Original Article

Dicle Dent J. 2024;25(2):38-45

Investigation of the effect of denosumab and ozone application on bone healing in critical size bone defects

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Cite this article as: Keskin E, Kaya B, Aktaş A. Investigation of the effect of denosumab and ozone application on bone healing in critical size bone defects. *Dicle Dent J*. 2024;25(2):38-45.

Received: 04/01/2024 ◆ **Accepted**: 15/06/2024 ◆ **Published**: 28/06/2024

ABSTRACT

Aims: In maxillofacial surgery, various drugs are used in order to accelerate the recovery of defects caused by any reason. Several studies have shown that denosumab from bisphosphonate group drugs used in osteoporosis patients has positive effects on new bone formation. In general medicine and dentistry, ozone is in widespread use as an alternative treatment and has positive effects on new bone formation. The aim of this study was to evaluate new bone formation by performing denosumab and ozone on critical-sized rat calvarium defects.

Methods: In our study, 40 Sprague Dawley rats were used. Rats were divided into 4 groups. Only grafts were placed in the control group. After applying graft to ozone group (O) and ozone and denosumab (O-D) groups, topical ozone was applied for 15 seconds. Denosumab group (D) and O-D group were injected subcutaneously (s.c) 10 mg/kg Prolia (denosumab) every 4 weeks for 8 weeks. 5 animals from each group at the end of week 4, while the other five animals in the group at the end of 8 weeks after being sacrificed for histopathological examination was performed. The differences between the groups were evaluated by statistical analysis.

Results: After histopathological examination, better bone formation was observed in the ozone and denosumab treated groups compared to the control group. There was no statistically significant difference between the groups except for the control group, however, new bone formation was determined in the groups treated with denosumab compared to the ozone group.

Conclusion: As a result of our study, we believe that the application of ozone and denosumab has a positive effect on the formation of new bone, but more comprehensive studies on the subject are needed.

Keywords: Rat, ozone, denosumab, new bone formation

INTRODUCTION

In oral and maxillofacial surgery, healing and bone formation in the operating area have been one of the most emphasized issues. Today, autogenous grafting is considered and used as the gold standard in bone healing. Although autogenous grafts are the gold standard, different graft materials (allografts, xenografts, alloplastic graft) can be used due to their disadvantages such as creating a second wound area, the risk of bone graft resorption, the risk of nerve injuries and the risk of infection.[1]

Ozone (O_3) is a natural compound consisting of three oxygen atoms. Ozone is not a radical molecule due to its chemical structure, but it is the third most powerful oxidant known. Since ozone cannot be stored and has a half-life of 40 minutes, it is an unstable gas that must be used all at once. O_3 has been frequently used in dentistry and oral surgery since 1993 due to its antimicrobial properties, analgesic effect, and regulatory effects on microcirculation and peripheral blood circulation.[2]

Bone tissue is considered one of the hardest tissues in our body. If bones are exposed to trauma, their repair abilities are highly developed, and therefore, new bone tissue is obtained in the affected area and the functions of the relevant area are restored.[3,4]

The disruption of the anatomical integrity of the bone due to external or internal forces is called a fracture. A number of physiological reactions begin to restore the damaged bone integrity. In bone healing, scar tissue does not form and it heals through restructuring. Fracture healing begins from the moment the fracture occurs and continues until the regular

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bone tissue and the fracture ends unite.[5] If fibrous tissue forms after the bone tissue heals, this indicates that the fracture has not healed.[6]

Denosumab is a recently FDA-approved monoclonal antibody for the treatment of osteoporosis in postmenopausal women at high risk of fracture.[7] It is also being evaluated for the prevention of osteoporosis in postmenopausal women, as well as for the treatment and prevention of bone loss in patients undergoing hormone ablation therapy for breast and prostate cancer.[8,9] The aim of this study was to evaluate new bone formation by performing denosumab and ozone on critical-sized rat calvarium defects.

METHODS

This study was supported by Dicle University Scientific Research Projects with project number DİŞ17.022. Ethics committee approval for our thesis study was received from Dicle University Animal Experiments Local Ethics Committee (Date: 30.05.2017, Decision No: 2017/09). The experimental stages of our study were carried out at Dicle University Prof. Dr. Sabahattin Payzın Health Sciences Research Center, and histopathological examinations were carried out at Dicle University Faculty of Medicine, Department of Histology and Embryology. This study was conducted on critical-sized bone defects opened experimentally in the rat calvarium.

In our study, 40 Sprague-Dawley male rats with an average weight of 350 grams were used. Sprague-Dawley rats aged 6 months were used in the study. During the experiment, all subjects were housed in special plastic cages in ventilated rooms with 12 hours of light and 12 hours of darkness, at a temperature of 24 degrees, free access to drinking water and standard food. Before starting our study, the health status of all subjects was checked by a veterinarian.

