Clinical and Experimental Health Sciences

Radiographic Evaluation of the Effect of Vitamin D3 Supplementation on Regeneration of Calvarial Bone Defects in Rats

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ABSTRACT

Objective: The present study was aimed to evaluate radiographically the effect of orally administered vitamin D3 on guided bone regeneration in calvarial critical size defects (CSD) in rats.

Methods: Two calvarial CSD were created in 12 male Sprague-Dawley rats. One of the defects was left empty (E defect), while the other one was treated with deproteinized bovine bone graft and collagen-based resorbable membrane (GM-filled defect). Following surgical intervention, rats were randomly assigned into two groups; the control group was administered distilled water, and the test group was treated with 2 µg /kg vitamin D3 by gavage once a day for 8 weeks. Radiological images were obtained from rats on 4th and 8th weeks. The area fraction of newly formed osteoid was determined using Image Fiji Analysis Software.

Results: The percentages of area fraction in the GM-filled defects were statistically higher than the E defects in both study groups at 4th and 8th weeks (p<.0001). In both E and GM defects, the percentage of area fraction was higher at weeks 4 and 8 in the test group compared to the control groups (p<.0001). In comparison to the other groups, the GM-filled defect in the test group had the highest mean percentage of area fraction (p<.0001).

Conclusion: This study demonstrated that healing of CSD could be evaluated by radiography and Vitamin D3 improves bone healing, particularly when guided bone regeneration is used in rats with CSD at the calvaria.

Keywords: Bone regeneration, radiography, Vitamin D3.

1. INTRODUCTION

Alveolar bone loss continues to be a concern in the field of oral rehabilitation since congenital illnesses, tumors, and trauma can all cause major face bone abnormalities that are difficult to correct both functionally and aesthetically (1, 2). As a well-known fact, if the optimal method is not used for bone formation, the natural structure of the bone cannot be accomplished, and as a result, unfavorable fibrous tissue forms during the healing process (3). Guided bone regeneration (GBR), is a well-established method for treating bone defects (4). This procedure allows for the filling of a space maintained by either resorbable or non-resorbable barrier membranes with bone, allowing for the regeneration of bone tissue (5, 6). An essential component of the procedure is the membrane inhibits apically downgrowth of epithelium. Biocompatibility, clinical management, integration by the host tissues, the capacity to create space, and acceptable mechanical and physical properties are positive attributes of the membrane used for GBR (7). The first generation of barrier membranes consisted of non-resorbable membranes. These membranes typically exhibit biocompatibility and the ability to create space (8). However, non-resorbable membranes require a second surgical procedure to be removed. A second generation of membranes made of resorbable materials as collagen-based membranes were established and widely used in a variety of clinical situations (7). Osteogenesis, osteoinduction, and osteoconduction are three different processes that bone regeneration can be achieved (9). Allografts, xenografts, alloplasts, and autogenous bone are the major types of bone graft materials (8). Xenografts are made by deproteinizing cow, horse, and pig bone tissue with the removal of organic material. Its benefits include having a porous structure that is similar to that of human cancellous bone, being high in osteoconduction because it acts as a support structure for the new bone formation and being reasonably priced when compared to other bone graft materials (8, 10). Deproteinized bovine bone material

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is the most widely used clinical product among xenograft materials due to its stable and excellent bone formation ability (11). According to research, bone graft materials covered by barrier membranes were well preserved and exhibited osteoconductive properties; additionally, the bone grafts could maintain stability enough to be successfully incorporated into the healthy bone as the membranes were employed in combination (12). This contributed to a positive regenerative outcome by providing sufficient space (13). According to the results of the prior studies, using collagen membrane in conjunction with xenografts may improve bone regeneration (14).

