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Effects of alloplastic graft material combined with a topical ozone application on calvarial bone defects in rats

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Background: *This study presents the evaluation of the damage in the bone tissue resulting from a calvarial defect in rats and the efficiency of exposure to an ozone application with an alloplastic bone graft on the calvarial bone damage.*
Materials and methods: *Wistar male rats (n = 56) were divided into four groups: a control group (n = 14), defect and ozone group (n = 14), defect and graft group (n = 14), and defect, graft, and ozone group (n = 14). Under anaesthesia, a circular full-thickness bone defect was created in all groups, and the experimental groups were further divided into two sub-groups, with 7 rats in each group sacrificed at the end of the 4th and 8th weeks. Bone samples were dissected, fixed in 10% formalin solution, and decalcified with 5% ethylene-diamine-tetraacetic acid (EDTA). After the routine follow-up on tissues, immunostaining of osteopontin and osteonectin antibodies was applied to sections and observed under a light microscope.*
Results: *The control group exhibited osteopontin and osteonectin expression in fibroblasts and inflammatory cells at the end of the 4th week with an acceleration at the 8th week. Ozone administration elucidated new trabecular bone formation by increasing osteoblastic activity. Lastly, our observations underscore that a combination of allograft and ozone application increased the osteoblast, osteocyte, and bone matrix development at the 4th and 8th weeks.*
Conclusions: *Exposure to an ozone application with an alloplastic bone graft on calvarial bone damage may induce osteoblastic activity, matrix development, mature bone cell formation, and new bone formation in rats.* (Folia Morphol 2020; 79, 3: 528–547)

Key words: calvarial defects, osteopontin, osteonectin, rat, immunohistochemistry

INTRODUCTION

Calvarial bone defects are associated with trauma, pathology, and non-union of a fracture and represent a significant clinical problem [13, 72]. The osteogenic potential of autograft and allografts is known in the field of orthopaedics, plastic surgery, and oral and maxillofacial surgery [41, 67]. Autograft is the current gold standard treatment for bone grafting; however,

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The experimental part of the study was conducted at The Experimental Research Centre of Dicle University, 21280, Diyarbakır, Turkey. Histopathological examinations of the tissues were evaluated in The Histology and Embryology Laboratory in Medical School of Dicle University, 21280, Diyarbakır, Turkey.

it is limited by the available volume of graft material, donor site morbidity, and unpredictable bone resorption [24, 27]. Allografts are good alternatives to bridge defects, but the risk of disease transmission and adverse host immune reactions limits the use of allografts. Therefore, improved strategies are urgently needed to better treat craniofacial bone defects [25, 48].

Antioxidants applied in defect-induced bone injuries alone do not prevent bone loss. It has also been reported that the effect of antioxidants is more prominent, especially after graft application. Experimental studies have indicated that osteoprogenitor cell activity is induced when the graft is applied for 4–6 weeks, depending on the type of graft material [66]. Several types of graft material are available. For instance, allografts are provided from donors of different genetic characteristics in the same species [26]. There are limitations in the convenience of graft types, such as the shortage in the usability of autografts, inappropriate characteristics of allografts, and risk of disease transmission in xenografts. For all these reasons, researchers have developed an approach of synthetic graft material use in bone defects.

In an experimental study, it has been reported that hyperbaric oxygen and ozone are equally effective in bone development in rats induced with calvarial defects [32]. In a clinical study with topical ozone application, alveolar bone healing is accelerated using postoperative long-term systemic ozone application [21]. Preparing a superhydrophilic titanium implant functionalised with ozone gas can modulate osteoconductivity and inhibit the inflammatory response to titanium implants. This superhydrophilic surface has been proposed to be useful as an endorsed implantable biomaterial and as a biomaterial for implantation in other tissues [64]. The effect of ozone treatment in combination with autogenous bone grafts on bone healing in rat calvaria has been investigated. Exposure to an ozone application increases new bone formation by autogenous bone graft in the rat calvarial defect model [45].

Clinically, the importance of ossification and fracture healing is well known in rats. It has been reported that exposure to ozone application provides good healing in diabetes [1], oral mucositis [5], surgical treatment of peri-implantitis [30], bone regeneration [20, 70], and osteogenesis with calvarial defects in experimental models [59]. Many rodent experimental animals, including rats, have been used to design bone defect models [7, 12, 50, 53]. Anatomically, these rodent models were studied in the calvarium, femur, mandible, and spine. The defect of the calvarium and femur in rats is defined as regions that do not require fixation for stabilisation. The calvarial defects are histologically considered an intramembranous model. The bone matrix is considered to be less applicable to biomaterials. In other words, it has been suggested that the bone matrix is more suitable for endochondral bone model studies. For this reason, calvarial defect models should be designed according to appropriate strategies [23]. A smaller size calvarial defect may recover spontaneously; thus, this was taken into account for the purposes of the study. As a result, an 8 mm calvarial defect was created [36].

Osteonectin protein is involved in the upregulation of mineralisation in osteoblast cells, and cell adhesion in osteoclast cells. Osteonectin is a single chain acidic glycoprotein that is rich in cysteine, which is synthesised by the cells of an osteoblastic lineage that is abundantly expressed in bones undergoing active remodelling. It is connected with type I collagen, calcium, and hydroxyapatite and therefore prevents mineralisation [34]. Osteopontin (also called BSP-1) protein located in the extracellular bone matrix is non-collagenous and acts in bone cell functions [38]. Osteopontin participates in the upregulation of cell adhesion and differentiation in osteoblast cells, and concomitantly in the upregulation of cell adhesion and bone resorption in osteoclast cells [51]. In a study on two different phases of bone belonging to rats, the distribution of N-linked glycoproteins was determined [28]. In another paper, it was noted that bone sialoprotein binds firmly to collagen type I and hydroxyapatite crystals, revealing that sialoprotein and osteopontin are involved in mineralisation [54, 68]. Bone sialoprotein is closely related to the nucleation of amorphous calcium phosphate [71]. In addition, mice with decreased levels of osteopontin in the bone were reported to be more prone to fractures, depending on the varying amount of calcium bound by osteopontin [65]. Bone, cartilage, dentin, cement, vascular tissue, and epithelial tissue cells express osteopontin [61]. Osteoclasts, differentiated osteoblasts, and osteocytes release osteopontin and act in osteoclast adhesion, resorption, formation, and migration [60]. It is known that in remodelling bone tissue, cells secrete osteopontin. In addition, osteopontin also contributes to the modulation of the inflammatory phase, acting as a pro-inflammatory cytokine [63].

In this study, we aimed to show the expression of osteonectin and osteopontin proteins in rats with calvarial defects by exposure to a topical ozone application with an alloplastic graft implant.

MATERIALS AND METHODS

Ethics and experimental procedure

Approval of the study was obtained from the Experimental Animal Ethics Committee of Dicle University. Experimental animals were obtained from the Health Sciences and Application Centre at Dicle University in Diyarbakir, Turkey. All operations on animals were performed according to the standards in the Guide for the Care and Use of Laboratory Animals (2011, 8th ed.) released by the National Research Council.

A total of 56 healthy male Wistar rats weighing 280 to 300 g were used for the study.

The individually housed animals were kept in suitable cages under the conditions of a 12 h light and 12 h dark cycle at temperatures of 22 ± 2°C at 50–70% humidity. They were fed a standard pellet diet and water *ad libitum*. At the end of the experiment, no difference was observed between experimental and control rats in terms of food/water consumption and body weight gain. At the end of the 4th week, 2 rats in the control group, 1 rat in the defect and ozone group, and 1 rat in the defect and graft group were seen to have died. At the end of the 8th week, 2 rats in the control group, 1 rat in the defect and ozone group, 2 rats in the defect and graft group, and 2 rats in the defect, graft and ozone group were found to be dead. The tissues of all dead rats were routinely examined histologically.