Study Groups Experimental animals were divided into 4 groups, with 10 rats in each group

Group 1 [Control Group (C)]: Only graft was applied to the rats in this group.

Group 2 [Ozone Group (O)]: Topical ozone and graft application was applied to the rats in this group.

Group 3 [Denosumab Group (D)]: Subcutaneous denosumab was given to the rats in this group and graft was applied.

Group 4 [Ozone and Denosumab Group (O+D)]: Topical ozone, subcutaneous denosumab and graft were applied to the rats in this group.

Graft was applied to the defect area of all rats in the study. Control group; The defect was opened only on the calvarium with a trephine drill and the graft was applied. In the ozone group; Topical ozone was applied to the rats in this group after the graft was placed in the defect area. Denosumab Group; Rats in this group were injected subcutaneous denosumab after placing the graft in the defect area. In the ozone and denosumab group; After the graft was placed in the defect area, topical ozone was applied to the rats in this group and subcutaneous denosumab was injected. 5 animals from each group were sacrificed at the end of the 4th week, and the other 5 animals in each group were sacrificed at the end of the 8th week. The removed tissues were stored in fixative solution and examined histopathologically.

Surgery Prodecure

General anesthesia was achieved by intramuscular injection using Ketamine (Ketalar®, Pfizer, Turkiye) 50 mg/kg and Xylazine (Rompun®, Bayer, Turkiye) 10 mg/kg in the anesthesia applied for surgical procedures in all rats. Asepsis and antisepsis criteria were complied with during surgical procedures in all subjects. All instruments to be used were sterilized before the operation. After the calvarial area of all subjects was shaved, they were stained with povidone-iodine and covered with a sterile cover, leaving the operation area exposed. To reach the calvarium, approximately 24 mm long skin and subcutaneous incisions were made in the anterior-posterior direction. By blunt dissection, the outer surface of the calvarium was reached by removing the soft tissues and stripping the periosteum. The defect was prepared with a 5 mm diameter trephine bur attached to a contra-angle handpiece under physiological saline cooling. Bovine xenograft (Hypro-Oss, Bioimplon GmbH, Gießen, Germany) was placed in the defect area. Topical ozone (W&H Prozone Ozone generator, Bürmoos, Austria) was applied for 15 seconds after the graft was applied to the ozone group (O) and ozone+denosumab (O+D) groups. Denosumab group (D) and ozone and denosumab (O-D) groups were injected subcutaneously (s.c) with 10 mg/kg Prolia (ready-to-use injector containing Denosumab 60 mg SC solution for 23 injections, Amgen, Turkiye) every 4 weeks for 8 weeks. After the operation, the working area was closed primarily with 3/0 silk suture. For infection control, 30 mg/kg Cefazolin was administered intramuscularly (i.m.) post-operatively. Subjects were taken to the recovery room in metal cages. In the post-operative period, 30 mg/kg Cefazol (i.m) and Carprofen 4 mg/kg subcutaneously were administered to the subjects who were fed orally, once a day, for the first five days after the surgery.

Histopathological Analyzes

In each group, half were sacrificed in the 4th week and the other half in the 8th week. At the end of the 4th and 8th weeks, the rats were given high doses of anesthesia and euthanized using the cervical blockade method. For histopathological analyses, the calvarial bone was resected, the calvarium sample of each subject was fixed in Zinc-Formalin for at least 8 hours, and following the fixation process, it was decalcified with 15% nitric acid solution for 72 hours. These fixed and decalcified samples were embedded in paraffin and sections with an average thickness of 4-5 µm were taken from the paraffin blocks of each sample. H-E, Masson Trichrome and Mallory-Azan staining methods were used as histochemical staining methods. The samples were scored and statistical analysis was performed considering the criteria of osteoblastic activity, bone trabeculae, collagen fiber distribution, connective tissue cells and osteocyte cells.

Semi-Quantitative Histologic Scoring

Scoring was performed using a 0 to 3 scoring system (0, none; 1, minimal; 2, moderate; 3, abundant).

Statistical Analysis

The data obtained in this study were analyzed with the IBM SPSS Statistics Version 22 package program. While investigating whether the variables came from a normal distribution, Shapiro Wilk's was used due to the number of units. While examining the differences between the groups, the Kruskal Wallis-H test was used because the variables did not come from a normal distribution. If significant differences were seen in the Kruskal Wallis-H test, groups with differences were determined with the PostHoc multiple comparison test. When examining the difference between two dependent variables, the Wilcoxon test was used because the variables did not come from a normal distribution. The significance level was set as $p<0.05$.

