

RESEARCH ARTICLE

The Effects of Allograft Combined with Ozone Therapy on Regeneration of Calvarial Defects in Rats

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ABSTRACT

Objective: Ozone accelerates wound healing and increases oxygen supply. The purpose of this study was to investigate the effect of ozone therapy combined with bone allograft on bone regeneration in rats with calvarial defects, histomorphometrically and histopathologically.

Material-Method: Twenty four male Wistar rats were used in this study. A 5 mm diameter critical-size defects were created in all rats using a trephine bur. The rats were divided into 4 groups; empty defect (control) (n=6), ozone application into the control defect (control+ ozone) group (n=6), defect filled with allograft (Allograft) group (n=6), ozone combined with allograft application (allograft + ozone) group (n=6). Gaseous ozone administered at 80% density for 14 days. The animals were euthanized at 8-week. Total bone area was measured histomorphometrically and osteoclast and osteoblast number were analyzed histopathologically.

Results: The osteoblast numbers increased in allograft + ozone group compared to the allograft group (p<0.05). The control defects showed significantly less new bone formation than those of the allograft groups at 8 weeks (p<0.05). Total bone area was significantly higher in allograft+ozone group than those of the allograft group (p<0.05).

Conclusions: Within the limitations of this study, we suggested that allograft+ozone combination increased osteoblast number and new bone area. However, further studies are needed to investigate the mechanisms of action of ozone on bone regeneration.

Keywords: Bone healing, allograft, ozone therapy, calvarial defect.

INTRODUCTION

The purpose of bone augmentation procedure is the formation of new bone after the healing in the area which was augmented.¹ There are several grafts materials used for regeneration the autogenous grafts are considered the "gold standart" due to their osteogenic, osteoinductive and osteoconductive features. Although potential favorable properties of autogenous bone grafts, a tendency toward unpredictable resorption and morbidity at the donor site represents disadvantages of this grafts.²

Allograft is a tissue transferred from a donor to a recipient of the same species but of nonidentical genetic composition.³ Allografts are used freeze-dried demineralized bone allograft (FDBA) and demineralized freeze-dried bone allograft form known as (DFDBA).⁴

FDBA provides an osteoconductive effect to bone regeneration while DFDBA is to allow an osteoconductive surface while maintaining the additional benefit of its functioning as a source for the osteoinductive factors⁵ and its these features can be achieved by using structural bone morphogenetic proteins (BMP) such as BMP-2, 4, 7, its own structure.⁶ In a study reported that the combination of DFDBA and platelet rich plasma accelerated bone regeneration in dog mandible.⁷

When DFDBA is transplanted into bone with good vascularity, it permits the mesenchymal cells to migrate to the area consequently increase the osteogenesis. These events are induced by the following functions of the BMP; (1) mitogenic effects on undifferentiated mesenchymal cells and osteoblast precursors; (2) Stimulation of the osteoblast phenotype

(increasing the alkaline phosphatase activity in the bone cells); (3) its functioning as chemo-attractant for mesenchymal cells, monocytes and Type 4 collagens that serve as cellular adhesion proteins.⁸ Studies suggested that during the preparation of allograft, the mechanical strength of the graft decreases in 50%.^{9, 10} Furthermore, in the previous studies that were conducted on DFDBA, it was indicated that the regeneration has not occurred a certain because the osteoinductive proteins were not preserved at sufficient level during the sterilization and therefore there were no clinical estimates on this issue.¹¹⁻¹³

Ozone is normally present as a gas made of three atoms of oxygen with a cyclic structure, a gaseous or aqueous form.¹⁴ The therapeutic effect of ozone is not only limited to the cell wall; therapeutic mechanisms is also related to bactericidal, virucidal and fungicidal action as well as having. Also, systemic hemostatic repair properties (enhancing the oxygen carrying capacity of the blood; optimization of pro- and anti-oxidative processes; regulating the microcirculation and peripheral blood circulation; dose-dependent effect on blood clotting; activation of the production of the biologically active factors), immunomodulating effect, analgesic effect, and detoxifying effect.¹⁵

There are a few study investigating the effects of ozone on bone regeneration in literature. Furthermore, the previous our study demonstrated that graft regeneration increased significantly when ozone therapy in conjunction with autogenous grafts in the rat calvarial defects.¹⁶ However, the influence of ozone therapy on allograft healing has not been studied previously. Therefore, the aim of the present study was

to examine the currency of this hypothesis in a critical size defect model experimentally created in rat calvaria and analyze the bone formation when ozone therapy in together with allograft

MATERIAL-METHOD

Twenty-four male Wistar rats were used in this study. The rats were 12-14-week old and were 210-220 grams. Rats were kept in individual plastic cages in an experimental animal room (21-C, 55%-70% humidity, 1 atm pressure, with a 12-hour day/night cycle). The animals were fed a standard laboratory pellet diet, and drinking water was available ad libitum during the experiment protocole. The approval for this study was received from the Local Ethical Committe for Animal Experiments at Cumhuriyet University.