The experimental design for the groups was as follows. For all groups, half of the rats were sacrificed at the end of the 4th week, and the remaining half were sacrificed at the end of the 8th week. The following are the group designs:

- control group (n = 14): a calvarial bone defect was created without any treatment, and the wound was sutured;
- defect and ozone group (n = 14): a calvarial bone defect was created and treated with ozone;
- defect and graft group (n = 14): a calvarial bone defect was created, and alloplastic bone grafts were applied to the defect;
- defect, graft, and ozone group (n = 14): calvarial bone defect was created, and alloplastic bone grafts plus ozone treatment was applied to the defect.

Calvarial defect model and surgical procedure

Anaesthesia was performed with 3 mg/kg xylazine (Rompun 2%; Bayer) and 90 mg/kg ketamine HCl i.p. (Eczacıbası, Istanbul) [46]. After the scalp was shaved and disinfected using 70% alcohol, the open frontal bone was uncovered by an incision. The periosteum was removed with a periosteal elevator and a trephine bur. Then, the full thickness of the calvarial bone defect was created at 8 mm in diameter. An alloplastic graft material (Bio-Graft-HT, IFGL Bio Ceramics, India) consisting of a combination of porous biphasic 60% synthetic hydroxyapatite granules and 40% beta-tricalcium phosphate bone graft granules with a diameter of 350–500 μm was applied in the defect area of the third and fourth groups. In the groups with exposure to ozone, prozone (W&H, Bürmoos, Austria) at a concentration of 80% was applied to the prepared graft area from a distance of 1 mm using a Coro tip applicator. Prozone was administered for 120 s, two times a week with a Coro tip applicator. Subcutaneous tissue was sealed with a 6/0 vicryl suture, and the skin was closed using a 5/0 silk suture. The skin on the calvarium was completely removed, and the defect was taken out with bone forceps [39].

In this experimental study, a calvarial defect model was created, and immunohistochemical observations were evaluated in the four groups. Calvarial defects with ozone and graft application were examined both separately and together. Our purpose was to investigate the relevance of exposure to the ozone application and healing of the calvarial defect at the tissue level immunohistochemically. To show this relationship immunohistochemically, osteonectin and osteopontin expressions were evaluated in all groups.

Immunohistochemical staining

Samples of calvarial bone were fixed with 10% formaldehyde solution, decalcified with 5% ethylene-diamine-tetraacetic acid (EDTA), dehydrated in a graded series of ethanol, and then embedded in paraffin wax. Then, 4–5 μm thick sections were cut with a microtome (Leica, Germany) and placed on coated slides. Sections were brought to distilled water and washed 3 times for 5 min in phosphate buffered saline (PBS, pH 7.4) (catalogue number # 10010023, Thermo Fisher Scientific, US). To unmask antigen sites, slides were incubated with EDTA solution in microvawe for 110 min at 3 × 90°C. The sections were washed in 3 times for 5 min in PBS and incubated with hydrogen peroxide (catalogue

TA-015-HP, Thermo Fisher Scientific, US) for 20 min. Ultra V block (TA-125-UB, Thermo Fisher Scientific, US) was applied to the sections for 8 min prior to the addition of the primary antibodies, which were left on overnight osteonectin (SPARC Monoclonal Antibody), catalogue # 33-5500, 1:100; and osteopontin monoclonal antibody, catalogue # MA5-17180, 1:100, both from Thermo Fisher Scientific, US). The sections were washed 3 times for 5 min in PBS and then were incubated with biotinylated secondary antibody (catalogue # TP-125-BN, Thermo Fisher Scientific, US) for 14 min. After washing with PBS, streptavidin peroxidase (catalogue # TS-125-HR, Thermo Fisher Scientific, US) was applied to the sections for 15 min. The sections were washed 3 times for 5 min in PBS. Diaminobenzidine (catalogue # TA-012-HDC, Thermo Fisher Scientific, US) was applied to sections for up to 20 min as a chromogen. Control slides were prepared using the same procedure, without primary antibodies. Counterstaining was done using Harris's haematoxylin for 45 s, dehydrated through ascending alcohol and cleared in xylene (Product Number: HHS32 Sigma, haematoxylin solution, Harris Modified, Sigma-Aldrich, 3050 Spruce Street, Saint Louis, MO, 63103, USA). Slides were mounted with Entellan® (lot: 107961, Sigma-Aldrich, St. Louis, MO, USA) and examined under a light microscope (Olympus, Germany).

Scoring of parameters for immunohistochemistry

Semi-quantitative scoring [22, 40, 42] was determined by examining osteoblastic activity, osteocytic activity, osteoclastic activity, and new bone formation in the bone tissue in 15 different regions within the microscope field, and 10 cells were counted in each area for osteonectin and osteopontin expression. These parameters were scored as: 0 = no change, 1 = too weak, 2 = weak, 3 = medium, and 4 = strong.

Statistical analysis

Statistical analyses were performed using the SPSS 22.0 programme. The Kruskal-Wallis variance analysis method was used for non-parametric tests in comparison between groups because the data were not distributed according to the Shapiro-Wilk test. According to the Kruskal-Wallis variance analysis, the difference between the groups was statistically significant at $p < 0.05$. The comparison of the groups with each other was compared with the Mann-Whitney U test with Bonferroni correction from multiple comparison tests. In the comparison of the pairs between groups, the Bonferroni-corrected Mann-Whitney U test was used for multiple comparison tests.

The statistical results of the scoring model of the histological sections of our study were evaluated in the Biostatistics Department of Dicle University.

RESULTS

Immunohistochemical results

For the control group at the 4th week, the osteopontin expression was positive in the inflammatory cells of the calvarial bone. The osteopontin positive expression was observed in some osteoclast cells (Fig. 1a, red arrow), and osteopontin negative expression was observed in newly formed small trabecular bone parts (Fig. 1a, yellow arrow). However, the matrix and osteoblastic activity have not yet increased in the newly formed bone (Fig. 1a, a*). For the 8th week of the control group, new bone formation was evidenced by the increased activity of the connective tissue cells and osteoblast cells within the defect area. The osteoblast cells around the bone trabeculae showed osteopontin positive expression (Fig. 1b, yellow arrow) at the site of the bone matrix (Fig. 1b, b*). Osteonectin expression in the fibroblast and osteoblast cells (Fig. 1c, yellow arrow) was found to be positive in the control rats for the 4th week. Bone trabeculae increased in the peripheral osteoblastic activity (Fig. 1c, c*). The expansion of bone trabeculae at the 8th week showed positive osteonectin expression in the osteoblast cells (Fig. 1d, yellow arrow) and matrix structure (Fig. 1d, red arrow, Fig. 1d*). In the defect and ozone group, osteopontin expression in the fibroblast, osteoclast, and osteoblast cells showed positive expression at the 4th week and showed osteopontin reaction in new bone trabeculae due to matrix development (Fig. 2a, yellow arrow, Fig. 2a*). For the 8th week, osteoblastic activity and osteopontin expression in osteocytes (Fig. 2b, yellow arrow) showed a positive reaction. Bone trabeculae and the matrix for new bone formation became evident (Fig. 2b, b*). An increase in connective tissue cells occurred between the calvarial bone and the defect area at the 4th week, whereas the osteonectin expression in the fibroblast and collagen fibres (Fig. 2c, red arrow) and osteoblast cells (Fig. 2c, yellow arrow) was positive (Fig. 2c, c*). For the 8th week, osteonectin expression increased in fibroblast macrophages and osteoblast cells. Expansion in new bone trabeculae (Fig. 2d, black star) became evident in osteocytes (Fig. 2d, blue arrow), osteon

