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Research Article

EVALUATION OF THE EFFICACY OF SYSTEMICALLY APPLIED DENOSUMAB IN THE REPAIR OF BONE DEFECTS

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ABSTRACT

This study investigated the effect of denosumab on new bone formation, both alone and in combination with different graft materials. To this end, four defects were created in the skulls of 20 New Zealand rabbits. One defect was left empty, while the other three were treated with autograft, xenograft, and calcium phosphate (BCP) grafts. The animals were divided into an experimental group (n=10), which received 10 mg/kg of denosumab subcutaneously once a month for 2 months, and a control group (n=10), which was administered the same dose of physiological saline. After 8 weeks, the animals were sacrificed, and their bone tissue samples were analyzed histomorphometrically. The percentage of bone volume (PBV) was calculated by dividing the area of mature bone tissue by the total tissue area. The PBV in empty defects treated with denosumab was significantly higher than in the control group ($p < 0.05$). Moreover, the combination of denosumab and autograft resulted in a significant increase in new bone formation (NBF) ($p < 0.05$), thus demonstrating the positive effect of denosumab on bone formation. However, further research with larger experimental groups would be needed to determine the practicality, dosage, and duration of this treatment before clinical application.

INTRODUCTION

Major goals of oral and maxillofacial surgery include restoring the anatomical form of the defects caused by various reasons in the mouth and jaw region and regaining the lost functions. Although some of these bone defects resolve through the self-repairing ability of the bone, in large bone defects, various bone graft materials are needed to support the repair of bone tissue or to ensure complete healing of the defects. The harvesting and structural properties of graft materials used for the treatment of bone defects, as well as their effectiveness in new bone formation, show remarkable variation. Among these materials, autogenous bone grafts are commonly used and are known to have osteoinductive and osteoconductive properties. However, the use of these grafts is limited by several factors such as the requirement for an additional surgery, recipient site infections, and inability to obtain sufficient amounts of graft material (Pandit et al., 2012). A viable alternative to autogenous grafts is allografts. However,

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while they proved to be are superior to autogenous grafts, theyir use is associated with several disadvantages, including debated osteoinductive capacity, potential for immunological response in the recipient tissue, and the risk of infectious diseases (Giannoudis et al., 2005). Furthermore, xenografts are widely used in oral surgery and bovine xenografts. In particular, xenografts are frequently preferred because of their similarity to human bone structure, biocompatibility, favorable osteoconductive structure (Stavropoulos & Karring, 2010). Tricalcium phosphate (TCP) and hydroxyapatite(HA) are synthetic bone graft substitutes that do not have an osteoinductive effect and serve only as a skeleton for new bone formation in the defect area (Erbe et al., 2001)

In recent years, various drug applications have become popular in the repair of defects. Of these, a commonly used application is denosumab, which has been extensive applied in the prevention of skeletal adverse events (pain and fractures in the bone) secondary to multiple myeloma or bone metastases from solid tumors (Ahern et al., 2018). More specifically, denosumab was used in adults and skeletally mature adolescents with an unresectable tumor or in cases where surgical resection may cause serious morbidity (Jamshidi et al., 2018), in postmenopausal osteoporotic women with a high risk of bone fracture (Tsourdi et al., 2019), in glucocorticoid (GC)-induced osteoporosis (GCOP) (Iwamoto et al., 2019), and in loss of bone mineral density (BMD) caused by androgen deprivation (Briot et al., 2019). Denosumab was originally approved in 2012 by the Food and Drug Administration (FDA) for the treatment of postmenopausal osteoporotic women at a high risk of fracture and unresponsive to other treatment applications, as well as for the treatment of male patients at a high risk of fracture (Goldhahn et al., 2012; Schwarz & Ritchlin, 2007;). Previosu research also demonstrated that denosumab increases BMD and reduces the risk of fractures (Bone et al., 2008; Miller et al., 2008; Lewiecki et al., 2007; Cummings et al., 2009).

Overall, to date, numerous studies have sought to improve the healing of graft materials (Özer et al., 2019, 2022). Contributing to this body of research in the present study, we aimed to investigate the effect of denosumab on bone regeneration by administering denosumab systemically using three different graft materials with osteoinductive or osteoconductive properties in the defects created in the rabbit calvaria, as well as to compare the effects of denosumab on grafts with different characteristics.

MATERIAL AND METHOD

Ethical Approval and Research Design

The study was approved by İnonuUniversity Medical School Experimental Animals Ethics Committee (Approval No: 2013/A-52). All experiments were conducted at İnonu University Experimental Animal Production and Research Laboratory. A total of 20 male New Zealand rabbits with an average weight of 3 kg and aged on average 7 months were used in the experiment. All rabbits were kept in experimental cages in a 12/12-h light/dark cycle at an average temperature of 24 °C and were given ad libitum access to standard food and tap water. The health status of the animals was monitored by a specialist veterinarian. The animals were divided into two groups: the experimental group (n=10) and the control group (n=10). A total of four bicortical defects of 6 mm in diameter were made in the calvarium of each rabbit using a trephine bur (Hokugo et al., 2007). Then, eEqual volumes of autograft, xenograft, and biphasic calcium phosphate (BCP) were administered to defects, while the remaining defect was left intact. The experimental group received subcutaneous injection of 10 mg/kg Prolia (Denosumab 60 mg solution for injection in pre-filled syringe, Amgen, Turkey) once a month for 2 months, while the control group received subcutaneous injection of 10 mg/kg saline during the same period of time. All animals were sacrificed 8 weeks later with intravenous sodium pentothal (Pentotal, Abbott, USA) and bone tissue samples were sent to the Histology and Embryology Laboratory of İnonuUniversity Faculty of Medicine for histological and histomorphometric examination. Defects in each rabbit were labeled as A, B, C and D. Defect A was left intact in each rabbit; Defect B was filled with the autogenous graft material formed as a result of grinding the bone obtained from the defect using a bone grinder; Defect C was filled with OsteoBiol®Gen-Os (OG) (TecnossDental, Turin, Italy) graft material, which is a bovine-derived xenograft. Finally, Defect D was filled with MİS®4BONE (MB) (MIS, Tel Aviv, Israel) graft material, a fully synthetic graft material made of hydroxyapatite (HA) (60%) and β-TCP (40%) (Figure 1).

