCl buffer (pH 7.4) for 14 days. The tissues were subsequently dehydrated, cleared and embedded in paraffin blocks. Five-micrometer-thick sections were cut from these blocks and stained using Gomori's method to determine ossification.

### ***Immunohistochemical staining***

Immunohistochemical investigations were performed on tissue preparations embedded in paraffin and by using a Zymed Histostain Plus Bulk kit (code: 85-9043, Histostain Plus Bulk Kit, Zymed, South San Francisco, CA, USA) and streptavidin-peroxidase (Akbalik and Ketani 2013) (19). Briefly, sections were deparaffinized, rehydrated and incubated for 15 min in 3% H<sub>2</sub>O<sub>2</sub> in methanol. After the sections were washed in phosphate-buffered saline (PBS), antigen retrieval was performed by boiling in 0.01 M citric buffer pH 6.0 for 30 min at 95 °C using a water bath and by cooling for 20 min prior to immunostaining. Sections were then washed in PBS and incubated in protein blocking solution (Ultra V Block) for 10 min at room temperature to prevent nonspecific binding. Subsequently, the preparations were incubated with primary antibodies for 20 h at +4°C (Table 1)

<b><i>Antibody</i></b>	<b><i>Clone, isotype</i></b>	<b><i>Host</i></b>	<b><i>Cellular localization</i></b>	<b><i>Dilution</i></b>	<b><i>Supplier (Catalog no.)</i></b>
<i>Osteopontin/OPN (human)</i>	<i>AKm2A1, monoclonal IgG</i>	<i>Mouse</i>	<i>Cytoplasmic</i>	<i>1:200</i>	<i>Santa Cruz (sc-21742)</i>
<i>Osteonectin/Sparc (human)</i>	<i>H-90, polyclonal IgG</i>	<i>Rabbit</i>	<i>Cytoplasmic</i>	<i>1:200</i>	<i>Santa Cruz (sc-25574)</i>
<i>Osteocalcin (human)</i>	<i>FL-100, polyclonal IgG</i>	<i>Rabbit</i>	<i>Cytoplasmic</i>	<i>1:200</i>	<i>Santa Cruz (sc-30044)</i>

**Table 1.** Details of antibodies used.

After being washed in PBS, the sections were incubated with biotinylated secondary antibodies for 20 min at room temperature and washed in PBS. Subsequently, the preparations were incubated in streptavidin peroxidase conjugate for 20 min at room temperature and were then washed with PBS. To visualize the reaction, the sections were treated with 3,3'-diaminobenzidine (DAB) for 5-15 min. After the reaction developed, the sections were counterstained with Gill's hematoxylin, dehydrated through an alcohol series, cleared in xylene, and finally mounted in entellan. Negative controls were used for the confirmation of the staining. As a negative control, the primary antibodies used for staining were replaced with PBS. OPN, OC and ON expression in bone tissue was examined microscopically at x200 magnification. Immunohistochemical staining results were evaluated semi quantitatively. The intensity of positive staining was defined as + weak, ++ medium, +++ strong, + / ++ weak to moderate, and ++ / +++ moderate to strong. The slides were examined and photographed using a Nikon Eclipse E400 (Nikon, Tokyo, Japan) microscope equipped with a digital camera (Nikon Coolpix-4500).

### ***Statistical analysis***

Kruskal Wallis and Mann-Whitney U tests were used as nonparametric statistical analyses. Values of  $p < 0.05$  were considered statistically significant.

## **RESULTS**

### ***Histological findings in calvarial bone tissue***

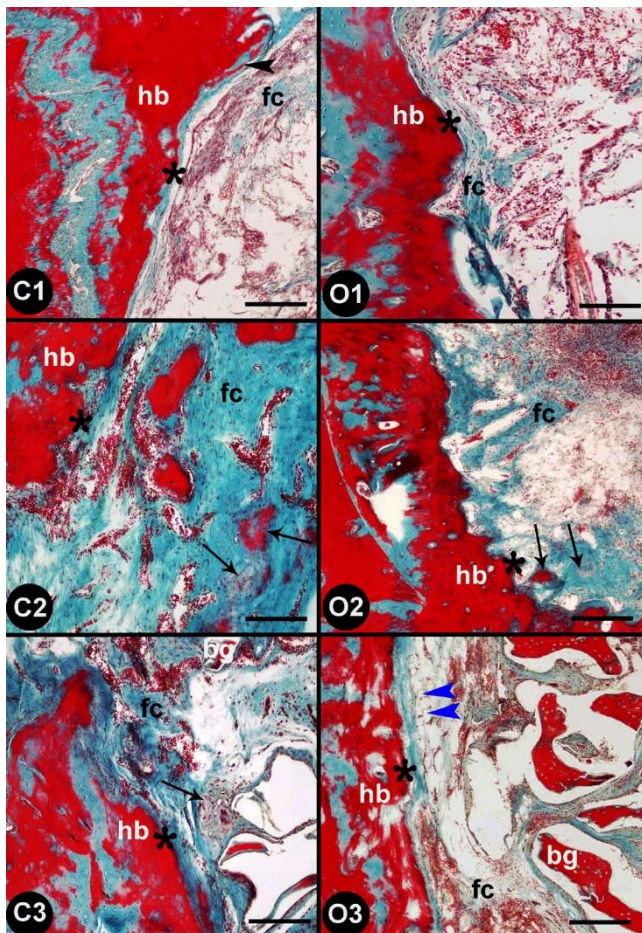
In C1 group (animals with cranial defect only, control group), structures (bone spicules) characterized by intramembranous ossification were observed along the border of the cavity. Furthermore, fibrous tissue was abundant in the cavity and vascularization (angiogenesis) had occurred in some areas. In O1 group (animals with cranial defect only, ovariectomy group), intramembranous ossification was observed neither in the cavity nor along the border of the cavity, and fibrous tissue was not diffusely distributed, but cellular structures were markedly abundant. No significant revascularization was observed between control (animals with cranial defect only) and ovariectomy group rats (animals with cranial defect only).