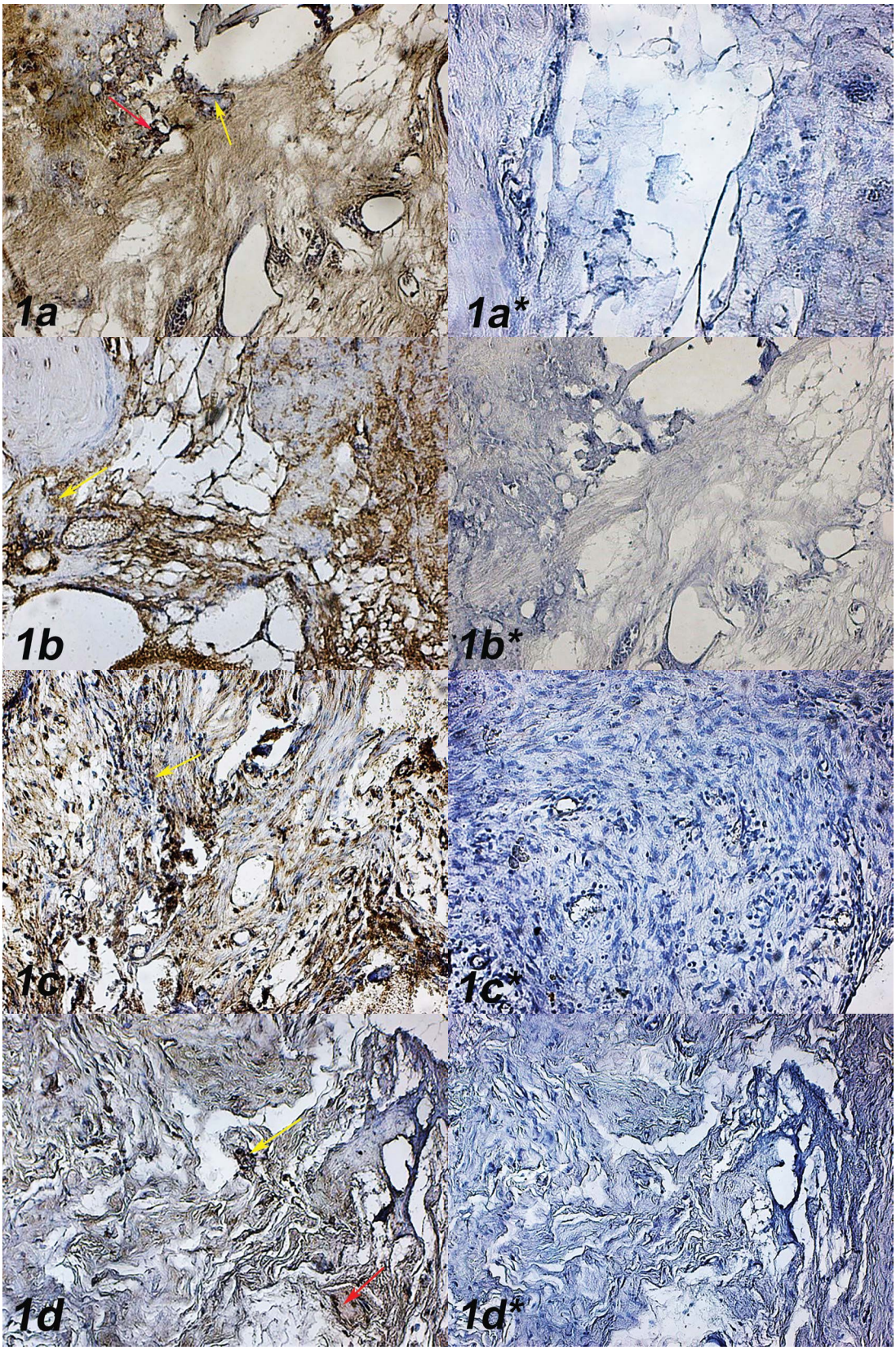

Figure 1. a. Osteopontin immunostaining, control group (4th week), scale bar = 50 μm; **a*.** Negative control group (4th week), haematoxylin staining, scale bar = 50 μm; **b.** Osteopontin immunostaining, control group (8th week); **b*.** Negative control group (8th week), haematoxylin staining, scale bar = 50 μm; **c.** Osteonectin immunostaining, control group (4th week), scale bar = 50 μm; **c*.** Negative control group (4th week), haematoxylin staining; scale bar = 50 μm; **d.** Osteonectin immunostaining, control group (8th week), scale bar = 50 μm; **d*.** Negative control group (8th week), haematoxylin staining, scale bar = 50 μm.

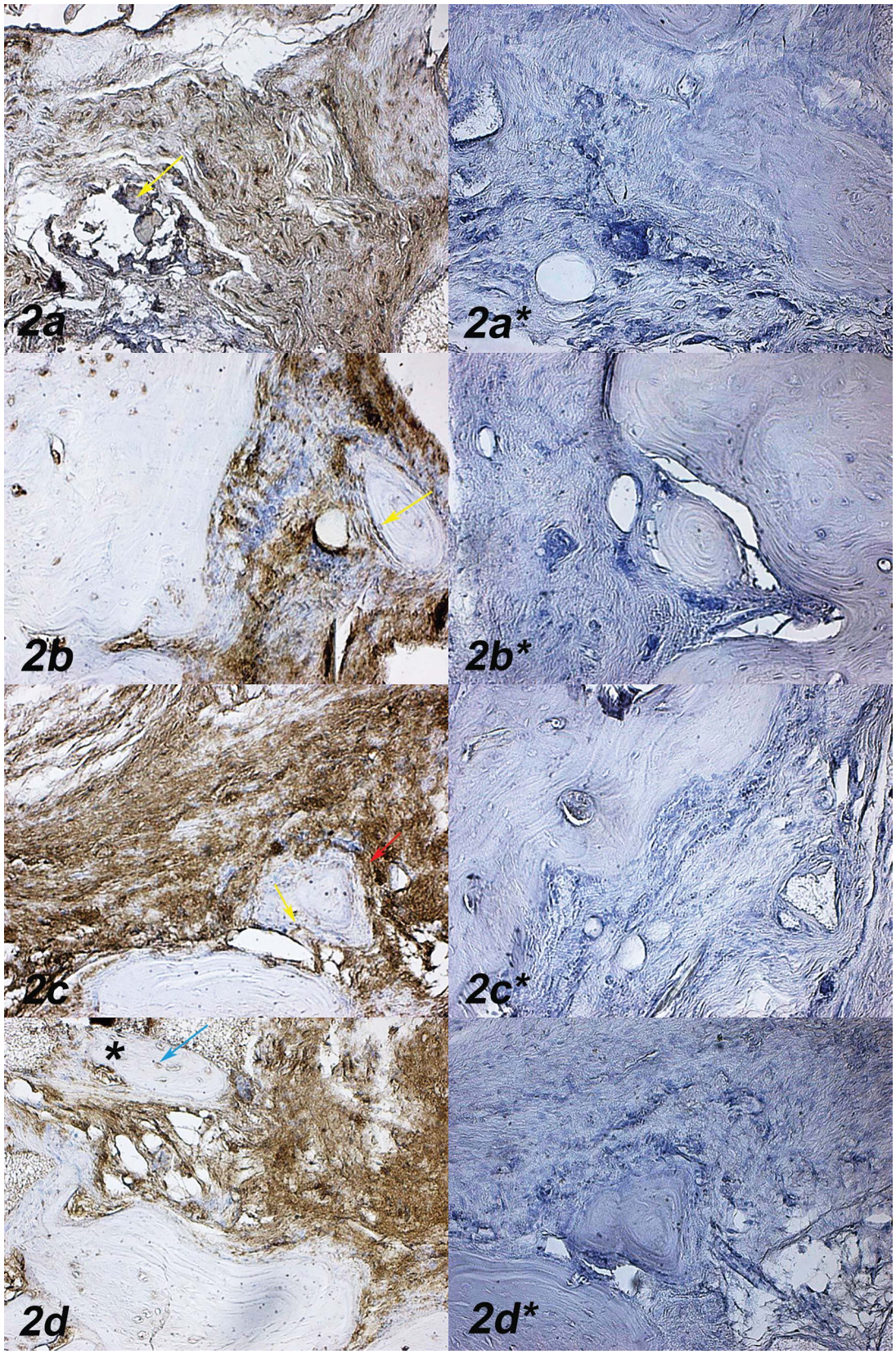

Figure 2. a. Osteopontin immunostaining, defect + ozone group (4th week), scale bar = 50 μm; **a*.** Negative control group (4th week); haematoxylin staining, scale bar = 50 μm; **b.** Osteopontin immunostaining, defect + ozone group (8th week), scale bar = 50 μm; **b*.** Negative control group (8th week), haematoxylin staining, scale bar = 50 μm; **c.** Osteonectin immunostaining, defect + ozone group (4th week), scale bar = 50 μm; **c*.** Negative control group (4th week), haematoxylin staining, scale bar = 50 μm; **d.** Osteonectin immunostaining, defect + ozone group (8th week), scale bar = 50 μm; **d*.** Negative control group (8th week), haematoxylin staining, scale bar = 50 μm.