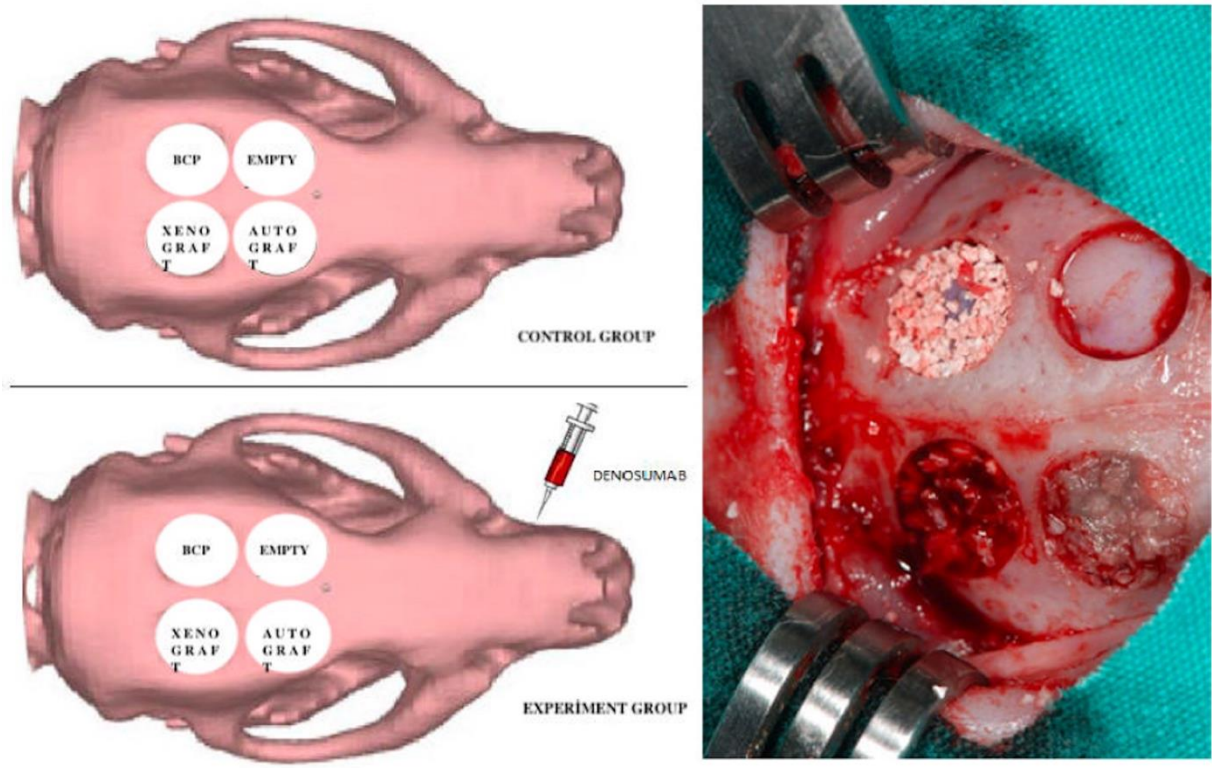

Figure 1.Denosumab-treated control and experimental groups

Histological and Histomorphometric Examination

Bone tissue samples were fixed in 10% formaldehyde for 72 h. After the fixation process, the samples were subjected to decalcification for 12 days in 10% formic acid solution, which was changed every other day. Following the decalcification process and after dehydration through increasing grades of ethanol (50%-99%) and xylene series, the samples were embedded in paraffin blocks. Subsequently, 6-µm thick sections were obtained from the paraffin blocks using a microtome and were mounted on slides. The sections were stained with hematoxylin-eosin(H&E) and Gomori's trichrome staining and then examined under a Leica DFC280 light microscope, with photographs taken using a Leica Q Win Plus V3 image analysis system (Leica Microsystems Imaging Solutions, Cambridge, UK). The total tissue area in each section and the mature bone tissue areas within this total tissue area were measured on the photographs. The newly formed bone percentage was calculated by dividing the mature bone tissue area to the total tissue area in each section (Gül et al., 2014).

Statistical Analysis

The data were analyzed using SPSS for Windows version 22.0 (Armonk, NY: IBM Corp.). Normal distribution of the data was assessed with Shapiro Wilks test. The groups were compared using Kruskal-Wallis test, in which a *p*-value of < 0.05 was considered to be

statistically significant, followed by post-hoc Mann Whitney U test with Bonferroni correction, in which a *p*-value of < 0.008 was considered significant.

RESULT

Histological sections of bone tissues in the defects were examined in both experimental and control groups. The results revealed d that the bone tissue showed osteoconductive activity in the site extending from the surgical margins to the defect area in all groups. In addition, fibrous connective tissue and bone tissue were observed in the defect areas in all groups (Figures 2, 3).