Studies on vitamins have expanded as it has been clear how beneficial nutrition is to human health. A fat-soluble hormone, vitamin D refers to two compounds: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol); it is converted by the liver and kidneys into an active form of Vitamin D3 (1,25(OH)2D3). The anabolic effect of 1,25(OH)2D3 on bone metabolism is well known (15). It is essential both for calcium and phosphorus homeostasis, which affects bone remodeling (16, 17). The discovery that vitamin D3 has receptors with a high affinity for osteoblastic cells provides more validity to the idea that vitamin D3 regulates bone production and mineralization. Several studies have demonstrated that vitamin D3 administration increases fracture healing (18), implant osseointegration (19), bone density and regeneration (20, 21). Hong et al. (21) concluded that vitamin D has a positive effect on bone regeneration in the study in which they examined the effects of topical and systemic vitamin D3 applications on bone density and regeneration. The findings demonstrated that topical treatment of D3 expedited the formation of new bone and increased bone density, but this method had a lower effect than systemic vitamin D3 administration. There has been also substantial research on the essential functions of vitamin D in the control of calcium homeostasis and bone metabolism. However, there is still a lack of comprehensive information on the effects of cholecalciferol on bone healing and regeneration in dentistry (20).

Radiographic methods, histologic and histomorphometric analyses can be used to evaluate the healing of bone regeneration. Radiographic assesments have been used to examine the effect of various treatment concepts on bone formation. They offer the potential benefit of being less expensive and time-consuming than histologic examination; however, the validity of these assessments has not yet been thoroughly investigated (22). A small number of studies have investigated the accuracy of the evaluation of bone regeneration using standardized conventional radiographs.

In the present study, we hypothesized that vitamin D3 and GBR can improve bone healing in a rat model. Thus, the purpose of this study was to evaluate radiographically the effect of orally administered vitamin D3 on GBR in a critical-size defect (CSD) model at the calvaria of rats.

2. METHODS

2.1. Animals

Our study was approved by the Acıbadem University Animal Experimentation Ethics Committee (protocol no. 2020/32). The authors followed the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines 2.0 from 2020. Twelve male, 4-month-old (mean weight 300–350 g), healthy Sprague Dawley rats were used in the study. The rats were placed in pairs in purpose-built cages at the Acıbadem University Experimental Animal Research Laboratory, with a 12-h light/dark cycle at 21°C ± 2°C and with ad libitum access to rat food and water during the entire experiment. Every cage had a limit of 2 rats to contain in the shelter. Surgical procedures were performed under general anesthesia, and all efforts were made to minimize animal suffering.

According to the power analysis performed by using the values obtained from an animal study (10) having similar defect size with the present study and comparing new bone formation between the groups, at a 80% power and 5% significance level, a minimum of 6 rats per group and a total of 12 rats were found necessary.

2.2. Surgical Procedures

To minimize suffering, animals were anesthetized with a combination of ketamine (10% Ketasol; Richter Pharma AG, Wels, Austria), and xylazine (Rompun, Bayer, Leverkusen, Germany) with 35 and 3 mg/kg respectively. The scalp was shaved and cleaned with povidone-iodine after general anesthesia. The skin, subcutaneous tissue, and periosteum were reflected, exposing the parietal bones, after a 2 cm-long midline incision was made along the sagittal suture (Figure 1a). For the 5 mm defects, two full-thickness, non-suture associated bone defects were trephined in the left and right sides of the parietal bone under constant normal saline irrigation. Surgery was performed carefully to prevent injury to the cranial dura mater. The one side defects were left empty (E defect) while a collagen-based resorbable membrane (circular membrane with a diameter of 6 mm on the midline) (BioGide®) and a bovine bone graft (BioOss®) were applied in the other side defects (GM-filled defect) of all animals (Figure 1 b,c,d,e). An absorbent suture (Vicryl 3-0, 4-0; Ethicon Inc., NJ, USA) was used to seal the subcutaneous tissue, and the skin was left to recover. Following surgery, animals were given intramuscular injections of the antibiotic Ceftriaxone (Rocephin, Roche, Nutley, New Jersey, USA), 25 mg/kg, for 3 days, and the analgesic Carprofen (Rimadyl, Pfizer, New York, USA), 4 mg/kg, 24 hours a day, for 3 days.

2.3. Experimental Groups

The animals were randomly divided into two groups by a researcher (HOO) after the rats awoke from anesthesia following the procedure; the control group (n=6) was given distilled water, and the test group (n=6) was given 2 µg /kg vitamin D3 by gavage once a day for 8 weeks. Twenty four

hours following the final vitamin dose, all animals were euthanized by anesthetic overdose and sacrificed at week 8.