structures, and osteonectin expression (Fig. 2d, d*). The defect and graft applied group revealed a significant increase in connective tissue cells in the graft site by the 4th week. Histologically, we found an increase in osteopontin expression of fibroblast cells (Fig. 3a, red arrow), inflammatory cells (Fig. 3a, green arrow), and osteoblast cells (Fig. 3a, yellow arrow). Small bone trabeculae were obviously seen (Fig. 3a, a*). For the 8th week, osteoblast cells (Fig. 3b, yellow arrows) close to the calvarial bone area showed the enlargement of new bone trabeculae (Fig. 3b, black star) with increased osteopontin expression (Fig. 3b, b*). Osteonectin expression in osteoblast cells (Fig. 3c, yellow arrows) was positive in the graft site for the 4th week of application (Fig. 3c, c*). For the 8th week, osteonectin expression increased in osteoblasts with new bone trabeculae in the graft area. Osteonectin reacted positively in osteoblast cells (Fig. 3d, yellow arrow), osteocytes (Fig. 3d, blue arrow), osteoclast cells (Fig. 3d, red arrow), and the bone matrix (Fig. 3d, d*). For the defect, graft, and ozone treated group at the end of 4th week, osteopontin expression was observed in osteoblast cells (Fig. 4a, yellow arrow), and osteocytes were peripherally seen in new bone trabeculae (Fig. 4a, black star, Fig. 4a*). For the 8th week, despite an increase in cells and collagen fibre structures within the graft site, osteopontin expression continued to increase. Mature bone trabeculae became evident. Osteopontin expressions were observed in Haversian canals (Fig. 4b, red arrow), osteocytes (Fig. 4b, yellow arrow), and the bone matrix (Fig. 4b, b*). For the 4th week, osteonectin expression was positive in cells between the calvarial bone and the defect area. In the connective tissue cells and fibrous structures, osteonectin was positively observed in osteocyte cells (Fig. 4c, yellow arrow) in trabeculae (Fig. 4c, c*). For the 8th week, new bone trabeculae enlarged, and the osteoblastic activity increased in the area and started to associate with the calvarial bone region. Thus, osteonectin expression is increased, and new bone formation is accelerated. Osteonectin expression in mature bone cells (Fig. 4d, black star) showed a positive reaction (Fig. 4d, d*). In addition, osteoblastic activity, osteocytic activity, osteoclastic activity, and new bone formation were statistically determined among all groups for osteonectin and osteopontin expressions at the 4th week (Tables 1, 2 and 5; Figs. 5, 6) and the 8th week (Tables 3, 4 and 6; Figs. 7, 8). Osteogenesis was eventually promoted in the ozone-treated graft application by the 8th week.

Quantification of immunohistochemistry

The quantification of osteonectin and osteopontin expression in the control, defect and ozone, defect and graft, and defect, graft, and ozone groups were performed by evaluating the osteoblastic activity, osteocytic activity, osteoclastic activity, and new bone formation at the 4th and 8th weeks. Results of the present study were given in Tables 1, 2 and 5 and Figures 5, 6 for the 4th week and in Tables 3, 4 and 6 and Figures 7, 8 for the 8th week.

DISCUSSION

Many clinical studies have been performed on calvarial defect models to evaluate bone regeneration related to different diseases [11, 56, 58, 69]. Histological techniques have been used to demonstrate tissue damage [2, 8, 17]. On the other hand, radiological and mechanical techniques have been used for structural and functional evaluations [15, 55, 62]. Different bone graft materials are currently available for regeneration of bone defects in oral and maxillofacial surgery, such as the closure of osteotomy openings and alveolar increment [4, 6, 10, 19, 52]. Synthetic bone graft materials are available in intra-bone defects, orthognathic surgery, facial bone defects, and maxillary sinus ground [3, 10, 16, 18, 31, 47, 49].

Insufficient work has been carried out to show a relationship between exposure to ozone application and calvarial defect in rats [1, 32, 33, 45]. Kan et al. [32] aimed to compare the efficacy of hyperbaric oxygen and systemic ozone, used separately and in combination, on the healing of bone defects. They showed that quantitative histological assessment of calvarial bone healing showed no total ossification of the critical-size cavity in any of the groups at the end of 30 days. However, they also indicated that partial intramembranous and endochondral ossification were observed during the calvarial bone defect healing process. Their histomorphometric analysis showed more new bone formation in all experimental groups compared with the control group on days 5, 15, and 30 [32]. Similarly, the effects of systemic and topical ozone applications on alveolar bone healing after tooth extraction were investigated. In the histomorphometric analysis, they concluded that measurements of mineralised and trabecular bone and osteocyte and osteoblast surfaces did not show a statistically significant difference between the sacrificed groups at the 14th day. They also suggested that