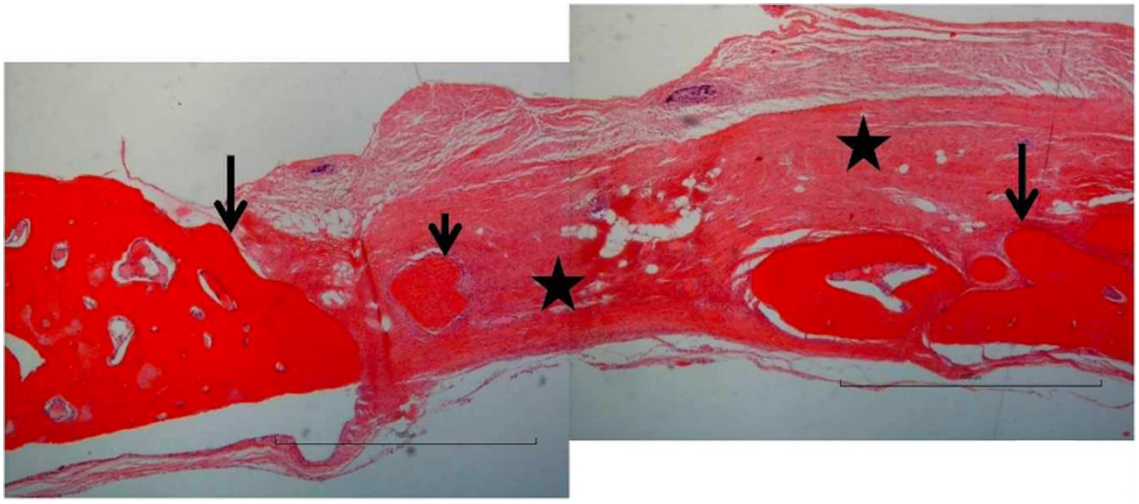

Figure 2a. General histological appearance of control group A (arrows: bone tissue and osteoconductive bone formation at surgical tips, star: fibrous connective tissue, arrowhead: mature bone tissue fragments. H-E, Scale = 1000 µm.)

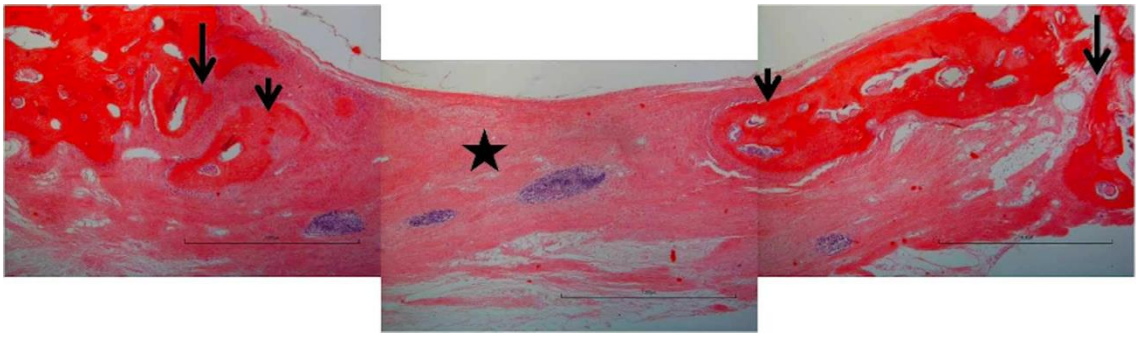

Figure 2b. General histological appearance of experimental group A (arrows: bone tissue and osteoconductive bone formation at surgical tips, star: fibrous connective tissue, arrowhead: mature bone tissue fragments. H-E, Scale = 1000 µm.)

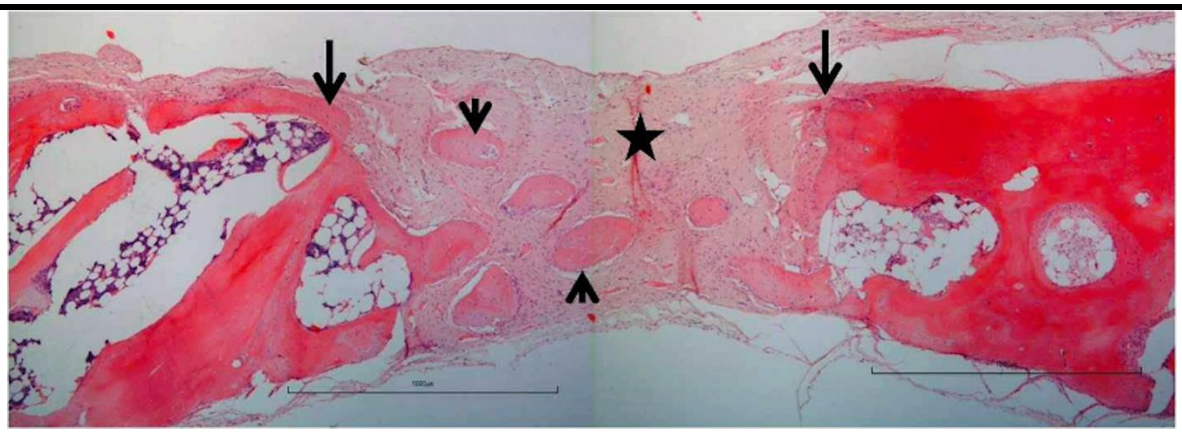

Figure 2c. General histological appearance of control group B (arrows: bone tissue and osteoconductive bone formation at surgical tips, star: fibrous connective tissue, arrowhead: mature bone tissue fragments. H-E, Scale = 1000 μm.)