RESULTS

In our research, a total of 40 Sprague-Dawley adult male rats with an average weight of 350 g were used, 10 in each group. Our study consisted of 4 groups: control group (C), ozone (O), denosumab (D), ozone and denosumab (OD), and 5 animals from each group were sacrificed at the end of the 4th week, and the other 5 animals in the group were sacrificed at the end of the 8th week. The removed tissues were subjected to histomorphological examination in fixative solution. During the experimental study, it was observed that the rats tolerated the surgical procedure well, there were no adverse effects in terms of their nutrition, no infection occurred due to the operation, and the general health of the subjects was good.

Histopathological Findings (Week 4)

1. Control group: In the 4th week, intense mononuclear cell infiltration was observed in the grafted area of the control group calvarial bone. In the graft area, small bone trabeculae with increased matrix, an increase in osteoblast cells, and a development in the direction of mature bone cells were observed. Thickening and irregular distribution of collagen fibers were observed (Figure 1).

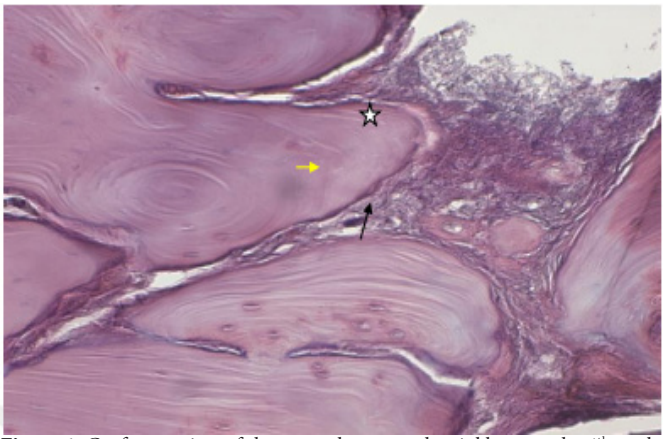

Figure 1. Graft area view of the control group calvarial bone at the 4th week. Dense mononuclear cell infiltration (*), small bone trabeculae (black →), Osteoblast cells (yellow →) (Staining: Hematoxylin-eosin (H-E), bar: 50µ

2. Ozone group: In the 4th week ozone group, in the area where the calvarial bone graft was applied, maturation of bone trabeculae and concentration in the matrix, an increase in osteoblastic activity around the bone trabeculae, and a prominence in mature osteocyte cells were observed. Within the graft area, an increase in normal connective tissue cells, hyperplasia in fibroblast cells, a decrease in inflammatory cells, thickening and irregular distribution in collagen fibers were observed (Figure 2).

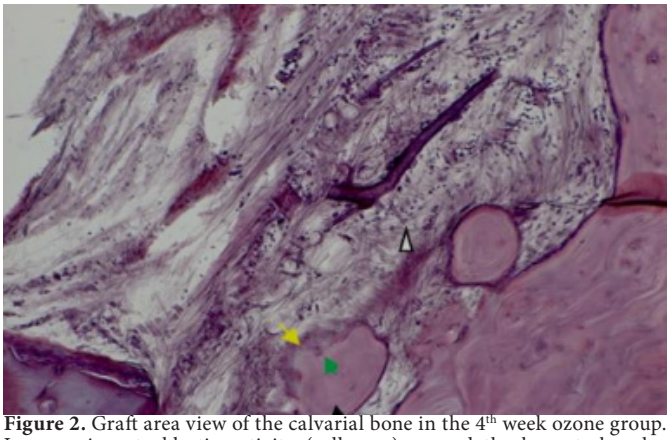

Figure 2. Graft area view of the calvarial bone in the 4th week ozone group. Increase in osteoblastic activity (yellow →) around the bone trabeculae (black →), prominence in osteocytecells (green →), increase in connective tissue cells (Δ) (Staining: Hematoxylin-eosin (H-E), bar: 50µ)

3. Denosumab group: In the 4th week denosumab group, in the area where the calvarial bone graft was applied, expansion of the bone trabeculae was observed due to the increase in matrix in the bone trabeculae, an increase in osteoblast cells in the periphery of the bone trabeculae, and a prominence in the bone lacunae. It was observed that there was a thickening of collagen fibers in the graft area and connective tissue cells with high mitotic activity due to a decrease in cell infiltration. It was observed that bone development accelerated and the number of bone cells increased due to the effect of the drug (Figure 3).