O1 (animals with cranial defect only, ovariectomy group), intramembranous ossification and osteogenesis had occurred locally, and fibrous tissue was diffuse and regularly distributed. Furthermore, vascularization was significantly increased. In group O2 (animals with cranial defect with MSCs, ovariectomy group), intramembranous ossification



was observed at a limited level and was localized to the border of the cavity. Furthermore, fibrous tissue was irregularly distributed in only some parts of the cavity. The vascularization in groups C2 (animals with cranial defect with MSCs, control group) and O2 (animals with cranial defect with MSCs, ovariectomy group) did not differ.

When compared to those in the other control groups, the fibrous structures of the matrix in group C3 (animals with demineralized freeze-dried bone allografts with MSCs, control group) displayed a compact structure as a result of a bone graft having been placed in the cavity, and these structures were also tightly adhered to the border of the cavity. Intramembranous ossification was observed between the portions of the bone graft. In group O3 (animals with demineralized freeze-dried bone allografts with MSCs, ovariectomy group), ossification was not observed. The fibrous tissue showed a weak distribution between the bone grafts and along the border of the cavity, in a strip-like formation along the latter. Furthermore, in contrast to those in the other groups, the stem cells had differentiated into adipose cells along the border of the cavity and were present in the form of infiltrating cells (Figure 1).



**Figure 1.** Histological analysis of the defect areas in the rat calvaria bone in the control (C1, C2, and C3) and ovariectomy (O1, O2, and O3) groups. The defect was animals with cranial defect only, as shown in Figures C1

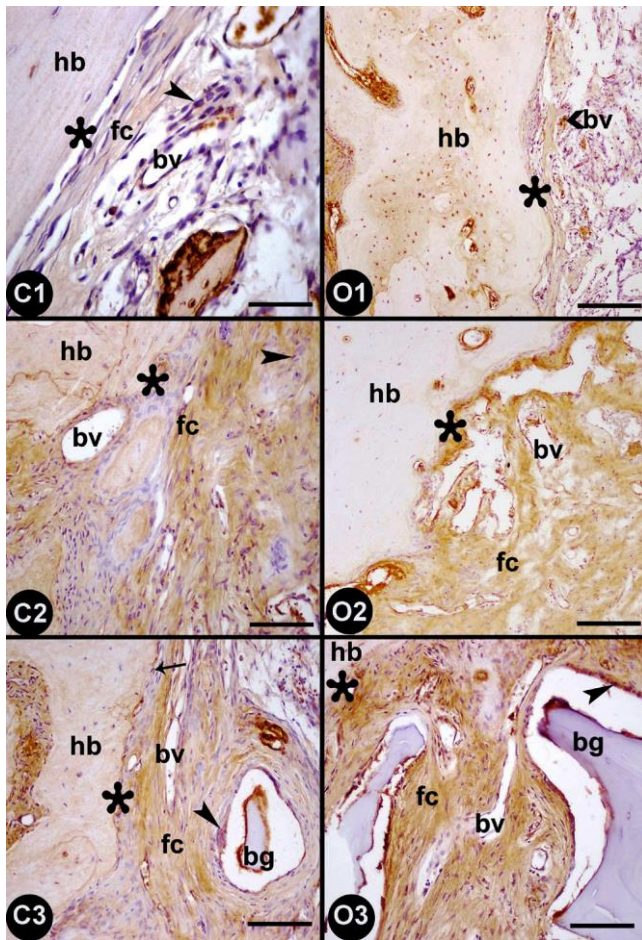
and O1, and the defect areas were treated with stem cells (C2, O2) and stem cells+bone grafts (C3, O3); **hb**: host bone, **fc**: fibrous connective tissue, **bg**: bone graft, **asterisk**: interface between host bone and defect, **arrow**: ossification areas, **black arrowhead**: bone spicule, **blue arrowhead**: adipocytes. Gomori's staining method. Scale bars: 100  $\mu\text{m}$  (C1-C2 and O1-O2) and 250  $\mu\text{m}$  (C3-O3).