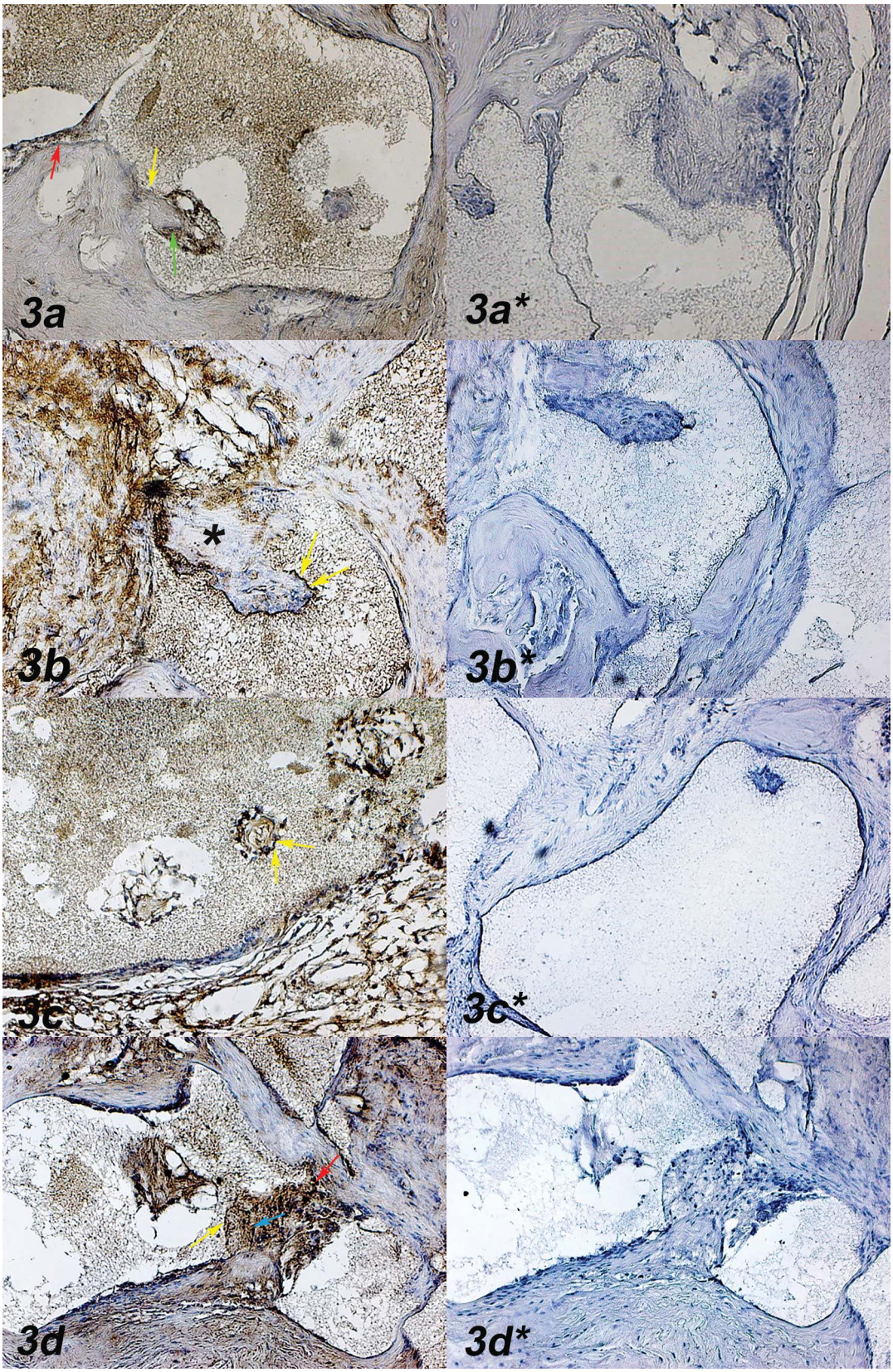

Figure 3. a. Osteopontin immunostaining, defect + graft group (4th week), scale bar = 50 μm; **a*.** Negative control group (4th week), haematoxylin staining, scale bar = 50 μm; **b.** Osteopontin immunostaining, defect + graft group (8th week), scale bar = 50 μm; **b*.** Negative control group (8th week), haematoxylin staining, scale bar = 50 μm; **c.** Osteonectin immunostaining, defect + graft group (4th week), scale bar = 50 μm; **c*.** Negative control group (4th week), haematoxylin staining, scale bar = 50 μm; **d.** Osteonectin immunostaining, defect + graft group (8th week), scale bar = 50 μm; **d*.** Negative control group (8th week), haematoxylin staining, scale bar = 50 μm.

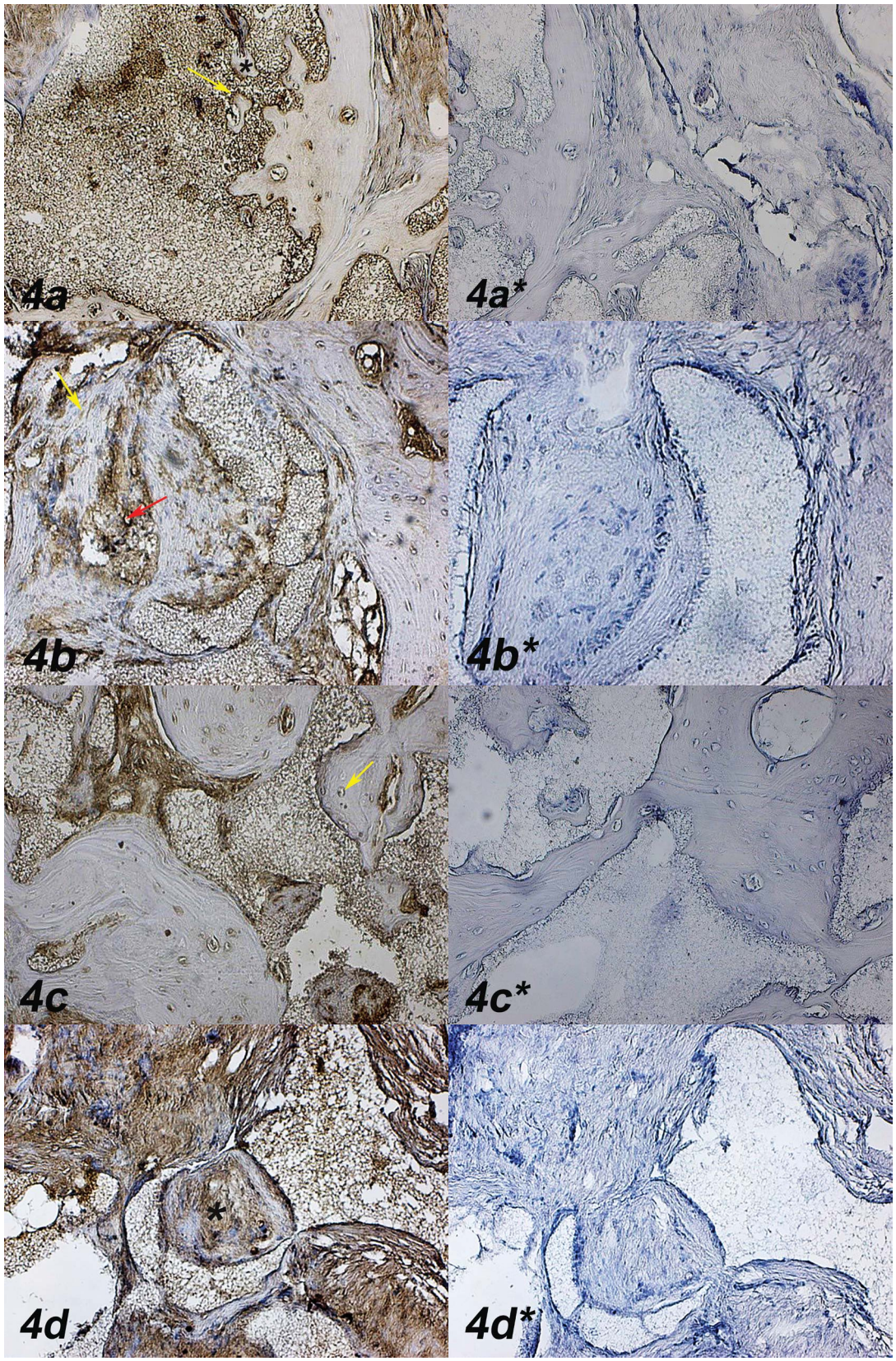

Figure 4. a. Osteopontin immunostaining, defect + graft + ozone group (4th week), scale bar = 50 μm; **a*.** Negative control group (4th week), haematoxylin staining, scale bar = 50 μm; **b.** Osteopontin immunostaining, defect + graft + ozone group (8th week), scale bar = 50 μm; **b*.** Negative control group (8th week), haematoxylin staining, scale bar = 50 μm; **c.** Osteonectin immunostaining, defect + graft + ozone group (4th week), scale bar 50 μm; **c*.** Negative control group (4th week), haematoxylin staining, scale bar = 50 μm; **d.** Osteonectin immunostaining, defect + graft + ozone group (8th week), scale bar = 50 μm; **4d*.** Negative control group (8th week), haematoxylin staining, scale bar = 50 μm.

Table 1. Evaluation of osteonectin expression results of parameters at 4th week by Kruskal-Wallis test (*statistically significant result)

Groups		**Osteonectin expression at 4th week**		
		Osteoblastic activity	**Osteocytic activity**	**Osteoclastic activity**
Control group	Minimum	0	0	2
	Maximum	1	1	4
	Range	1	1	2
	Median	1.00	0.00	3.00
Defect + ozone group	Minimum	1	2	2
	Maximum	3	3	3
	Range	2	1	1
	Median	2.00	2.00	2.00
Defect + graft group	Minimum	1	2	2
	Maximum	2	3	3
	Range	1	1	1
	Median	2.00	2.00	2.00
Defect + graft + ozone group	Minimum	2	3	0
	Maximum	3	4	2
	Range	1	1	2
	Median	3.00	3.00	1.00
	Groups	**N**	**Mean rank**	
Osteonectin expression at 4th week (Osteoblastic activity)	Control group	7	5.43	
	Defect + ozone group	7	16.79	
	Defect + graft group	7	12.71	
	Defect + graft + ozone group	7	23.07	
	Total	28		
Osteonectin expression at 4th week (Osteocytic activity)	Control group	7	4.00	
	Defect + ozone group	7	15.57	
	Defect + graft group	7	15.57	
	Defect + graft + ozone group	7	22.86	
	Total	28		
Osteonectin expression at 4th week (Osteoclastic activity)	Control group	7	22.50	
	Defect + ozone group	7	14.71	
	Defect + graft group	7	16.07	
	Defect + graft + ozone group	7	4.71	
	Total	28		
Osteonectin expression at 4th week	**Osteoblastic activity**	**Osteocytic activity**	**Osteoclastic activity**	
Chi-square	18.932	20.803	18.443	
df	3	3	3	
Asymp. Sig.	0.000*	0.000*	0.000*	

there have been no statistically significant findings in the mineralised bone and osteoblast field areas between the groups sacrificed on day 28. However, the comparison of the histomorphometric parameters of the trabecular bone between days 14 and 28 showed higher values on day 28. In addition, they observed statistically significant decreases in mineralised bone and osteocyte-osteoblast surfaces for all of the groups sacrificed on day 28 [21]. Accordingly, the effects of exposure to ozone application on calvarial defects and