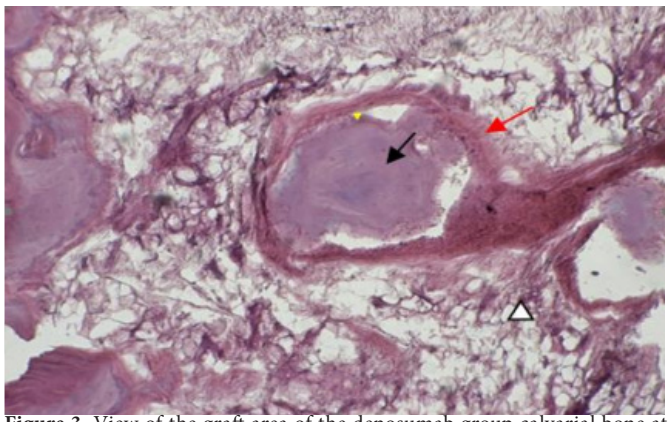

Figure 3. View of the graft area of the denosumab group calvarial bone at the 4th week. Expanded bone trabeculae (black →), dense collagen bands around the bone trabeculae (red →), increase in osteoblast cells (yellow →), significant increase in connective tissue cells (Δ) (Staining: Hematoxylin-eosin (H-E), bar: 50µ)

4. Ozone and Denosumab group: In the 4th week, in the ozone and denosumab group, it was observed that in the area where the calvarial bone graft was applied, collagen fiber synthesis increased, thickening of the fibers became evident, hypertrophy in fibroblast cells and an increase in connective tissue cells. Increased bone matrix development, hyperplasia of osteoblast cells in the periphery, and prominence of osteocyte cells within the lacuna were observed. It has been observed that new bone development accelerates further and there is a significant increase in the number of mature bone cells due to drug and ozone application (Figure 4).

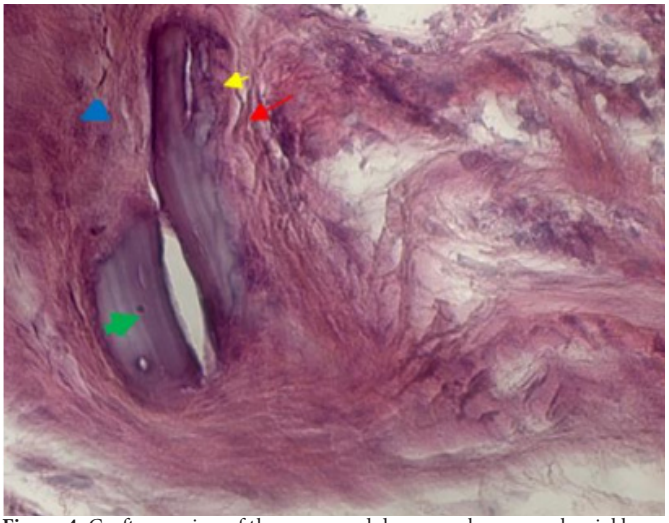

Figure 4. Graft area view of the ozone and denosumab group calvarial bone at the 4th week. Increase in collagen fiber synthesis (red →), prominence in osteocyte cells within the lacuna (green →), hypertrophy in fibroblast cells (blue →), increase in osteoblast cells (yellow →) (Staining: Hematoxylin -Eosin (H-E), bar: 50μ)

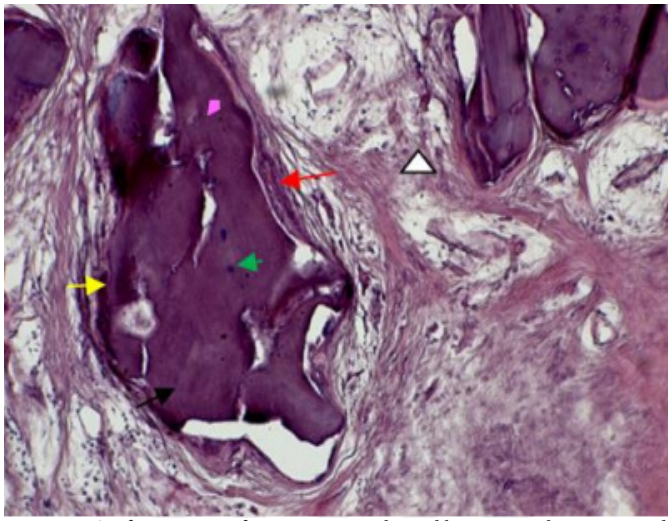

Figure 6. Graft area view of ozone group calvarial bone at week 8. Increased bone trabeculae (black →), dense collagen bands (red →), connective tissue cells (Δ), Osteocyte cells (green →), Osteoblast cells (yellow →), Osteon (pink →) (Staining: Hematoxylin-eosin (H-E), bar: 50μ)

Histopathological Findings (Week 8)

1.Control group: In the area where the calvarial bone graft was applied in the control group at the 8th week, the bone trabeculae in the graft area expanded due to the increase in osteoblastic activity, the amount of matrix increased, and the amount of osteocyte cells began to increase with the lacunae becoming evident in some places. It was observed that collagen bands were quite thick and connective tissue cells were diffusely increased (Figure 5).