### ***Immunohistochemistry for osteopontin (OPN)***

In C1 group (animals with cranial defect only, control group), the osteoprogenitor cells along the border of the cavity were not immunoreactive for OPN. However, while the osteoblasts and extracellular matrix in the areas of intramembranous ossification along the border of the cavity showed weak OPN immunoreactivity, the newly formed blood vessels in the cavity were positively stained for OPN (Figure 2-C1). In O1 group (animals with cranial defect only, ovariectomy group), the normal bone tissue showed positive immunoreactivity for OPN, but OPN immunoreactivity was limited to the blood vessels in the newly formed tissue in the cavity (Figure 2-O1).

In group C2 (animals with cranial defect with MSCs, control group), similar to group C1, the osteoprogenitor cells along the border of the cavity did not show OPN immunoreactivity. Although the osteoblasts in the cavity presented weak OPN staining, OPN expression in the blood vessels was increased (Figure 2-C2). In group O2 (animals with cranial defect with MSCs, ovariectomy group), staining for OPN was observed neither in the osteoprogenitor cells along the border of the cavity nor in the osteoblasts in the cavity, but the extracellular matrix and blood vessels were positive for OPN (Figure 2-O2).

In group C3 (animals with demineralized freeze-dried bone allografts with MSCs, control group), the osteoprogenitor cells along the border of the cavity and the newly formed osteoblasts and extracellular matrix in the periphery of the bone graft demonstrated moderate OPN immunoreactivity, whereas the blood vessels showed strong OPN immunoreactivity (Figure 2-C3). In group O3 (animals with demineralized freeze-dried bone allografts with MSCs, ovariectomy group), the newly formed osteoblasts in the periphery of the bone graft presented moderate immunoreactivity for OPN, whereas the staining of the blood vessels was found to be similar to that observed in group C3 (Figure 2-O3).



**Figure 2.** Osteopontin expression in the rat calvaria bone defect areas in the control (C1, C2, and C3) and ovariectomy (O1, O2, and O3) groups. The formation of the groups is shown in figure 1; **hb**: host bone, **fc**: fibrous connective tissue, **bv**: blood vessel, **bg**: bone graft, **asterisk**: interface between host bone and defect, **arrow**: osteoprogenitor cell, **arrowhead**: osteoblast. Scale bars: 25  $\mu\text{m}$  (C1), 50  $\mu\text{m}$  (C2-C3-O3), and 100  $\mu\text{m}$  (O1-O2).

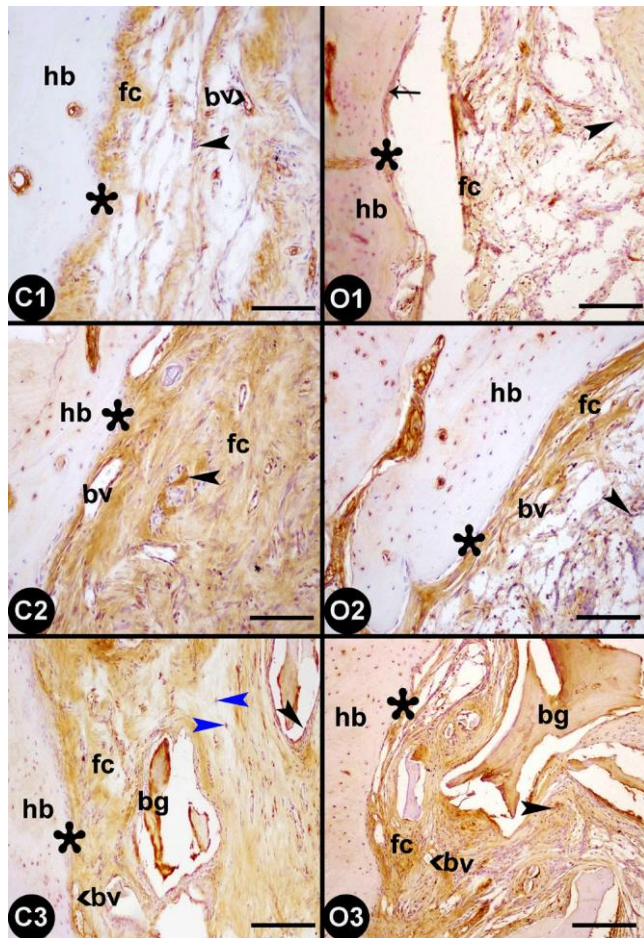
### ***Immunohistochemistry for osteocalcin (OC)***

In C1 group (animals with cranial defect only, control group), the osteoprogenitor cells along the border of the cavity did not show immunoreactivity for OC, but the osteoblasts presented weak OC immunoreactivity. The extracellular matrix in the border of the cavity showed moderate staining, and the blood vessels stained positively for OC (Figure 3-C1). In O1 group (animals with cranial defect only, ovariectomy group), the osteoprogenitor cells also showed OC immunoreactivity, and when compared to that of group C1, the OC immunoreactivity of the osteoblasts and extracellular matrix was weaker (Figure 3-O1).