Table 2. Evaluation of osteopontin expression results of parameters at 4th week by Kruskal-Wallis test (*statistically significant result)

Groups		Osteopontin expression at 4th week			
		Osteoblastic activity	Osteocytic activity	Osteoclastic activity	New bone formation
Control group	Minimum	0	0	2	0
	Maximum	1	1	4	1
	Range	1	1	2	1
	Median	1.00	0.00	3.00	0.00
Defect + ozone group	Minimum	1	2	2	1
	Maximum	3	3	3	2
	Range	2	1	1	1
	Median	2.00	2.00	2.00	2.00
Defect + graft group	Minimum	1	2	2	1
	Maximum	2	3	3	3
	Range	1	1	1	2
	Median	2.00	2.00	2.00	2.00
Defect + graft + ozone group	Minimum	2	3	0	3
	Maximum	3	4	2	4
	Range	1	1	2	1
	Median	3.00	3.00	1.00	3.00

	Groups	N	Mean rank
Osteopontin expression at 4th week (Osteoblastic activity)	Control group	7	5.43
	Defect + ozone group	7	16.79
	Defect + graft group	7	12.71
	Defect + graft + ozone group	7	23.07
	Total	28	
Osteopontin expression at 4th week (Osteocytic activity)	Control group	7	4.00
	Defect + ozone group	7	15.57
	Defect + graft group	7	15.57
	Defect + graft + ozone group	7	22.86
	Total	28	
Osteopontin expression at 4th week (Osteoclastic activity)	Control group	7	22.50
	Defect + ozone group	7	14.71
	Defect + graft group	7	16.07
	Defect + graft + ozone group	7	4.71
	Total	28	
Osteopontin expression at 4th week (New bone formation)	Control group	7	4.21
	Defect + ozone group	7	12.79
	Defect + graft group	7	16.86
	Defect + graft + ozone group	7	24.14
	Total	28	

Osteopontin expression at 4th week	Osteoblastic activity	Osteocytic activity	Osteoclastic activity	New bone formation
Chi-square	18.932	20.803	18.443	22.612
df	3	3	3	3
Asymp. Sig.	0.000*	0.000*	0.000*	0.000*

Table 3. Evaluation of osteonectin expression results of parameters at 8th week by Kruskal-Wallis test (*statistically significant result)

Groups		**Osteonectin expression at 8th week**		
		Osteoblastic activity	**Osteocytic activity**	**Osteoclastic activity**
Control group	Minimum	1	0	0
	Maximum	2	2	2
	Range	1	2	2
	Median	2.00	1.00	1.00
Defect + ozone group	Minimum	2	2	0
	Maximum	3	4	1
	Range	1	2	1
	Median	3.00	3.00	1.00
Defect + graft group	Minimum	2	2	1
	Maximum	3	4	2
	Range	1	2	1
	Median	2.00	3.00	1.00
Defect + graft + ozone group	Minimum	3	3	0
	Maximum	4	4	1
	Range	1	1	1
	Median	4.00	4.00	.00
		Groups	**N**	**Mean rank**
Osteonectin expression at 8th week (Osteoblastic activity)		Control group	7	5.71
		Defect + ozone group	7	16.00
		Defect + graft group	7	13.00
		Defect + graft + ozone group	7	23.29
		Total	28	
Osteonectin expression at 8th week (Osteocytic activity)		Control group	7	4.43
		Defect + ozone group	7	15.86
		Defect + graft group	7	14.79
		Defect + graft + ozone group	7	22.93
		Total	28	
Osteonectin expression at 8th week (Osteoclastic activity)		Control group	7	18.00
		Defect + ozone group	7	11.86
		Defect + graft group	7	19.71
		Defect + graft + ozone group	7	8.43
		Total	28	
Osteonectin expression at 8th week		**Osteoblastic activity**	**Osteocytic activity**	**Osteoclastic activity**
Chi-square		18.450	19.208	10.621
df		3	3	3
Asymp. Sig.		0.000*	0.000*	0.014*

their healing time are not well known. However, it is well known that ozone accelerates wound healing and increases blood flow. It is dissolved in water or gas for medical purposes. Exposure to ozone application can induce several biological responses, such as improving blood circulation and accelerated oxygen capacity in ischaemic tissue, upregulating cellular antioxidant enzymes, facilitating the activation of the immune system, and supporting the secretion of growth factors [57].

Table 4. Evaluation of osteopontin expression results of parameters at 8th week by Kruskal-Wallis test (*statistically significant result)

Groups		Osteopontin expression at 8th week			
		Osteoblastic activity	Osteocytic activity	Osteoclastic activity	New bone formation
Control group	Minimum	2	1	1	1
	Maximum	3	3	3	3
	Range	1	2	2	2
	Median	3.00	2.00	2.00	2.00
Defect + ozone group	Minimum	2	2	0	3
	Maximum	4	4	1	4
	Range	2	2	1	1
	Median	3.00	3.00	0.00	4.00
Defect + graft group	Minimum	1	2	0	3
	Maximum	3	4	2	4
	Range	2	2	2	1
	Median	2.00	3.00	1.00	3.00
Defect + graft + ozone group	Minimum	3	3	0	3
	Maximum	4	4	1	4
	Range	1	1	1	1
	Median	4.00	4.00	0.00	4.00

	Groups	N	Mean rank
Osteopontin expression at 8th week (Osteoblastic activity)	Control group	7	12.29
	Defect + ozone group	7	16.50
	Defect + graft group	7	7.07
	Defect + graft + ozone group	7	22.14
	Total	28	
Osteopontin expression at 8th week (Osteocytic activity)	Control group	7	8.50
	Defect + ozone group	7	16.00
	Defect + graft group	7	11.86
	Defect + graft + ozone group	7	21.64
	Total	28	
Osteopontin expression at 8th week (Osteoclastic activity)	Control group	7	23.93
	Defect + ozone group	7	10.50
	Defect + graft group	7	14.57
	Defect + graft + ozone group	7	9.00
	Total	28	
Osteopontin expression at 8th week (New bone formation)	Control group	7	5.29
	Defect + ozone group	7	17.57
	Defect + graft group	7	14.29
	Defect + graft + ozone group	7	20.86
	Total	28	

Osteopontin expression at 8th week	Osteoblastic activity	Osteocytic activity	Osteoclastic activity	New bone formation
Chi-square	14.479	11.272	15.729	16.237
df	3	3	3	3
Asymp. Sig.	0.002	0.010	0.001	0.001

Table 5. Comparison of osteonectin and osteopontin expressions at the 4th week as a binary group with the Bonferroni correction Mann-Whitney U test (*p < 0.05 is statistically significant result by Bonferroni correction Mann-Whitney U test)