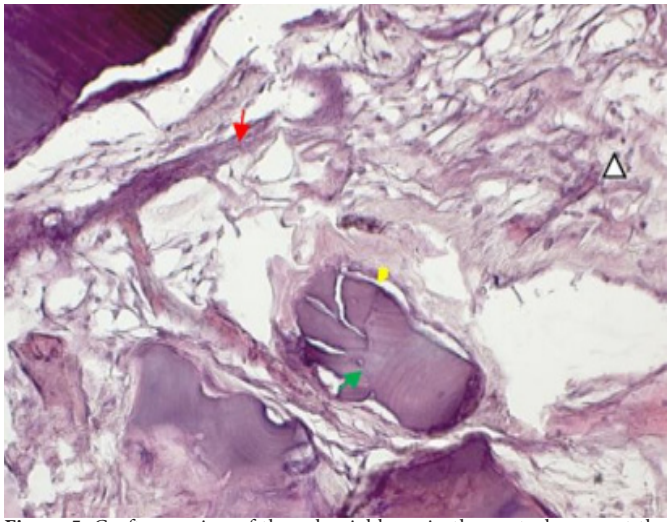

Figure 5. Graft area view of the calvarial bone in the control group at the 8th week. Increase in osteoblastic activity (yellow →) Increase in the number of osteocytes (green →), collagen fibers in the form of thick bands (red →), significant increase in connective tissue cells (Δ) (Staining: Hematoxylin-eosin (H-E), bar: 50μ)

2. Ozone group: In the 8th week ozone group, in the grafted area of the calvarial bone, bone trabeculae of varying sizes that had completed bone development were observed within the graft area. An increase in osteoblastic activity, prominence in osteocyte cells and an increase in the number of osteon structures were observed within these trabeculae. It was determined that collagen bands around the trabeculae in the graft area were extremely concentrated and the number of connective tissue cells increased (Figure 6).

3. Denosumab group: In the 8th week denosumab group, in the area where the calvarial bone graft was applied, a significant expansion of the bone trabeculae, a significant increase in osteoblastic activity in the periphery, an increased number of osteocyte cells and osteon structures located in the lacuna began to appear. In the graft area, it was observed that collagen fibers were formed in the form of tight and thick bands and there was an intense increase in connective tissue cells between them. New bone formation took shape (Figure 7).

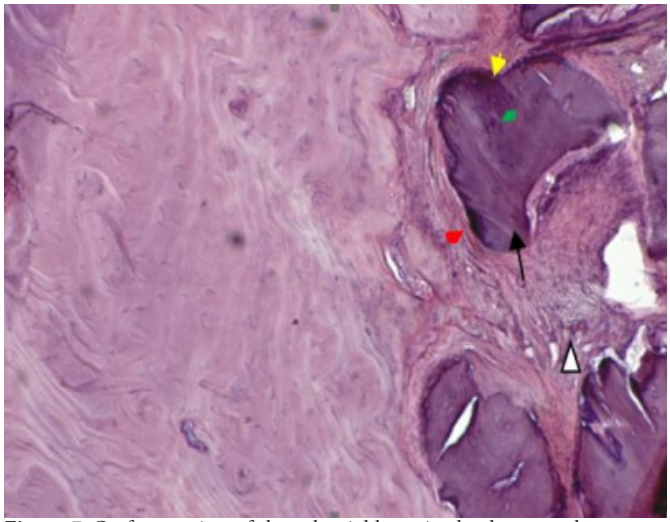

Figure 7. Graft area view of the calvarial bone in the denosumab group at the 8th week. Expanded bone trabeculae (black →), dense collagen bands (red →), connective tissue cells (Δ), Osteocyte cells (green →), Osteoblast cells (yellow →), (Staining: Hematoxylin-eosin(H-E), bar: 50μ)

4. Ozone and Denosumab group: In the 8th week, in the ozone+denosumab group, all elements suitable for new bone formation began to form in the expanded bone trabeculae in the area where the calvarial bone graft was applied. A significant hypertrophy of osteoblastic cells and an increased number of osteocyte cells settled in the lacuna were observed. Although osteon structures appear distinct, bone marrow has begun to form within the trabecular areas. Collagen fibers were arranged in thick and tight bands around the trabeculae,

and connective tissue cells increased in clusters. All findings regarding new bone formation were seen as positive in this group (Figure 8).