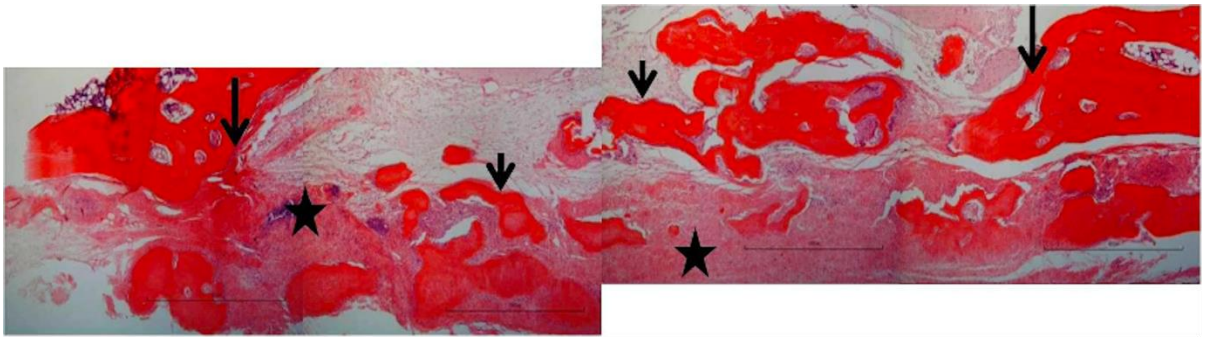

Figure 2d. General histological appearance of experimental group B (arrows: bone tissue and osteoconductive bone formation at surgical tips, star: fibrous connective tissue, arrowhead: mature bone tissue fragments. H-E, Scale = 1000 μm.)

Figure 2e. General histological view of control group C (arrows: bone tissue and osteoconductive bone formation at surgical tips, star: fibrous connective tissue, arrowhead: mature bone tissue fragments. H-E, Scale = 1000 μm.)

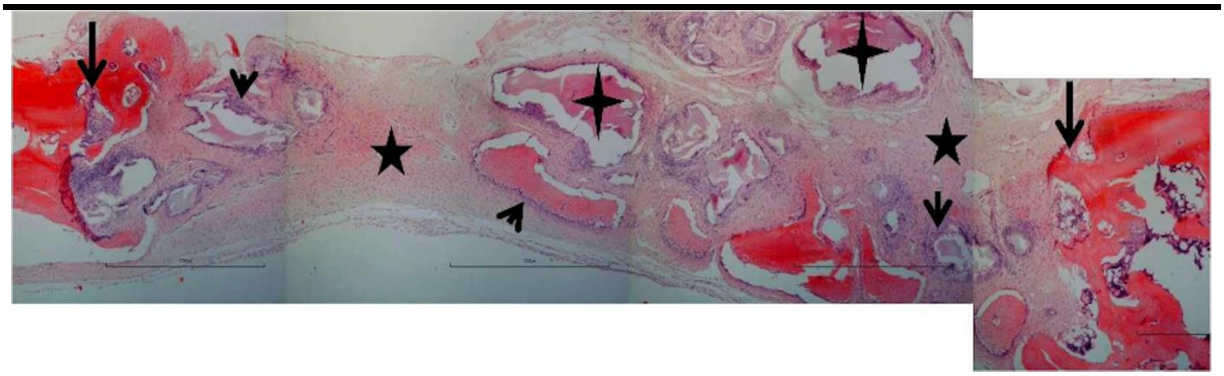

Figure 2f. General histological view of experimental group C (arrows: bone tissue and osteoconductive bone formation at surgical tips, star: fibrous connective tissue, four-point star: graft areas arrowhead: osteoblastic-osteogenic activity around graft areas. H-E, Scale = 1000 µm.)

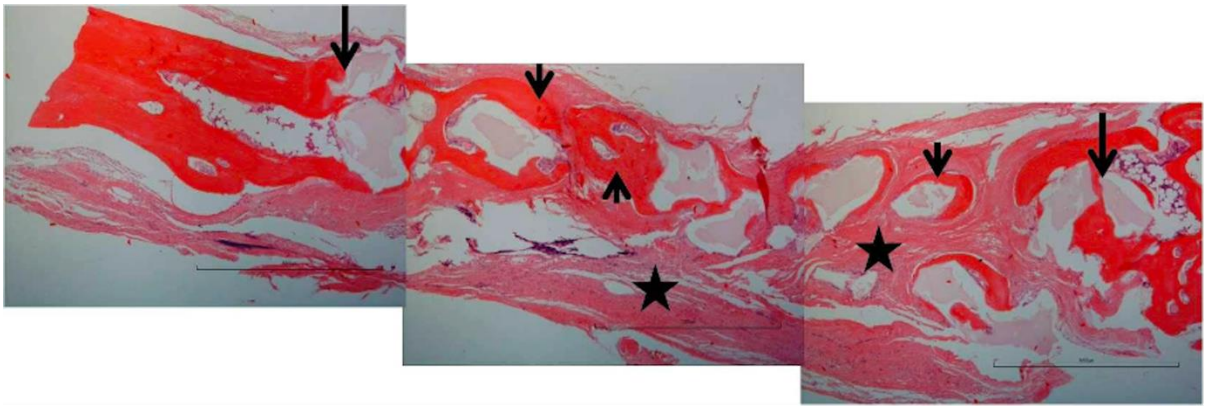

Figure 2g. General histological appearance of control group D (arrows: bone tissue and osteoconductive bone formation at surgical tips, star: fibrous connective tissue, arrowhead: mature bone tissue fragments. H-E, Scale = 1000 µm.)