Osteonectin expression at 4th week	P value	
Osteoblastic activity		
1–2	0.038	< 0.05*
1–3	0.481	> 0.05
1–4	0.000	< 0.05*
2–3	1.000	> 0.05
2–4	0.077	> 0.05
3–4	0.787	> 0.05
Osteocytic activity		
1–2	0.034	< 0.05*
1–3	0.034	< 0.05*
1–4	0.000	< 0.05*
2–3	1.000	> 0.05
2–4	0.491	> 0.05
3–4	0.491	> 0.05
Osteoclastic activity		
1–2	0.381	> 0.05
1–3	0.752	> 0.05
1–4	0.000	< 0.05*
2–3	1.000	> 0.05
2–4	0.103	> 0.05
3–4	0.007	< 0.05*
Osteoblastic activity		
1–2	0.0038	< 0.05*
1–3	0.481	> 0.05
1–4	0.000	< 0.05*
2–3	1.000	> 0.05
2–4	0.077	> 0.05
3–4	0.787	> 0.05
Osteocytic activity		
1–2	0.034	< 0.05*
1–3	0.034	< 0.05*
1–4	0.000	< 0.05*
2–3	1.000	> 0.05
2–4	0.491	> 0.05
3–4	0.491	> 0.05
Osteoclastic activity		
1–2	0.381	> 0.05
1–3	0.752	> 0.05
1–4	0.000	< 0.05*
2–3	1.000	> 0.05
2–4	0.103	> 0.05
3–4	0.041	< 0.05*
New bone formation		
1–2	0.272	> 0.05
1–3	0.019	< 0.05*
1–4	0.000	< 0.05*
2–3	1.000	> 0.05
2–4	0.048	< 0.05*
3–4	0.533	> 0.05

1 — control group; 2 — defect + ozone group; 3 — defect + graft group; 4 — defect + graft + ozone group

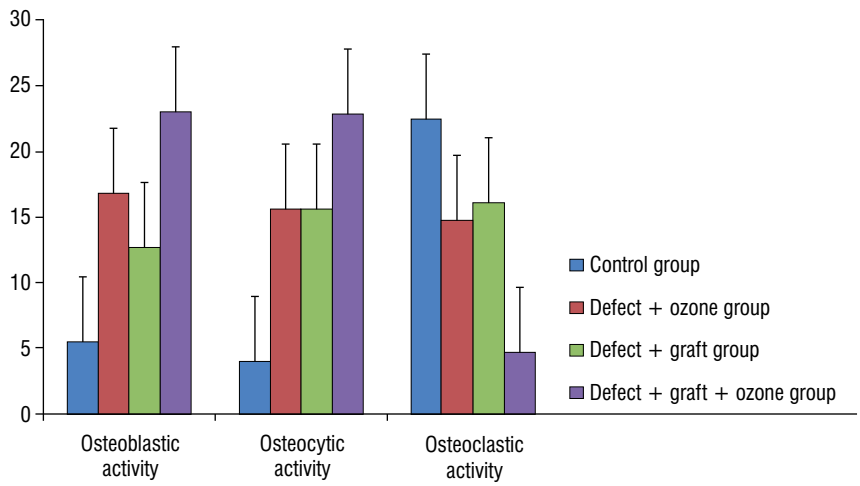

Figure 5. Graphic showing the evaluation of osteonectin expression results for parameters at the 4th week in the control and experimental groups.

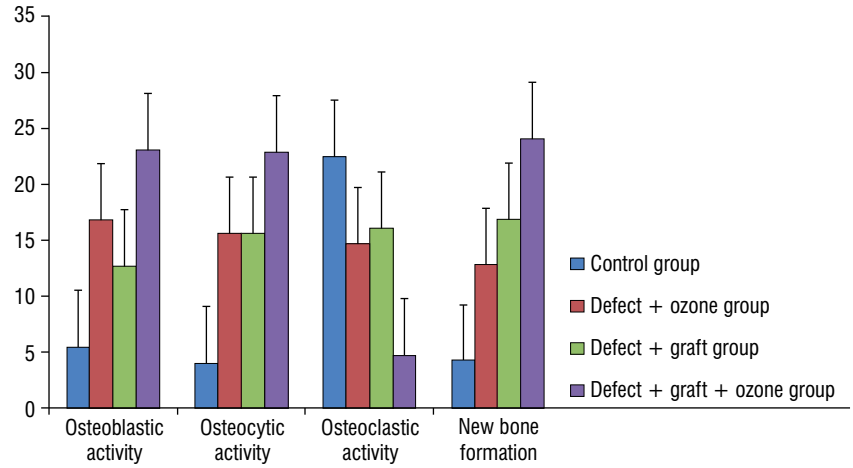

Figure 6. Graphic showing the evaluation of osteopontin expression results for parameters at the 4th week in the control and experimental groups.

Table 6. Comparison of osteonectin and osteopontin expressions at the 8th week as a binary group with the Bonferroni correction Mann-Whitney U test (*p < 0.05 is statistically significant result by Bonferroni correction Mann-Whitney U test)

Osteonectin expression at 8th week	P value	
Osteoblastic activity		
1–2	0.079	> 0.05
1–3	0.475	> 0.05
1–4	0.000	< 0.05*
2–3	1.000	> 0.05
2–4	0.475	> 0.05
3–4	0.079	> 0.05
Osteocytic activity		
1–2	0.044	< 0.05*
1–3	0.090	> 0.05
1–4	0.000	< 0.05*
2–3	1.000	> 0.05
2–4	0.336	> 0.05
3–4	0.582	> 0.05
Osteoclastic activity		
1–2	0.725	> 0.05
1–3	1.000	> 0.05
1–4	0.094	> 0.05
2–3	0.283	> 0.05
2–4	1.000	> 0.05
3–4	0.026	< 0.05*
Osteoblastic activity		
1–2	1.000	> 0.05
1–3	1.000	> 0.05
1–4	0.099	> 0.05
2–3	0.131	> 0.05
2–4	1.000	> 0.05
3–4	0.001	< 0.05*
Osteocytic activity		
1–2	0.417	> 0.05
1–3	1.000	> 0.05
1–4	0.009	< 0.05*
2–3	1.000	> 0.05
2–4	1.000	> 0.05
3–4	0.107	> 0.05
Osteoclastic activity		
1–2	0.007	< 0.05*
1–3	0.144	> 0.05
1–4	0.002	< 0.05*
2–3	1.000	> 0.05
2–4	1.000	> 0.05
3–4	1.000	> 0.05
New bone formation		
1–2	0.015	< 0.05*
1–3	0.163	> 0.05
1–4	0.001	< 0.05*
2–3	1.000	> 0.05
2–4	1.000	> 0.05
3–4	0.641	> 0.05

1 — control group; 2 — defect + ozone group; 3 — defect + graft group; 4 — defect + graft + ozone group

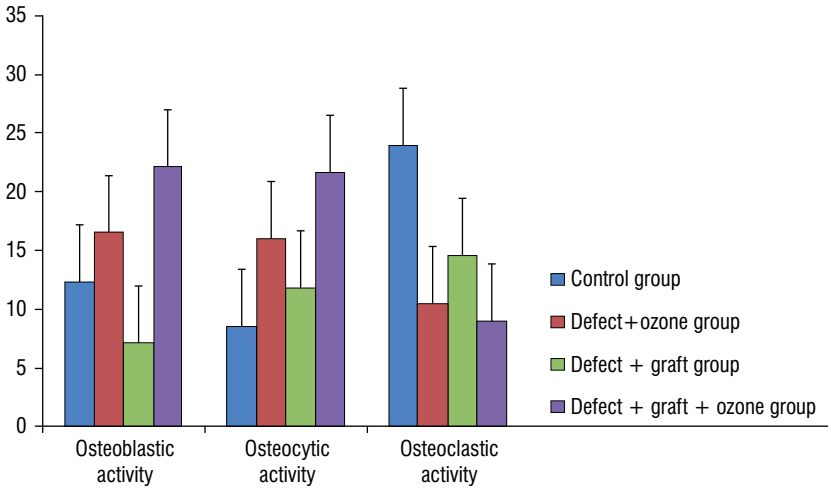

Figure 7. Graphic showing the evaluation of osteonectin expression results for parameters at the 8th week in the control and experimental groups.