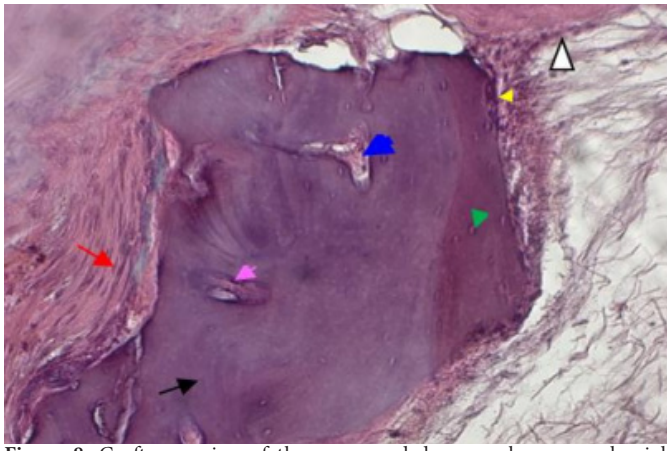

Figure 8. Graft area view of the ozone and denosumab group calvarial bone at the 8th week. Expanded bone trabeculae (black →), collagen fibers in the form of thick and tight bands (red →), increased connective tissue cells (Δ), a large number of increased osteocyte cells (green →) and Osteoblast cells (yellow →), prominent Osteon (pink →), formed bone marrow (blue →) (Staining: Hematoxylin-eosin (H-E), bar: 50μ)

There is a statistically significant difference between the groups in terms of bone formation 4th week scores ($p<0.05$). The bone formation score of the control group (K) at week 4 was significantly lower than the ozone and denosumab group (O+D) (Table1) (Figure 9).

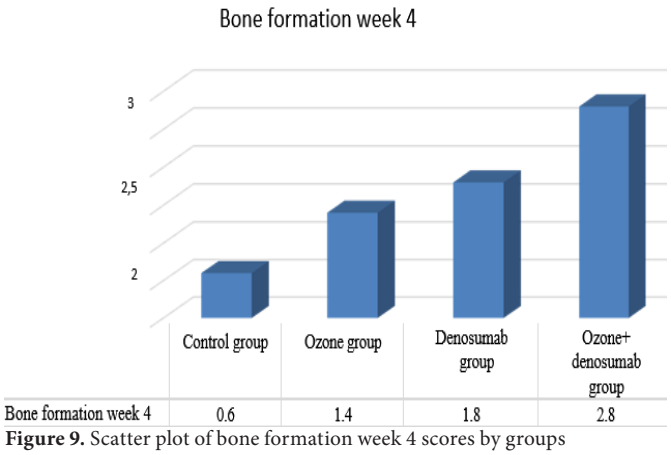

Figure 9. Scatter plot of bone formation week 4 scores by groups

There is a statistically significant difference between the groups in terms of bone formation 8th week scores ($p<0.05$). The bone formation 8th week score of the control group is significantly lower than the ozone and denosumab group (Figure 10) (Table 2).

There is statistically significant difference between the 4th week and 8th week findings in terms of bone formation scores in the control group ($p<0.05$). In the control group, the bone formation score at week 4 was significantly lower than the score at week 8. There is a statistically significant difference between the findings of the 4th week and the 8th week in terms of bone formation scores in the ozone group ($p<0.05$). In the ozone group, the bone formation score at week 4 was significantly lower than the score at week 8. There is a statistically significant difference between the 4th week and 8th week findings in terms of bone formation scores in the denosumab group ($p<0.05$). In the denosumab group, the bone formation score at week 4 was significantly lower than the score at week 8. There is a statistically significant difference between the 4th week and 8th week findings in terms of bone formation scores in the ozone and denosumab groups ($p<0.05$). In the ozone and denosumab group, the bone formation score at week 4 was significantly lower than the score at week 8 (Table 3) (Figure 11).