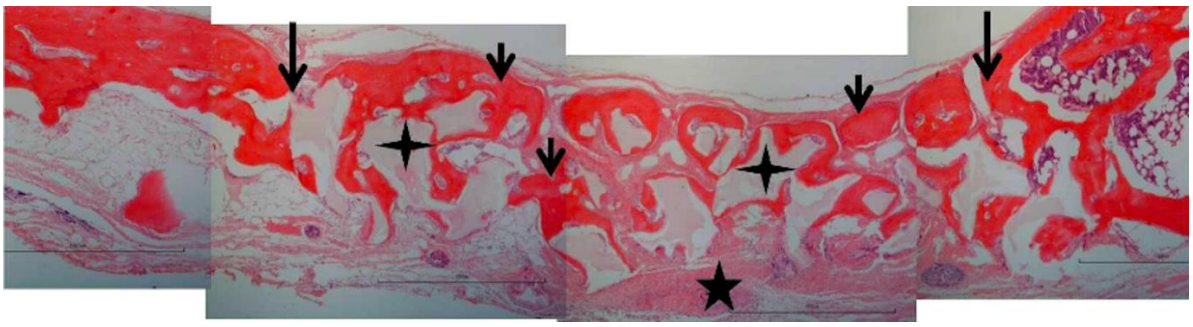

Figure 2h. General histological appearance of experimental group D (arrows: bone tissue and osteoconductive bone formation at surgical tips, star: fibrous connective tissue, four-point star: graft areas arrowhead: mature bone tissue fragments in trabecular structure H-E, Scale = 1000 µm .)

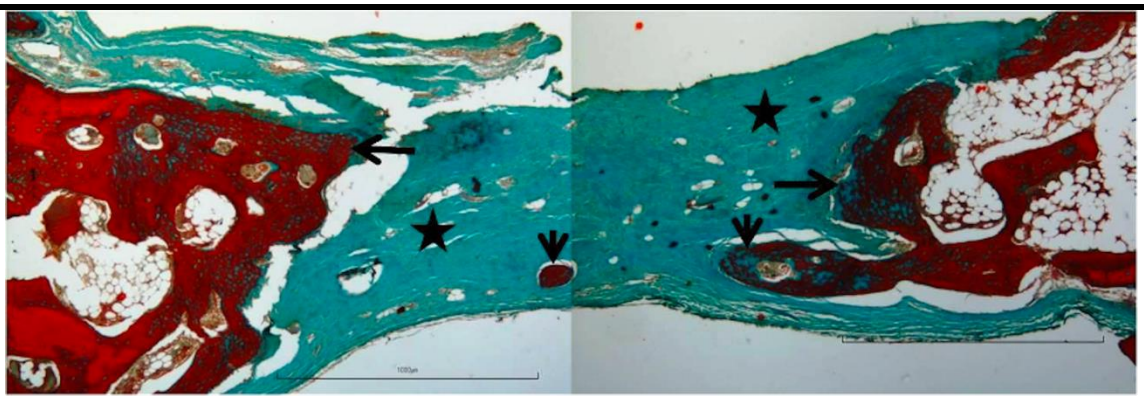

Figure 3a. Example photograph of control group A (arrows: bone tissue and osteoconductive bone formation at surgical tips, star: fibrous connective tissue, arrowhead: mature bone tissue pieces Trichrome, Scale = 1000 µm.)

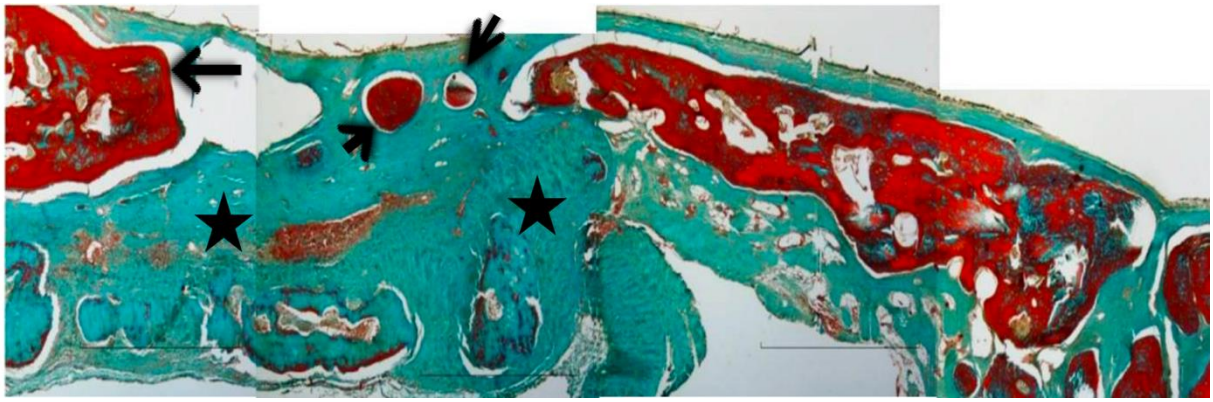

Figure 3b. Example photograph of experimental group A (arrows: bone tissue and osteoconductive bone formation at surgical tips, star: fibrous connective tissue, arrowhead: mature bone tissue fragments, double-headed arrow: osteoid tissue Trichrome, Scale = 1000 µm.)