Compared with that in the control group, the OC immunoreactivity of the osteoprogenitor cells along the border of the cavity in group C2 (animals with cranial defect with MSCs, control group) was found to be negative, but the staining of the extracellular

matrix and the blood vessels localized to the border of the cavity and the cavity was more intense. Furthermore, the osteoblasts in the cavity displayed weak immunoreactivity for OC (Figure 3-C2). In group O2 (animals with cranial defect with MSCs, ovariectomy group), the osteoprogenitor cells did not show any OC immunoreactivity, and the osteoblasts displayed weak OC immunoreactivity. The extracellular matrix of the border of the cavity stained strongly for OC and the staining in the cavity was observed to be weak. The OC immunoreactivity of the blood vessels was also determined to be strong (Figure 3-O2).

In group C3 (animals with demineralized freeze-dried bone allografts with MSCs, control group), the newly formed osteoblasts in the periphery of the bone graft displayed OC immunoreactivity ranging from moderate to strong, whereas the osteocytes in the ossified areas displayed weak OC immunoreactivity. The OC immunoreactivity of the extracellular matrix was more homogenous than that in the other groups. Blood vessels also stained positively for OC (Figure 3-C3). In group O3 (animals with demineralized freeze-dried bone allografts with MSCs, ovariectomy group), the osteoprogenitor cells displayed negative staining and the osteoblasts and extracellular matrix were stained moderately. However, the blood vessels displayed strong immunoreactivity for OC (Figure 3-O3).



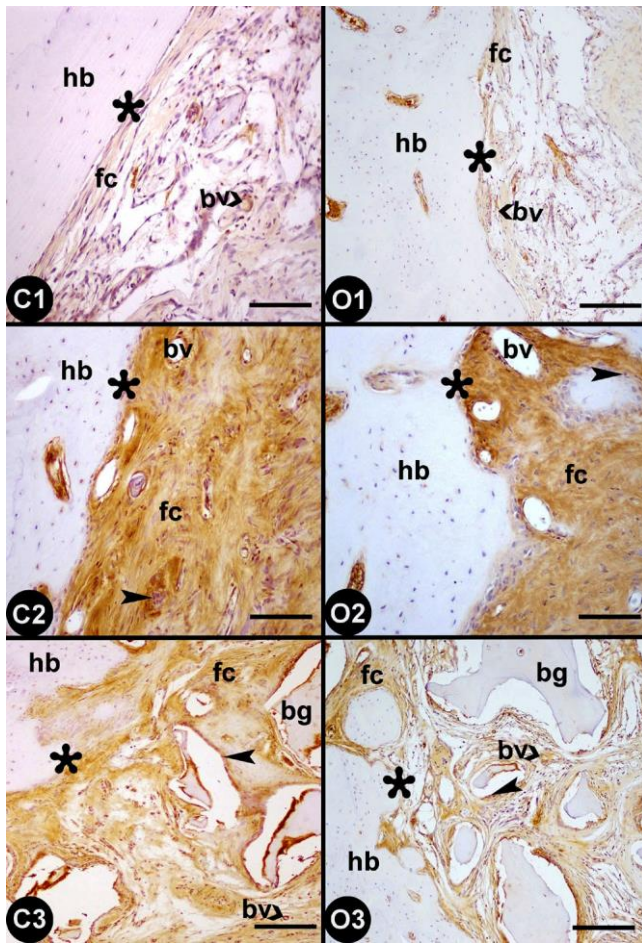
**Figure 3.** Osteocalcin expression in the rat calvaria bone defect areas in the control (C1, C2, and C3) and ovariectomy (O1, O2, and O3) groups. The formation of the groups is shown in Figure 1; **hb:** host bone, **fc:** fibrous connective tissue, **bv:** blood vessel, **bg:** bone graft, **asterisk:** interface between host bone and defect, **arrow:** osteoprogenitor cell, **black arrowhead:** osteoblast, **blue arrowhead:** osteocyte. Scale bars: 50  $\mu\text{m}$  (C1-C2-O2), and 100  $\mu\text{m}$  (C3-O1-O3).

### ***Immunohistochemistry for osteonectin (ON)***

In C1 group (animals with cranial defect only, control group), while the osteoprogenitor cells along the border of the cavity and the osteoblasts in the cavity stained negatively for ON, the extracellular matrix showed weak immunoreactivity, and the blood vessels showed moderate immunoreactivity for ON (Figure 4-C1). Group O1 (animals with cranial defect only, ovariectomy group) showed staining results similar to those of group C1, but the extracellular matrix stained weakly (Figure 4-O1).

In group C2 (animals with cranial defect with MSCs, control group), the osteoprogenitor cells along the border of the cavity were stained negatively, and the osteoblasts in the cavity showed weak ON expression. In contrast, the extracellular matrix and blood vessels were strongly stained (Figure 4-C2). In group O2 (animals with cranial defect with MSCs, ovariectomy group), the osteoprogenitor cells along the border of the cavity did not stain, the osteoblasts in the cavity showed weak immunoreactivity for ON. The extracellular matrix and blood vessel findings were similar to those of group C2 (Figure 4-O2).