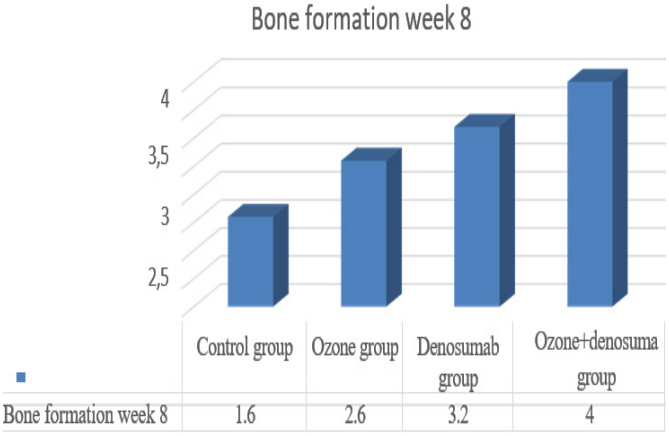

Figure 10. Scatter plot of bone formation week 8 scores by groups

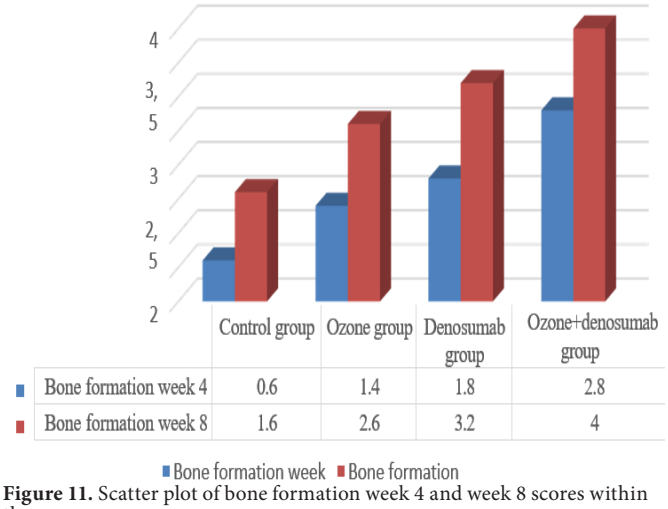

Figure 11. Scatter plot of bone formation week 4 and week 8 scores within the group

Table 1. Kruskal Wallis h test result regarding the difference between groups in terms of bone formation 4th week scores

		Group					Kruskal Wallis h Test		
		n	Mean	Median	Min	Max	SD	Mean Rank	h	p
Bone formation week 4	Control group	5	0.6	1	0	1	0.55	4.2	14.388	0.002
	Ozone group	5	1.4	1	1	2	0.55	8.8		
	Denosumab group	5	1.8	2	1	2	0.45	11.6		
	Ozone-denosumab group	5	2.8	3	2	3	0.45	17.4		
	Total	20	1.65	2	0	3	0.93	1-4		

Min: Minimum, Max: Maximum, SD: Standart deviation

Table 2. Kruskal Wallis h test result regarding the difference between groups in terms of bone formation 8th week scores

		Group					Kruskal Wallis h Test		
		n	Mean	Median	Min	Max	SD	Mean Rank	h	p
Bone formation week 8	Control group	5	1.6	2	1	2	0.55	3.6	16.139	0.001
	Ozone group	5	2.6	3	2	3	0.55	8.6		
	Denosumab gruop	5	3.2	3	3	4	0.45	12.3		
	Ozone+denosumab group	5	4	4	4	4	0	17.5		
	Total	20	2.85	3	1	4	0.99	1-4		

Min: Minimum, Max: Maximum, SD: Standart deviation

Table 3. Wilcoxon test result for the difference between bone formation 4th week and 8th week scores within the group

								Wilcoxon Test	
		n	Mean	Median	Min	Max	SD	Mean Rank	z	p
Control group	Bone formation week 4	5	0.6	1	0	1	0.55	0	-2.236	0.025
	Bone formation week 8	5	1.6	2	1	2	0.55	3		
Ozone group	Bone formation week 4	5	1.4	1	1	2	0.55	0	-2.121	0.034
	Bone formation week 8	5	2.6	3	2	3	0.55	3		
Denosumab group	Bone formation week 4	5	1.8	2	1	2	0.45	0	-2.07	0.038
	Bone formation week 8	5	3.2	3	3	4	0.45	3		
Ozon+denosumab group	Bone formation week 4	5	2.8	3	2	3	0.45	0	-2.121	0.034
	Bone formation week 8	5	4	4	4	4	0	3		

Min: Minimum, Max: Maximum, SD: Standart deviation

DISCUSSION

One of the most important areas of maxillofacial surgery is the restoration of bone volume lost for any reason. Since 1950, the use of modern medicine as well as regenerative and complementary medicine approaches on some disease groups has increased significantly. Ozone therapy, one of the popular applications of regenerative and complementary medicine, has been applied in private treatment centers in our country for many years. Ozone is a naturally occurring compound containing three oxygen atoms. It is normally found in the atmosphere and filters out the sun's harmful ultraviolet rays. Ozone therapy is the application of an ozone/oxygen mixture to the circulation or body cavities. Ozone therapy has been applied in medicine and dentistry for many years. Ozone has various effects such as antimicrobial, antihypoxic, immunomodulatory, biosynthetic and analgesic.[10]

Most published articles considering the use of ozone in dentistry concern the antimicrobial effects of ozone.[11-14] Additionally, there is not enough evidence for ozone application in oral and maxillofacial surgery.[15] We chose to use ozone in our thesis due to its therapeutic effect, which facilitates wound healing and improves blood flow.[16,17]