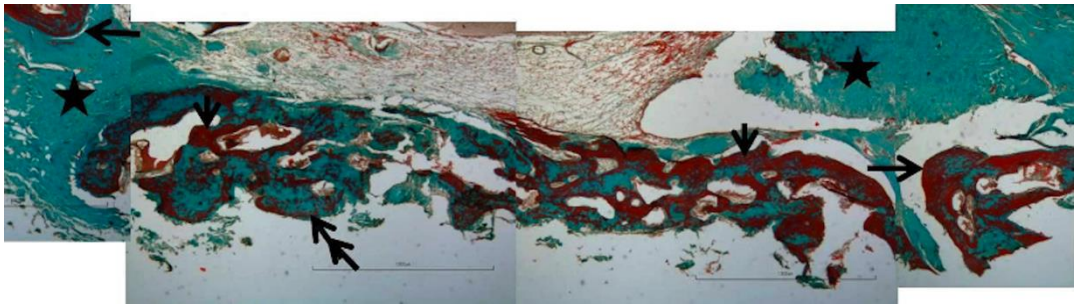

Figure 3c. Example photograph of control group B (arrows: bone tissue and osteoconductive bone formation at surgical tips, star: fibrous connective tissue, arrowhead: mature bone tissue fragments, double-headed arrow: immature bone tissue trabecular Trichrome, Scale = 1000 µm.)

Figure 3d. Example photograph of experimental group B (arrows: bone tissue and osteoconductive bone formation at surgical tips, star: fibrous connective tissue, arrowhead: mature bone tissue pieces Trichrome, Scale = 1000 µm.)

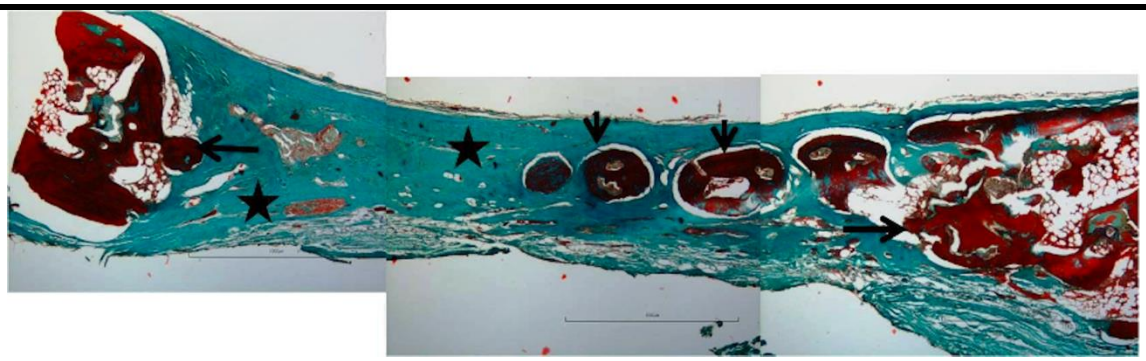

Figure 3e. Example photograph of control group C (arrows: bone tissue and osteoconductive bone formation at surgical tips, star: fibrous connective tissue, arrowhead: mature bone tissue pieces Trichrome, Scale = 1000 µm.)

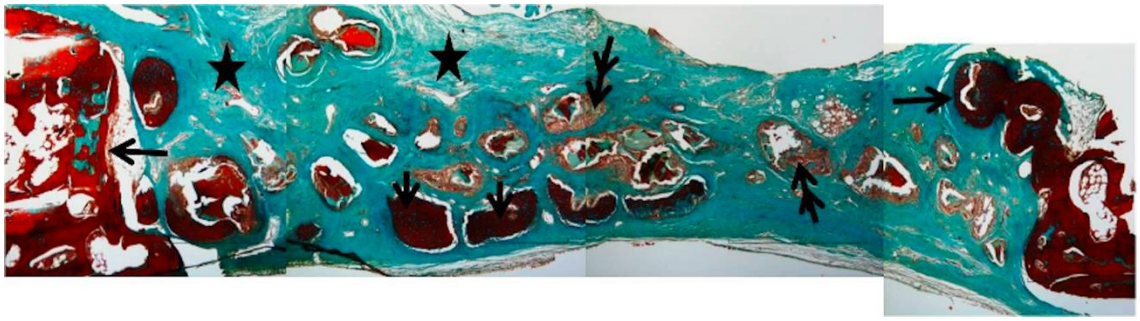

Figure 3f. Example photograph of experimental group C (arrows: bone tissue and osteoconductive bone formation at surgical tips, star: fibrous connective tissue, arrowhead: mature bone tissue fragments, double-headed arrow: osteogenic activity around graft areas Trichrome, Scale = 1000 µm.)

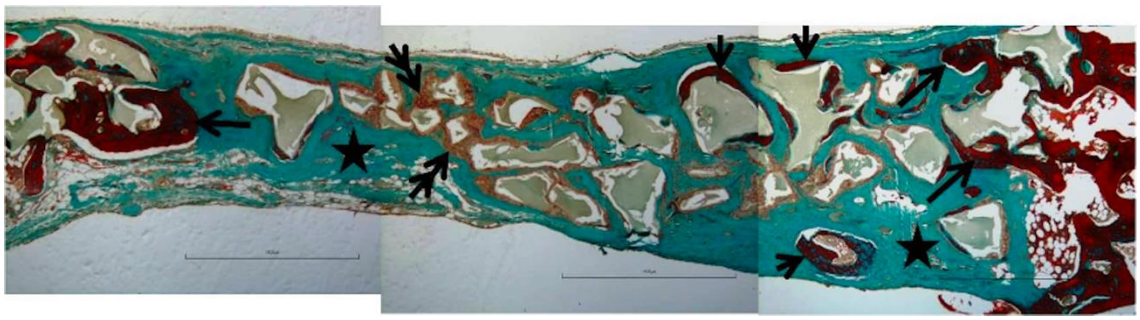

Figure 3g. Example photograph of control group D (arrows: bone tissue and osteoconductive bone formation at surgical tips, star: fibrous connective tissue, arrowhead: mature bone tissue fragments, double-headed arrow: osteogenic activity at the periphery of graft areas Trichrome, Scale = 1000 µm.)