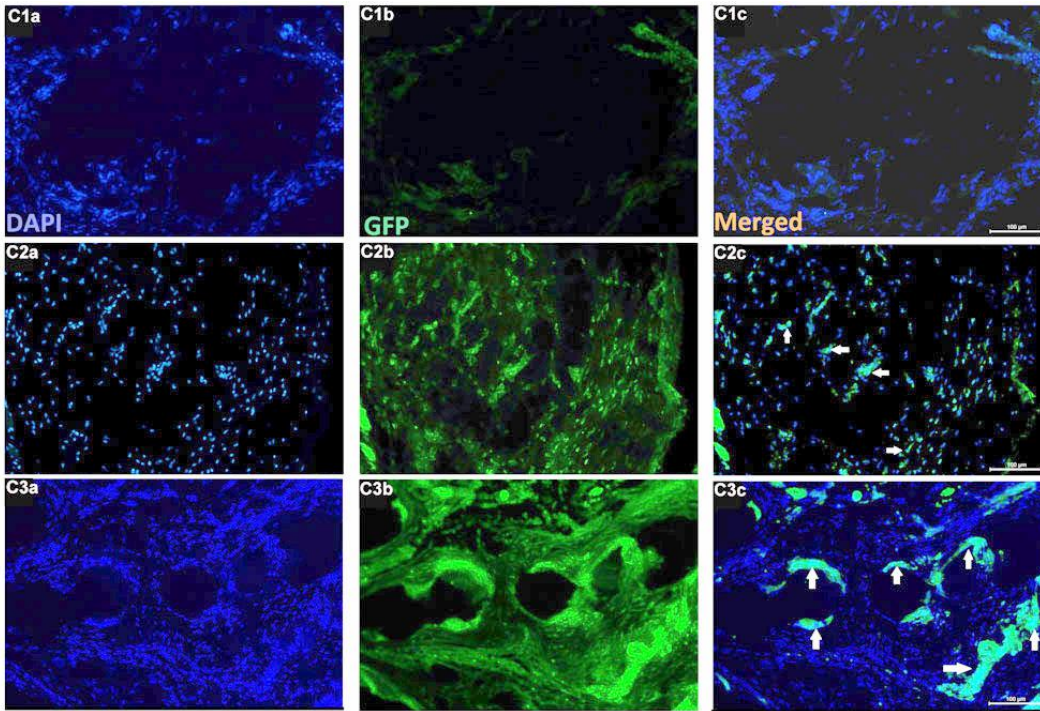
In group C3 (animals with demineralized freeze-dried bone allografts with MSCs, control group), the osteoprogenitor cells along the border of the cavity did not express ON, but the newly formed osteoblasts in the periphery of the bone graft displayed moderate staining. In contrast, the extracellular matrix and blood vessels displayed strong ON expression (Figure 4-C3). In group O3 (animals with demineralized freeze-dried bone allografts with MSCs, ovariectomy group), the osteoprogenitor cells showed no staining and the osteoblasts in the periphery of the bone graft displayed moderate ON expression. It was observed that adipose cells had formed in the cavity, yet these cells displayed no immunoreactivity. However, the extracellular matrix and blood vessels showed strong ON immunoreactivity (Figure 4-O3).



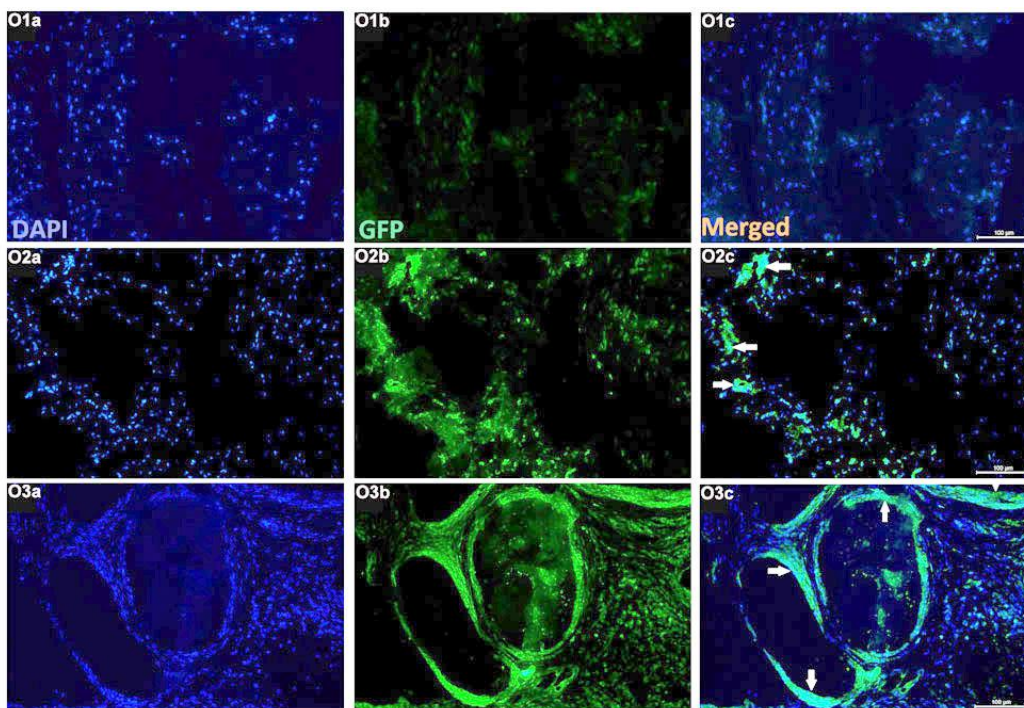
**Figure 4.** Osteonectin expression in the rat calvaria defect areas in the control (C1, C2, and C3) and ovariectomy (O1, O2, and O3) groups. The formation of the groups is shown in Figure 1; **hb**: host bone, **fc**: fibrous connective tissue, **bv**: blood vessel, **bg**: bone graft, **asterisk**: interface between host bone and defect, **arrowhead**: osteoblast. Scale bars: 50  $\mu\text{m}$  (C1-C2-O2) and 100  $\mu\text{m}$  (C3-O1-O3).