2.4. Radiographic Analysis

An X-ray machine (Siemens Arcadis Avantic C-Arm, Berlin, Germany) was used to take radiographs of the samples collected in week 4 and 8 (Figures 1f and 1g). The radiographic images were taken under exposure parameters of 7 mA, 0.03 s, and 70 kV with the X-ray beam perpendicular to the bone defect areas parallel to the floor. A standard threshold was used to include all areas of high density in order to quantitatively calculate the entire area of the newly produced osteoid. Each defect's osteoid region and the bone defects (n=12) were detected. Using Image Fiji Analysis Software (Olympus Image Analysis Software 5.0, Tokyo, Japan), the newly formed osteoid area fraction was determined. The radiographic evaluation is performed by the same researcher (GNV).

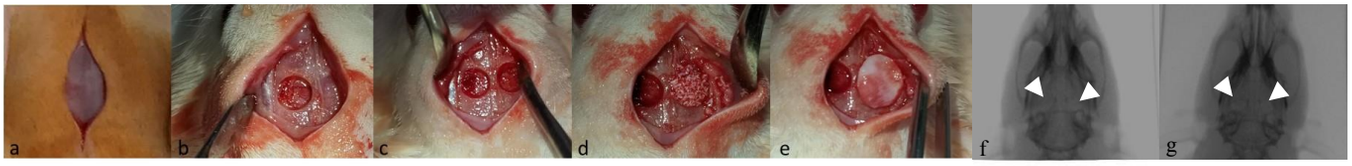

Figure 1: *Creation of the defects.* ***a)*** *Midline incision design from the frontal to the occipital region,* ***b)*** *Critical size defect on the left side,* ***c)*** *Creation of two critical size defects of 5 mm,* ***d)*** *Empty defect (Left) and defect filled with deproteinized bovine bone graft (Right),* ***e)*** *Empty defect (Left) and defect covered by collagen-based resorbable membrane after filling with deproteinized bovine bone graft (Right),* ***f)*** *Radiographic image of the Control group and* ***g)*** *of the Test group at week 8.*

2.5. Statistical Analysis

GraphPad Prism 8.0.2 (GraphPad Software Inc., San Diego, USA) was used for data analysis. Each quantitative result was presented as the mean ± standard deviation (SD). The intragroup comparisons were performed with the two-way ANOVA with post-hoc test, the differences between study groups were determined with two-way ANOVA supplemented by Tukey's multiple comparison test. P values of <.05 were as statistically significant.

3. RESULTS

No postoperative complications, infections, changes in animal behavior, body weight, or general health issues were seen in any of the rats following surgery or over the course of the study (up to 8 weeks). In all rats, healing of the tissue at the surgical sites was uneventful. Radiographic images of the rat calvarias from both groups were obtained at weeks 4 and 8. The percentage of the area fraction according to newly formed osteoid values at weeks 4 and 8 are presented in Figure 2. At week 4, the area fraction in the control group for the GM-filled defect was 13.11±0.87%, but it was only 11.0±0.94% for the E defect. At week 8, the percent area fraction in the control group was 14.15±0.69% for GM-filled defects and 12.19±0.39% for E defects. In the test group, at both 4th and 8th weeks, GM-filled defect (18.62 ± 0.49 and 19.81 ± 0.70%, respectively) showed higher area fraction than E defect (15.83 ± 0.41 and 17.10 ± 0.66%, respectively). It was determined that the values at 8th week were statistically higher than 4th week in both E defect and GM-filled defect in study groups (p<.05). In addition, the values in the GM-filled defect were statistically higher than the E defect in the study groups both at week 4 and 8. The percentage of area fraction, in both E and GM defects was higher at weeks 4 and 8 in the test group compared to the control groups (p<.0001). GM-filled defect in test group exhibited the highest mean percentage of area fraction between all groups (p<.0001).