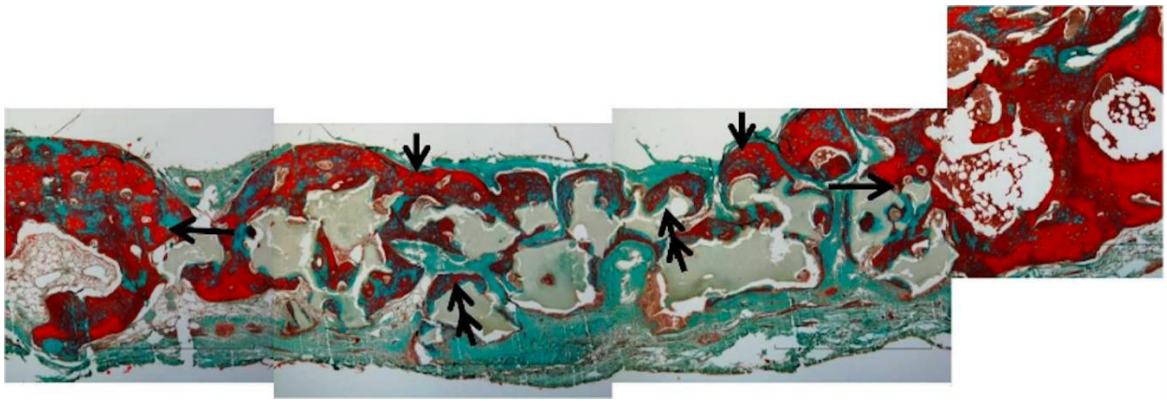

Figure 3h. Example photograph of experiment group D (arrows: bone tissue and osteoconductive bone formation at surgical tips, star: fibrous connective tissue, arrowhead: mature bone tissue pieces, double-headed arrow: immature bone tissue pieces around graft areas Trichrome, Scale = 1000 µm.)

The results of histomorphometric analysis of bone defects did not reveal a statistically significant difference between the averages of new bone formation obtained in the defects in the control group (p = 0.068) (Table 1). However, we found a statistically significant difference between the averages of new bone are (NBF) in the defects in the experimental group (p = 0.015). The averages of new bone area (NBF) obtained in Experimental groups C and D, as well as in Control group B, were significantly lower than in Experimental group B (p = 0.08, p = 0.007, p = 0.032, respectively). The mean new bone area obtained in Experimental group A was significantly higher than in Control group A (p = 0.021), while Experimental group B was significantly higher than Control group B (p = 0.032) (Table 1). Finally, the percentage of new bone formation (PBV) values obtained by proportioning the newly formed bone to the total tissue area were statistically significantly higher in Experimental group A than in Control group A (p = 0.037) (Table 2).

Table 1. New bone formation (μm²)

	Control	**Experimental**	
	(Mean±SD)	**(Mean±SD)**	**[1]*p***
Group A	308794.43±534242.25	862607.75±528867.54[a]	0.021*
Group B	629952±441453.39[b]	1280422±472561.26[c]	0.032*
Group C	1004034.8±554413.45	518225.88±461998.31[b]	0.107
Group D	642232.57±380579.12	432666.14±504658.1[b]	0.225
[2]*p*	**0.068**	**0.015***	

[1]Mann-Whitney U test [2]Kruskal-Wallis Test *$p < 0.05$
[a]: Significantly higher than in Control group A [b]: Significantly lower than in Experimental group B
[c]: Significantly higher than in Control group B.
SD: Standard deviation

Table 2. Percent bone volume

	Control	**Experimental**	
	(Mean±SD)	**(Mean±SD)**	**[1]*p***
Group A	15±17	33±15[a]	0.037*
Group B	27±13	38±2	0.199
Group C	36±2	19±18	0.187
Group D	24±12	14±14	0.180
[2]*p*	**0.173**	**0.060**	

[1]Mann-Whitney U test [2]Kruskal-Wallis Test *$p < 0.05$
[a]: Significantly higher than in Control group A
SD: Standard deviation

DISCUSSION

Over time, bones can lose density or undergo various injuries. Therefore, reconstruction of bone defects has been extensively studied both in orthopedics and maxillofacial surgery

research (Becker et al., 1994; Raghoebar et al., 2001; Von Arx & Buser, 2006) were graft materials were widely used in the treatment of bone defects. Graft types commonly used in maxillofacial surgery include autografts, xenografts, allografts, and synthetic grafts, which have differing properties and may have at least one or more of the features such as osteogenesis, osteoconduction, and osteoinduction (Kahnberg, 2008). Autografts, which are the first choice in the repair of bone defects, are the golden standard for bone transplantation, mainly because this graft type contains osteogenic cells and does not cause immunological reaction (Banwart et al., 1995; Younger & Chapman, 1989). In the present study, we assumed that systemically applied denosumab would lead to faster ossification by inducing osteoblastic activity in autogenous, xenogenous, and allogeneic graft applied defects. The results revealed that denosumab increased ossification.