#### ***Findings of green fluorescent protein (GFP) labeling of RBM-MSCs***

In the control group, no immune reaction was observed for GFP in animals with cranial defect only, without MSCs, whereas GFP and cells (arrows) were observed in C2 (animals with cranial defect with MSCs, control group) and C3 (animals with demineralized freeze-dried bone allografts with MSCs, control group). Animals with demineralized freeze-dried bone allografts in the control group, the MSCs transplanted with the graft show the new tissue formation (Figure 5). In the ovariectomy group, no immune reaction was observed for GFP in the non- MSCs group (O1), whereas GFP and cells (arrows) were observed in O2 (animals with cranial defect with MSCs, ovariectomy group) and O3 (animals with demineralized freeze-dried bone allografts with MSCs, ovariectomy group). MSCs transplanted with the graft in O3 were involved in new tissue formation (Figure 6).

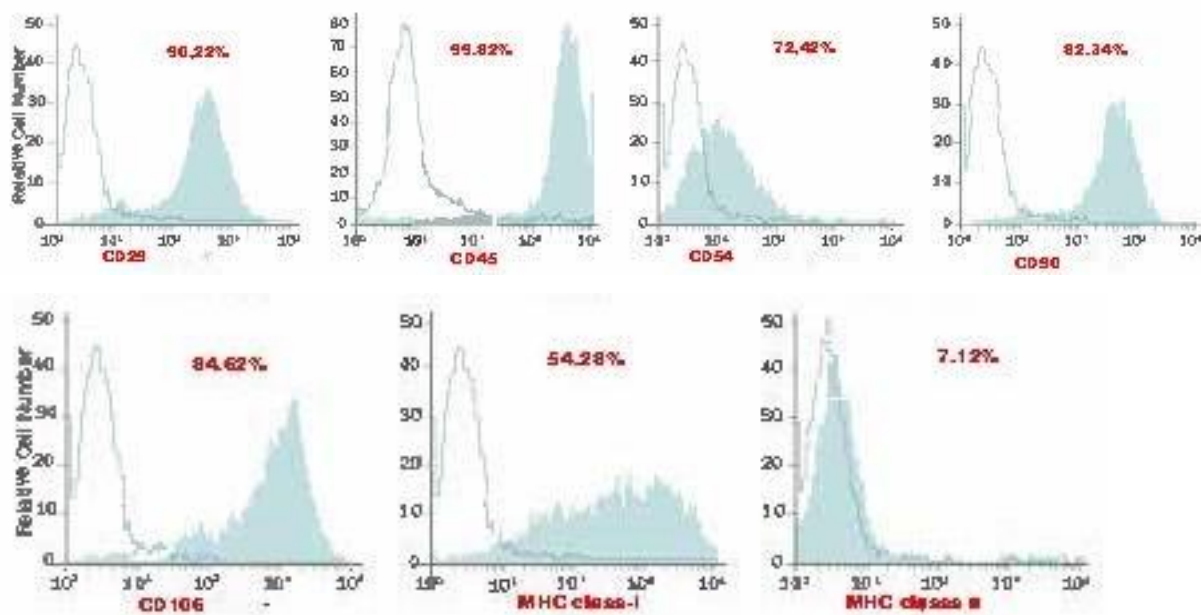


**Figure 5.** GFP fluorescence after immunostaining of the tissue sections from the control group. **C1**; animals with cranial defect only, **C2**; animals with cranial defect with MSCs, **C3**; animals with demineralized freeze-dried bone allografts with MSCs. Immune reaction (white arrows) was observed in GFP and cells belonging to C2 and C3 groups. MSCs transplanted in C3 with the graft showed new bone tissue formation. Scale bars:100  $\mu$ m.



**Figure 6.** GFP fluorescence after immunostaining of the tissue sections from the ovariectomy group. **O1**; animals with cranial defect only, **O2**; animals with cranial defect with MSCs, **O3**; animals with demineralized freeze-dried bone allografts with MSCs. Immune reactions (white arrows) were observed in GFP and cells belonging to O2 and O3 groups. Graft transplanted MSCs in O3 appeared to participate in new bone tissue formation. Scale bars:100  $\mu$ m

In addition, the cell expression of each marker is given in Table 2 for fluorescence intensity.