Various animal species are used in thesis studies in the field of maxillofacial surgery. When we look at the literature, animal studies conducted with denosumab were mostly found in rat, mouse, rabbit and monkey species.[18-21] We chose to use rats in our study in accordance with the literature.[18-21]

Denosumab is a new agent with antiresorptive potential used in the treatment of osteoporosis, which has been proven to increase bone mass, microarchitecture and durability in human and animal studies. The drug developed against RANKL (receptor activator of nuclear factor-κB-ligand) is a human monoclonal antibody. Besides these; It also acts to inhibit the differentiation and function of osteoclasts. Studies have reported that denosumab application has an antiresorptive effect on bone remodeling.[22] Denosumab acts as a decoy receptor by binding to RANKL, preventing it from binding to RANK. When the RANKL/RANK connection is blocked, the differentiation of preosteoclasts into mature osteoclasts, their activation and survival are inhibited, and RANKL cannot produce bone resorption. Thus, it reduces resorption in cortical and trabecular bone. Denosumab drug shows its antiresorptive feature by blocking the life and production of osteoclasts that cause bone destruction. It also reduces bone turnover and increases bone mineral denalsity.[23]

Alpan et al.[24] they placed a xenograft into the calvarial defect they created in diabetic rats. And they examined the effects of ozone on bone regeneration. According to the results of the study, it was determined that ozone applied in gaseous form increased xenograft resorption and accelerated early bone healing in diabetic rats.[24] In our study, it was determined that when ozone and denosumab were used together, they showed a synergistic effect and provided better results in new bone formation.

Buyuk et al.[25] examined the effects of ozone at various concentrations on bone healing in their study with 48 rats. The rats were divided into 4 groups, 10, 25, 40 µg/ml ozone was injected into the expansion area made in the premaxillary suture, and 1 ml saline solution was injected into the control group. Bone regeneration in the suture area was examined histomorphometrically and new bone area, fibrotic area, osteoblast, osteoclast number and vascularization were examined. All parameters examined were found to be significantly higher in the experimental groups than in the control group. It was reported that the values in the 25 µg/ml ozone applied group were higher than both the other experimental groups and the control group.[25] In our study, it was observed that ozone application alone increased new bone formation in the reconstruction of bone defects.

Ominsky et al.[19] they investigated the effect of denosumab on ovariectomized monkeys. They reported that denosumab reduced biological markers of bone remodeling and increased cortical and trabecular bone mass. They reported that denosumab increases bone etstrength by preserving bone quality and increasing bone mass.[19] In our study, it was observed that denosumab, which has antiresorptive properties in the repair of bone defects, increased new bone formation both alone and in combination with ozone.

Kostenuik et al.[21] reported that, as a result of their study, they detected an increase in both trabecular and cortical bone mass in monkeys treated with denasumab. In our study, when the control and denosumab groups were compared, it was observed that the drug denosumab significantly increased the formation of new bone area.

Gerstenfeld et al.[26] in their study, they investigated the effects of alendronate and denosumab on fracture healing in a mouse model. A fracture line was created in the femur region and denosumab (10 mg/kg) and alendronate (0.1 mg/kg) were injected in groups. They found that the bone volume at the fracture line was higher and the bone structures were harder and more durable in the denosumab-treated group.[26] The results obtained from our study are in line with the literature.

Bernhardsson et al.[27] they investigated the effect of denosumab on screw fixation in their study on rats. They found that the drug denosumab increased bone density more than alendronate. Additionally, they reported that denosumab increased screw fixation in cancellous bone more than bisphosphonate.[27] In our study, it was observed that the application of denosumab, which has antiresorptive properties, in the repair of bone defects, increased new bone formation both alone and in combination with ozone.

CONCLUSION

When the findings obtained as a result of our study were evaluated, the following conclusions were reached:

- It has been observed that ozone application alone increases new bone formation in the reconstruction of bone defects.
- It has been observed that the application of denosumab, which has antiresorptive properties in the repair of bone defects, increases new bone formation when used alone.
- It was determined that when ozone and denosumab were used together, they showed a synergistic effect and gave better results in new bone formation.
- There are many different studies in the literature showing that ozone has a positive effect on new bone formation. The results in our study are similar to other studies.
- In line with the results, we think that denosumab application can be used in operations to increase bone volume. Studies investigating long-term results can be conducted on this subject. On the other hand, it was deemed necessary to evaluate this treatment in terms of dose and duration with animal and clinical studies involving larger experimental groups before clinical applications.
- When we look at the results of our thesis, we think that it will benefit clinical studies, but it should be supported by additional studies.

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