Von Arx and Buser (2006) obtained autogenous bone grafts from the symphysis or ramus region to achieve horizontal ridge augmentation in 42 patients and suggested that intraoral autogenous graft application can be successfully used to achieve ridge augmentation. In another relevant study, Raghoebar et al. (2001) performed maxillary sinus augmentation by using autogenous bone grafts obtained from the iliac bone, symphysis region, and maxillary tuber in 99 patients. The authors reported that the use of autogenous bone graft in maxillary sinus augmentation can provide favorable and reliable long-term outcomes (Raghoebar et al., 2001). Furthermore, Becker et al. (1994) compared autogenous bone grafts with demineralized freeze-dried bone allografts (DFDBAs) and concluded that, although new bone formation was observed after 3 months in the areas where autogenous grafts were inserted, new bone formation was not observed in six out of seven areas where DFDBAs were inserted. The authors also noted that DFDBA could disrupt bone healing (Becker et al., 1994). In the present study, we induced a bone defect by placing an autograft in the defect area after grinding the harvested autogenous bone in a bone grinder. We also found that the mean rate of bone healing was significantly higher in the experimental group than in the control group.

Despite all these positive features, autogenous bone graft materials have several limitations, such as insufficient amount of graft material, requirement of a second surgical site, and prolonged postoperative recovery(David et al., 1990). Because of these disadvantages, allografts, xenografts, and alloplastic materials have become prominent alternatives to autogenous grafts in regenerative treatment of bone defects. The primary aim in using these materials is to facilitate bone regeneration and to use the material that is most similar to bone tissue (Kent et al., 1987). Fresh and freeze-dried allografts are highly antigenic, while freeze-dried and demineralized allografts are minimally antigenic or non-antigenic (Friedlaender et

al., 1976). Xenografts are widely used in oral surgery and appear as a good alternative to other bone grafts. Deproteinized bovine bone grafts are commonly used in maxillofacial surgery, sinus floor augmentation, alveolar crest augmentation, treatment of bone defects around teeth and implants, repair of mandibular and maxillary defects(Hollinger, 1986). Alloplastic grafts, which can be produced in unlimited quantities, are a promising alternative to allografts. These grafts are composed of ceramics, hydroxyapatites derived from sea corals, and bioactive glasses. Synthetic grafts made of hydroxyapatite and tricalcium phosphate are widely used in cranio-maxillofacial, orthopedic, and oral surgery. These graft materials are biocompatible and function as an effective skeleton in new bone formation (Antunes et al., 2013; Cordaro et al., 2008). For instance, Nemcovsky and Serfaty (1996) used hydroxyapatite crystals in 23 extraction sockets, closed the socket primarily by rotating the flap, and followed up the patients for 24 months. The authors indicated that alveolar ridge preservation with minimal ridge deformation provided beneficial outcomes (Nemcovsky & Serfaty, 1996).

Furthermore, Artzi et al. (2004) created a defect in the canine mandible, applied xenograft and BCP grafts to the defects, and observed the bone healing in the grafts at different time points. The results indicated that the xenograft was not completely resorbed at the end of 6 months, while the BCP graft was completely resorbed in 24 months. At the end of the study, complete bone healing was achieved in all grafted defects (Artzi et al., 2004). Of note, no significant difference between the control and experimental groups (in which xenograft and BCP graft were applied) was observed with regard to mean percentage of new bone formation. This could be due to the fact that this study period was not sufficient for the resorption of these grafts and that the drug we applied reduced the osteoclastic activity, thereby preventing resorption.

Denosumab, an agent with antiresorptive potential, has long been used in the treatment of osteoporosis in humans and animals, showing a positive effect on bone microarchitecture, such as increasing bone mass, microarchitecture, and strength (Deeks, 2018). Denosumab exerts its antiresorptive properties by blocking the maturation, function, and survival of osteoclasts that cause bone resorption. Moreover, it was also reported to improve bone turnover and increase BMD (Suresh & Abrahamsen, 2015). However, while numerous osteoporotic or non-osteoporotic animal studies documented positive effects of denosumab on bones, to the best of our knowledge, none of the previous studies evaluated the effect of denosumab in the healing of grafted defects. A previous study investigated the effect of denosumab and alendronate in the healing of femoral fracture in mice and reported that the callus tissue obtained from mice treated with denosumab had a significantly higher percent bone volume (PBV) and

one mineral density (BMD) as compared to the control and alendronate-treated groups at both 21 and 42 days (Gerstenfeld et al., 2009). In another pertinent study on the effect of denosumab on ovariectomized monkeys, Ominsky et al. found that denosumab reduced the level of biological markers of bone remodeling and increased the cortical and trabecular bone mass. The authors also reported that denosumab improved the bone strength by preserving bone quality and increasing the bone mass (Ominsky et al., 2011). Similarly, Kostenuik et al. (2009) reported that both trabecular and cortical bone mass increased in monkeys treated with denosumab. The autyhors found that denasumab decreased the bone resorption, increased the cortical and cancellous bone mass, and increased the trabecular structure on the micro level. In a 2022 study on the effects of a combined use of denosumab with xenogenic bone grafts on the healing of the defect, Özer et al. (2022) found that the combination therapy had no direct effect on new and total bone volume. In the present study, a comparison of the unfilled defects of the control and experimental groups revealed that denosumab significantly increased the new bone formation. In addition, the new bone formed in autografted defects was found to be significantly greater in the experimental group than in the control group.

CONCLUSION

The results of the present study revealed that the administration of denosumab, which has antiresorptive properties in the repair of bone defects, increased the new bone formation. Prior to clinical use of denosumab treatment, further animal and clinical studies involving larger experimental groups would be needed to substantiate practicality, dosage, and duration of this treatment.

Ethics Committee Approval: This study was approved by the İnonuUniversity Medical Faculty Experimental Animals Ethics Committee with the decision dated 20-06-2013 and numbered 2013/A-52.

Conflict of Interest: The authors confirm that they have no conflict of interest.

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