**Table 2.** Fluorescence intensity. Numbers in panels represent mean fluorescent intensity of the cells expressing each marker.

### Statistical findings

Groups	p	Parameter	n	Average Rank	Different (p<0.05) from factor nr
(1) C1	0,0001	1	7	7,50	(3)
(2) C2			7	7,50	(3)
(3) C3			7	18,00	(1)(2)
(1) C1	0,0109	2	7	7,36	(3)
(2) C2			7	9,71	(3)
(3) C3			7	15,93	(1)(2)
(1) C1	ns	3	7	11,00	ns
(2) C2			7	11,00	
(3) C3			7	11,00	
(1) C1	0,0007	4	7	4,86	(2)(3)
(2) C2			7	16,57	(1)(3)
(3) C3			7	11,57	(1)(2)
(1) O1	ns	1	7	11,00	ns
(2) O2			7	11,00	
(3) O3			7	11,00	
(1) O1	0,001	2	7	7,50	(3)
(2) O2			7	7,50	(3)



(3) O3			7	18,00	(1)(2)
(1) O1	ns	3	7	11,00	ns
(2) O2			7	11,00	
(3) O3			7	11,00	
(1) O1	0,0004	4	7	4,00	(2)(3)
(2) O2			7	15,00	(1)
(3) O3			7	14,00	(1)

**Table 3.** Comparisons of the parameters within the groups (ns; non-significant ;  $p>0,05$ )

1; osteoprogenitor cells, 2; osteoblasts, 3;osteocytes, 4;extracelluler matrix

Parameter	Groups	p	Groups	p	Groups	p
1	C1	ns	C2	ns	C3	0,001
	O1		O2		O3	
2	C1	0,007	C2	0,002	C3	ns
	O1		O2		O3	
3	C1	ns	C2	ns	C3	ns
	O1		O2		O3	
4	C1	0,002	C2	ns	C3	0,015
	O1		O2		O3	

**Table 4.** Comparisons of the parameters between the control and test groups (ns; non-significant ;  $p>0,05$ ) 1; osteoprogenitor cells, 2; osteoblasts, 3; osteocytes, 4; extracelluler matrix

When osteoprogenitor cells were evaluated between control (animals with demineralized freeze-dried bone allografts) and ovariectomy groups (animals with demineralized freeze-dried bone allografts), a significant statistical difference was found (Table 3 and 4). When osteoblast cells were evaluated between control and ovariectomy groups, the difference between C1 (animals with cranial defect only, control group)-O1 (animals with cranial defect only, ovariectomy group) and C2 (animals with cranial defect with MSCs, control group) -O2 (animals with cranial defect with MSCs, ovariectomy group) groups was found to be statistically significant (Table 3 and 4). And, when the extracellular matrix was evaluated, the difference between C1(animals with cranial defect only, control group)-O1 (animals with cranial defect only, ovariectomy group) and C3 (animals with demineralized freeze-dried bone allografts with MSCs, control group)-O3 (animals with demineralized freeze-dried bone allografts with MSCs, ovariectomy group) groups was found to be statistically significant (Table 3 and 4).

## DISCUSSION

We aimed to investigate the healing capacity of BM-MSCs used with DFDBAs for the treatment of Calvarial bone defects, assuming that BM-MSC combined with bone grafts would facilitate bone repair under conditions of osteoporotic bone injury. Therefore, the effects of BM-MSCs and allografts on bone healing in ovariectomized rats were concluded by evaluating immunohistochemical results.

Sethi et al. (20) evaluated and interpreted both clinically and radiographically by studying the changes post 1 week, 1 month, 3 months, and 6 months, respectively. It was stated that there was evidence of trabecular formation and calcification. They concluded that platelet-rich plasma-enriched DFDBA was a superior inoculant in terms of other available inoculants in patients (20). In this experimental study, we observed the successful results of demineralized freeze-dried bone allografts. Called “Mesenchymal Stem Cell Chondrocytes technique” was used to reconstruct a 15 mm massive femoral defect (approximately 50% of rat femur shaft length) in an experimental study. According to their results, considering the high repairability and the excellent biomechanical forces of the repaired femora, they concluded that the reconstruction of the large bone defect may be possible (21).

In an experimental study in which 8 mm defect was applied to one of the groups in which decalcified freeze-dried bone allograft was applied, statistically significant results were obtained when compared with the other groups (22). In an experimental study, ovariectomy was performed methodologically as in our study. It has been stated that limited proteins are known to be involved in the subsequent stages of bone formation and maturation in the proteomes of animals (23)

As a result of these cellular changes, osteogenesis becomes impaired and the bone formation period is insufficient to repair the increased bone destruction in estrogen deficiency-related osteoporosis (24,25). He et al. (8) also found that the femurs of mice with OVX-induced osteoporosis showed impaired angiogenesis, osteogenesis, and remodeling in their study (8). Calciolari et al. (23) observed immature bone formation both in OVX and control calvarial CSDs over a 30-day period (23). Stockmann et al. (26) also observed a similar result during the early stage of bone healing on the 30th day. There were no differences between the test and control groups, but at 60 days, new bone formation was achieved in the MSC group; however, significant pig calvarial bone regeneration was measured at day 90 (26). In our study, we observed intramembranous ossification in animals with cranial defect only of the healthy control rats, after 60 days. Consistent with other OVX-induced osteoporosis rat model studies, we did not observe intramembranous ossification

either in the cavity or along the border of the cavity of animals with cranial defect only with ovariectomy group after 8 weeks. However, in cortical bones, such as the calvarial bone, the healing process is slower than that in cancellous bone, with a poorer blood supply and less bone marrow; thus, much more than 60 days might be necessary for mature bone formation.