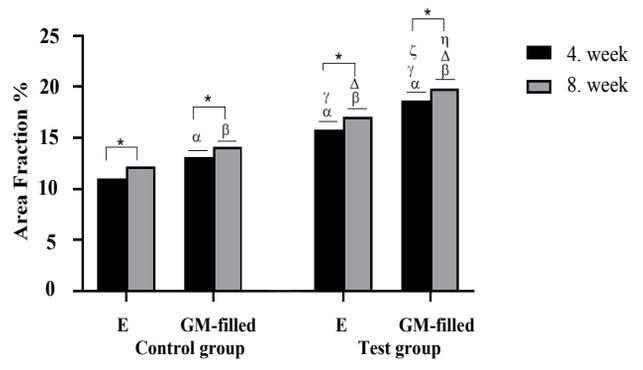

Figure 2. *The percentage of area fraction for all groups. [α]Compared to the Control group E defect at week 4 with p<.0001, [β]Compared to the Control group E defect at week 8 with p<.0001, [γ]Compared to the Control group GM-filled defect at week 4 with p<.0001, [Δ]Compared to the Control group GM-filled defect at week 8 with p<.0001, [ζ]Compared to the Test group E defect at week 4 with p<.0001, [η]Compared to the Test group E defect at week 8 with p<.0001; *p<.05, 8th week versus 4th week in all groups, Two-Way ANOVA test.*

4. DISCUSSION

The present study was designed to observe the effect of vitamin D3 on bone regeneration using a rat calvarial critical size defect model during instead of for 4 and 8 weeks and

evaluate new bone formation through radiological analysis. The outcomes showed that the administration of 2 µg/kg vitamin D from the first postoperative day could actually accelerate new bone formation in calvarial defect areas. Radiographic images taken at weeks 4 and 8 following surgery showed higher new osteoid formation.

Since it provides crucial data on physiological and pathological circumstances that could be used to develop better clinical interventions, the use of animal models for in vivo research has been favored (23). When using bone substitutes in an animal model to evaluate bone regeneration, it is essential to verify that the substituted material conformed to the concept of CSD. It was identified by Schmitz and Hollinger in 1986 as the smallest intraosseous diameter that will never spontaneously heal over the course of the animal's life or the research (24). According to the species of the animal and the site of the defect, this concept has several thresholds. Critical size defect has been utilized extensively in the improvement and establishment of a wide variety of regenerative materials and procedures, and is one of the most reliable and popular in vivo models in the field of bone regeneration. A CSD is one that does not repair during the period of the investigation (25). The diameter of the rat calvarial CSD has been the point of contention in the literature, with reports ranging from 4 mm to 8 mm, emphasizing the requirement of a control group in every investigation (23). The 5 mm diameter, however, has been widely accepted as a critical-size calvarial defect in healthy rats (26, 27). The use of a 5 mm CSD has the advantage of allowing for the establishment of two defects per animal, therefore allowing fewer animals to be included in the experiment; avoiding the inclusion of the sagittal suture, hence reducing the potential of midsagittal sinus lesions (28). The use of standard calvarial defects with a diameter of 5 mm in rats allows evaluation of the effects of bone substitutes used in GBR.

The follow-up periods in this experiment were only 4 and 8 weeks. In the study of Gosain et al. (29) on critical size calvarial defects in rats, the recovery in the 8th week after surgery was 30.1% greater than in the 4th week; they stated that it was only 7.7% greater at the 12th week compared to the 8th week. Accordingly, in a rat model, the critical period between the 4th and 8th week after injury was found to be sufficient for evaluating total recovery (30). Besides, 8 weeks was the right amount of time to evaluate late repair, including bone remodeling, bone regeneration, and graft material absorption by new bone tissue (31). Consistent with the literature, in this study, as the results after 4 weeks demonstrated less newly formed osteoid at the defect sites in both the control and test groups, suggesting that 4 weeks was insufficient time to complete the bone healing process.