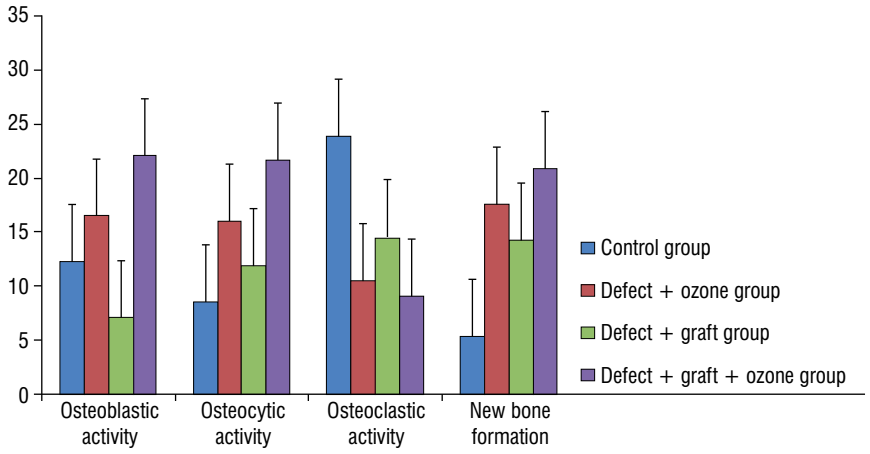

Figure 8. Graphic showing the evaluation of osteopontin expression results for parameters at the 8th week in the control and experimental groups.

Alpan et al. [1] suggested that treatment with ozone supports the consistency of xenograft and promotes regeneration of bone on a model of a calvarial defect in diabetic rats. In an experimental study, they asserted that both hyperbaric oxygen and ozone depicted the same efficacy to augment bone healing and that the concomitant use of them would be more effective [32]. Laçin et al. [39] confirmed that ozone has healing effects on bone defects at 4- and 8-week intervals, indicating that graft and ozone application is the best practice for new bone formation. They emphasised that ozone treatment establishes homeostasis by supporting angiogenesis and cell proliferation and induces the synthesis of a group of cytokines, such as leukotrienes, interleukins, and prostaglandins. Ozone treatment triggers macrophage activation and expedites phagocytosis. It also has a definite effect on bone formation compared to the controls in the calvarial defect rate model [33]. Ozdemir et al. [45] investigated the effect of exposure to ozone application on autogenous bone graft healing in calvarial defects. The authors indicated that ozone treatment increased the formation of fresh bone with the application of an autogenous bone graft in the calvarial defect rat model. Histomorphometrically, they observed that the total bone area in the autogenous bone graft with exposure to ozone application group was significantly higher than that of the autogenous bone graft group. They also supported that the exposure to the ozone application group significantly increased the percentage of total bone area, osteoblast numbers, and new bone formation compared to the autogenous bone graft group. In another study, alternative ozone thera-

pies on bone regeneration were investigated in the inter-premaxillary suture expansion on rats. Ozone treatment expedited the acceleration in the formation of fresh calcified bone in the area of suture [9].

Osteopontin promotes the early differentiation of osteoblasts, their adhesion to the bone, and bone formation. Bone cells secrete osteopontin during the process of bone remodelling and increase osteopontin expression in response to mechanical stimuli [14, 37]. Choi et al. [14] proposed that patients with ankylosing spondylitis with had significantly higher plasma osteopontin, tumour necrosis factor-alpha, and interleukin-6 levels and more mRNA expression than healthy controls. Klein-Nulend et al. [37] suggested that osteopontin appeared to stimulate adhesion, migration, and bone resorption by osteoclasts. They found that severe loss of osteopontin expression in primary bone cells cultured without mechanical stimulation down-regulated conditions of use to differentiate the osteoblastic phenotype [37]. To understand the interplay between systemic and local signalling in bone, they examined the effects of deficiency of the bone matrix protein osteopontin on the systemic effects of the parathyroid hormone, specifically within osteoblastic cell lineages. Parathyroid hormone receptor (PPR) in transgenic mice expressing a constitutively active form of the receptor (caPPR), specifically in cells of the osteoblast lineage, has a high bone mass phenotype. In these mice, osteopontin deficiency further increased bone mass. They indicated that this increase was associated with the conversion of the major intertrabecular cell population from haematopoietic cells to stromal/osteoblastic cells and with parallel elevations in the histomorphometric and biochemical parameters of bone formation and resorption [43]. Ihara et al. [29] aimed to obtain insight into the cellular mechanism underlying the phenomena observed in the osteopontin-deficient bone. They investigated the number of tartrate-resistant acid phosphatase (TRAP)-positive cells in the bones subjected to parathyroid hormone treatment in cultures. They found that the number of TRAP-positive cells increased significantly by parathyroid hormone in wild type bone; however, no such parathyroid hormone-induced increase in the TRAP-positive cells was observed in osteopontin-deficient bones. Their results indicated that the absence of osteopontin suppressed the parathyroid hormone-induced increase in bone resorption by preventing the increase in the number of osteoclasts in the local milieu of bone [29]. Khojasteh et al. [35] compared culture-expanded bone marrow-derived mesenchymal stem cells and platelet-rich plasma loaded to natural bone mineral (bio-oss) and beta-tricalcium phosphate for rat calvarial bone repair. According to their immunohistochemical observation, they concluded that the positive immunoreaction for osteopontin and osteonectin was significant in all groups. Based on these positive expressions in the defect area, they concluded that bone formation and mineralisation proceeded from the defect border to the centre [35]. Double administration of platelet-rich plasma has been reported to have no additional benefits for osteonectin and osteocalcin expression levels during bone healing in a rabbit cranial defect model. Furthermore, the findings revealed that the presence of beta-tricalcium phosphate affects osteonectin and osteocalcin expressions, suggesting the potential for beta-tricalcium phosphate to support early bone healing [44].

Limitations of the study

Despite the results, there are a few limitations in clinical contribution. Very few experimental studies exist on the use of alloplastic graft material with ozone application. Graft materials are used to prevent damage to the bones caused by defects in patients, inducing osteoblastic activity in bone repair and stimulating osteocyte and trabecular bone development. Whereas graft application has a stimulating and curative effect on bone tissue, the use of ozone is thought to induce alloplastic graft application by accelerating calvarial bone development in rats.

CONCLUSIONS

In our study, the group with 4 weeks of ozone application revealed a positive reaction in osteoclast cells and osteopontin expression in inflammatory cells. Osteoblastic activity began to increase within the defect area at the 8th week, and osteopontin expression reacted positively in the osteoblasts and bone matrix.

At the end of the 4th week in the defect and ozone group, fibroblast osteoclasts in the defect area and osteopontin expression in the inflammatory cells reacted positively. At week 8, new bone trabeculae were formed, osteoblastic activity increased, and osteopontin expression in osteoblast cells became evident. In the immunohistochemical examination of the defect and ozone group, an increase in the connective tissue between the defect area and the calvarial bone was observed (Fig. 2c, c*). Osteonectin

was accelerated in fibroblasts and osteoblasts. In the 8th week, fibroblast macrophages and positive osteonectin expression in osteoblast cells were observed, but new bone trabeculae, osteocytes, and osteon structures became evident (Fig. 2d, d*).

Positive expression was observed in the osteoblast cells at the graft site at the 4th week in the sections of the defect and graft group (Fig. 3c), whereas osteoblast cells with new bone trabeculae and bone matrix showed an increased expression of osteoblast cells at the 8th week (Fig. 3d, d*).

In the defect, graft, and ozone group (4th week), osteopontin expression was positive in osteoblast osteocytes, whereas new bone was visible in the trabeculae. At the 8th week, mature bone trabeculae developed Haversian ducts and increased osteopontin expression in osteocytes. In addition, we observed the positive expression of osteonectin in fibroblasts and some osteoblasts in the control group (4th week; Fig. 1c, c*). The defect, graft, and ozone application resulted in new bone trabeculae at the 4th week (Fig. 4c, c*). Osteoblast cells showed positive osteonectin expression, and in 8 weeks, the osteon tissues and osteocytes reacted positively (Fig. 4d, d*). Exposure to ozone application is used in combination with grafts in rat calvarial defects, and mature bone structure develops after about 8 weeks of bone matrix formation and new bone remodelling.

In the control group, osteonectin expression in the osteoblast cells and bone matrix increased in new bone trabeculae at the 8th week (Fig. 1d, d*).

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