Recently, it has been stated that the advantages and disadvantages of autogenous, allogeneic, xenogenic and alloplastic materials have gained meaning in periodontal treatment. (27). Kurkalli et al. (28) concluded that the placement of the osteogenic composite in a large deficient area of the parietal bone of the skull of rats resulted in a large demineralized bone matrix particle structure, fully reconstituted hematopoietic microenvironment within thirty days, and a well-integrated normal smooth bone. (28). Intini et al. (29) found that demineralized freeze-dried bone was not effective enough to induce bone formation in rat calvaria 8 weeks after surgery (29). Caplanis et al. (30) did not find any histological effects on bone formation in canine defects treated with demineralized freeze-dried bone allografts after 4 weeks (30). Bertolai et al. (31) have successfully used freeze-dried bone as a graft material in the treatment of maxillary atrophy, as in our study. (31).

In the meta-analysis, the authors concluded that MSCs improved bone regeneration, and it is preferable to use MSCs with an appropriate scaffold. Koob S et al. mentioned that mesenchymal stem cells (MSCs) enhance bone formation in calvarial defects (32). Moreover, in large animal studies, autologous MSCs transplanted alone or in combination with different bone substitutes were found to significantly increase bone formation in critical-sized defects (9). Kandal et al. suggested that the combined use of demineralized bone matrix with MSCs increases the osteoinductive responses in the frontal bone of rats. They suggested that this combination can provide enhanced craniofacial bone reconstruction results at the end of 12 weeks (33). In another experimental study, the utilization of mesenchymal stem cells with platelet-rich plasma and synthetic bone substitutes was found to enhance new bone formation (34). Semyari et al. observed the overall recovery of a bony defect treated with mesenchymal stem cells on different scaffolds with membranes after 8 weeks of calvarial surgery in rabbits (35).

Osteopontin has been implicated as being an important factor in bone remodeling. Research suggests it plays a role in attaching osteoclasts to the mineral matrix of bones and in the regulation of normal mineralization within the extracellular matrices of bones and teeth. Osteocalcin and osteonectin are not observed during initial crystal formation but are seen in the later stages of bone formation (36). Therefore, we chose these three bone markers to assess the bone formation activity. We also observed the effect of estrogen

deficiency on bone healing via the expression of these bone markers, as there are few studies on this issue. In our study, the osteoblasts and extracellular matrix staining for these proteins was weak in animals with cranial defect only in the control and ovariectomy groups without MSCs. Histological and immunohistochemical evaluation revealed that the findings obtained for the extracellular matrix, ossification and blood vessels were similar between groups O2 (animals with cranial defect **with MSCs**, ovariectomy group) and C2 (animals with cranial defect with MSCs, control group). Thus, estrogen deficiency may not influence the expression of bone markers, which is consistent with the findings of Tera Tde et al. (36). According to the results of this study, more new bone formation was observed in defects treated with MSCs alone than was observed in animals with cranial defect only. However, the combination of DFDBA/MSCs in animals with demineralized freeze-dried bone allografts of ovariectomy group was not as effective on compact intramembranous ossification at the end of the 8 weeks as expected.

Akita et al. found that there were no significant differences in 4 mm cranial rat defects among groups treated with MSCs only or MSCs with FGF-BMP at 8 weeks after transplantation (17). Similar to our study, Wang et al. created bony defects in ovariectomized rabbits and treated the defects with mesenchymal stem cells/decalcified bone matrix. Three months later, the authors concluded that the defect treatment was ineffective for the osteoporotic state and that the bone formation was significantly worse than that of the control group (37).

The properties of scaffolds are important for the migration, proliferation and differentiation of living cells during bone regeneration. In this study, the combination of human (DFDBAs) and animal (rBM-MSCs) scaffolds may be biologically incompatible. However, the osteogenic potential of the DFDBA may be diminished during the production process. Additionally, bone healing may have been negatively affected by the absence of a collagen membrane in the scaffold.

There are some limitations to the current study. It would be better to evaluate bone formation with histomorphometric parameters than histochemical staining. In addition, a collagen membrane may be used with the scaffold for 12 weeks to achieve complete bone regeneration. In conclusion, stem cell therapy could be an option to manage impaired bone formation. However, to achieve compact bone formation it is preferential to use proper scaffolds loaded with BM-MSCs for the appropriate healing time. Because there are limited studies in this field, further studies are required to investigate the proliferation and

differentiation of MSCs in different scaffolds for the enhancement of impaired bone formation.

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