The gold standard in regeneration is autologous bone grafting, but this method has limitations, such as longer recovery periods for graft harvesting, volume restrictions for the bone, restrictions in supply and donor site morbidity (32). Furthermore, using autogenous bone frequently necessarily requires a second surgical site and prolonged perioperative time (33). Advances in the use of bone substitutes to replace autogenous grafts improve both the patient's and the surgeon's operating conditions. Allograft also regularly has supply limitations (34). It can cause an immunogenic reaction, has a less consistent clinical outcome, and is only available in limited quantities. A xenograft is derived from a nonhuman species. As a result, antigenicity is significantly higher than that of allografts; it requires more sterile processing, which may result in decreased osteoinductive properties. These grafts may be less expensive and more readily available due to the abundance of donors. Additionally the shelf life is also generally long because of the extensive sterilization processes (35). Bio-Oss® is a deproteinized bovine bone mineral that is biocompatible and has low resorption and excellent bone conduction. Therefore, the slow degradation process of this product may help to maintain the stability of regenerated bone (36). Additionally, it has 60–70% porosity per unit volume and no organic components. No immunological responses have been reported to its clinical use (37). Bio-Oss® has been extensively researched in several studies over the last few decades, with several authors confirming its osteoconductive potential in animal or clinical studies (10, 38). Bio-Gide® is a natural bilayer collagen membrane that has a fibrous surface in addition to a cell-occlusive surface to protect the wound site during healing and enable protein deposition (33). It promotes consistent bone regeneration and perfect tissue connection (39). Comparing collagen membranes to non-resorbable membranes, several studies have found that collagen membranes may support even greater bone regeneration and wound healing (40). In order to protect the initial coagulum, the Bio-Gide® membrane combines with the surrounding tissues. After that, it optimally dissolves to enable the series of biological events that lead to regeneration (14). These membranes are among the most studied in the literature because they play important structural support roles, and collagen is the primary component of connective tissues. (41). The combination of Bio-Oss® and Bio-Gide® significantly lowers graft resorption, enables uncomplicated recovery, decreases morbidity, and minimizes patient discomfort (42). Considering the properties of these materials, in the present study in which these materials were used in GBR, there was no immune reaction in the GM-filled defects in both the vitamin-administered and non-vitamin-treated groups. Our study also demonstrated that new bone formation was higher in GM-filled defects compared to E defects at 4th and 8th weeks. In addition, new bone formation at week 8 was higher in both groups compared to week 4. Similar to our study, Fadel et al. (33) found that new bone formation was significantly higher in defects treated with the combination of Bio-Oss® and Bio-Gide® at both 4 and 8 weeks compared to empty defects, in bone regeneration in rats. Moreover, new bone formation was higher at week 8 than week 4 in both groups.

Studies on the administration of vitamins for bone healing and formation have become more popular in recent years. A limited number of studies on the effects of vitamin

administration on guided bone regeneration are available. However, there is currently insufficient evidence to support the idea that administering of vitamin D3 may have a positive effect on osteoblasts and stimulate bone regeneration. Animal studies revealed that administering vitamin D3, either systemically or locally, had a positive effect (21, 43, 44). Hong et al. (21) demonstrated that orally administering vitamin D3/Ca in addition to alloplastic grafts enhanced new bone formation and bone volume in dogs. Cignachi et al. (45) discovered that vitamin D3 helps to improve bone regeneration including in rats with induced diabetes. In one single study by Han et al. (46) the effect of eldecalcitol (ELD), an active vitamin D3 analog, on bone regeneration in 64 rats was investigated; the results showed that the systemic administration of ELD could improve new bone formation as evidenced by an increased bone volume and speeded mineralization. In another study on rats, it was observed that systemic administration of vitamin D3 increased the osseointegration of implants (47). The results of our study, which showed that orally administered vitamin D3 increases new bone formation radiographically in rats, is consistent with previous studies showing that dietary vitamin D3 consumption increases bone formation. In this study, it was also found that vitamin D3 promoted increased bone production, particularly in GM-filled defects.

The effect of various treatment concepts on bone formation has been evaluated using radiographic evaluations. Many animal studies have used different radiography techniques to assess bone regeneration (48-50). All of these studies used radiographic analysis without any histologic components. In this study, we were able to evaluate new bone formation radiographically in all defects at 4 and 8 weeks. However, to the best of our knowledge, no study that radiographically examines the effect of orally vitamin D3 administration on bone regeneration is present in the literature. One limitation of this study is that only radiographic analysis was performed to assess the effect of vitamin D3 supplementation on bone regeneration in rats.

6. CONCLUSION

Based on the results of this study, we can conclude that at week 4 and 8, radiographic evaluation can be utilized to identify new bone formation in CSD in rats and orally administered vitamin D3 enhances bone formation in CSD at the calvaria of rats.

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Experimentation Ethics Committee (approval date 11/06/2020 and number 2020/32)
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Author Contributions:
Research idea: GNV, HOO
Design of the study: GNV, HOO
Acquisition of data for the study: GNV
Analysis of data for the study: GNV
Interpretation of data for the study: GNV, HOO
Drafting the manuscript: GNV, HOO
Revising it critically for important intellectual content: HOO, SDD, HSY, ÖBA, LK
Final approval of the version to be published: GNV, HOO, HSY, ÖBA, LK, SDD

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