Surgical procedure

Animals were anesthetized preoperatively with an intramuscular injection of ketamine hydrochloride (40 mg/kg body weight, 10% Ketazol; Richter Pharma AG, Wels, Austria). The surgical site was shaved and disinfected. An incision was made in the scalp in the sagittal plane longitudinally the cranium, allowing reflection of a full-thickness flap in a posterior direction. A 5 mm diameter critical size defect was made on the right side of the parietal bone with a trephine bur (MIS Implant Tech, Shlomi, Israel) used in a low-speed handpiece under continuous irrigation with sterile saline attention was paid not to perforate the underlying dura mater and not to involve the sagittal suture. (Figure-1.A)

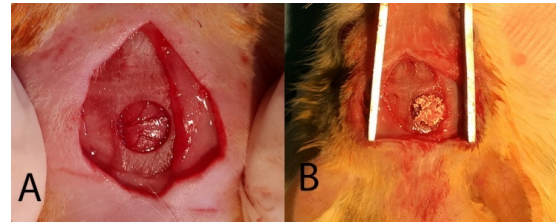


Figure 1: A: Critical-size defect (5 mm diameter) created on the parietal part of the rat calvarium. B: Graft material applied to the defect.

Animals were randomly divided into four groups as follows:

- 1- Empty defect (control) group (n=6)
- 2- Ozone application into the empty defect (control+ozone) group (n=6)
- 3- Allograft group (n=6)
- 4- Ozone Combined with Allograft application (allograft+ozone) (n=6).

After preparing the recipient site, allograft (0,5-1mm particle size, cortical bone) (Maxeus dental, Ohio, ABD) was implanted into the bone defect (Figure-1.B). The soft tissues were then sutured to achieve primary closure (4-0 catgut; Dogsan Sanayi, Istanbul, Turkey). To prevent postoperative infection, ceftriaxone (Roche, Basel, Switzerland) was given to the animals as intramuscular injections for 3 days (30 mg/kg). They were also given an intramuscular analgesic, 4 mg/kg carprofen (Pfizer, New York, NY, USA), every 24 h for 3 d, starting immediately after the operation .

Ozone therapy was performed using an ozone generator (Biozonix, Munich, Germany). It was applied 60 s, 80% oxygen (2100 ppm) every day for 14 days.

Histologic evaluation

At the end of the 8 week follow-up, animals were killed overdose of 200 mg/kg, iv, pentobarbital (Abbott Diagnostic Division, Abbott Park, IL, USA). The area of the original surgical defect and the surrounding tissues were removed en bloc. The blocks were fixed in

10% neutral formalin, rinsed with water and then demineralized in 10% formic acid. After decalcification, each specimen was divided longitudinally into two blocks in sagittal direction and embedded in paraffin. Serial sections (5 µm thick) were cut in a longitudinal direction, beginning at the center of the original surgical defect. The sections were stained with hematoxylin and eosin for analysis under light microscopy (Nikon, Eclipse 80i, Tokyo, Japan). Histological analysis was performed by a single examiner who was also blinded to the identity of samples. To measure bone formation, the number of osteoblasts was counted in all defect area.

Histomorphometric evaluation

The images of the histologic sections in all groups were captured by a digital camera connected to a light microscope with an original magnification (X 4). The digital images were saved on a computer. The Clemex Vision-Lite 5.0 software (Clemex Technologies, Quebec, Canada) was used for used for total bone area (mm²) as the histomorphometric analysis.

Statistical analyses

All statistical analyses were performed using a commercial computer program (SPSS system version 22; SPSS Inc., Chicago, IL, USA). The datas were subjected to statistical analysis with the Mann–Whitney U-test following the Kruskal–Wallis test (intergroup comparison). Differences of $p < 0.05$ were considered significant.

RESULTS

All rats survived to the end of the study, and no postoperative complications were noticed such as inflammatory tissue responses, exposure of graft material, or allergic reaction.

Histological sections were evaluated with a light microscope. In the control group, the defects generally were filled by thin fibrous connective tissue and the edges of defects were observed as thickening of the cortex. Also, it was observed that the number of the osteoclasts were lower in both control groups compared with the other groups ($p < 0.05$). In comparisons among the groups, it was observed that there were no significant differences in osteoclast numbers between the allograft and allograft+ozone groups at 8th week ($p > 0.05$).

The osteoblast numbers were not significantly different between the control and control+ozone groups at 8th week. However, numbers of osteoblast in allograft+ozone group were found statistically higher than those of the other groups ($p < 0.05$) (Figure-2).

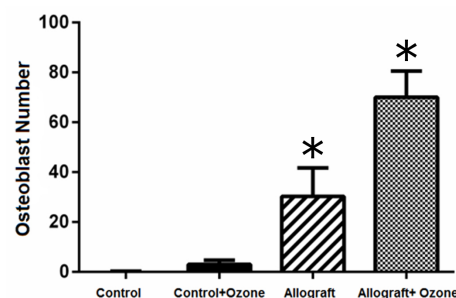


Figure 2: Osteoblast numbers for the study groups. * $p < 0.05$ vs. the other groups

New bone area measurements were statistically analyzed among the groups at 8th week. It was observed that the allograft+ozone group showed a significant increase in total bone area compared to other groups ($p < 0.05$) (Figure-3), while no statistical significance were observed between control and control+ozone group ($p > 0.05$) (Figure-4). Also, new bone formation were observed less in the control and control+ozone groups.

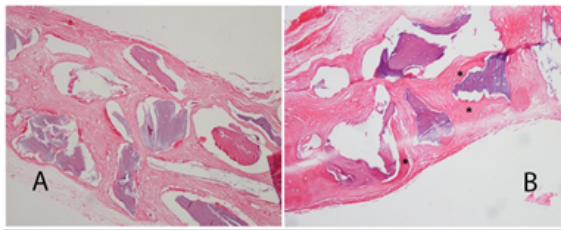


Figure 3: Histological sections in the allograft (A) and allograft+ozone (B) group, (*) indicates newly formed bone. (hematoxylin and eosin stain, original magnification x100).

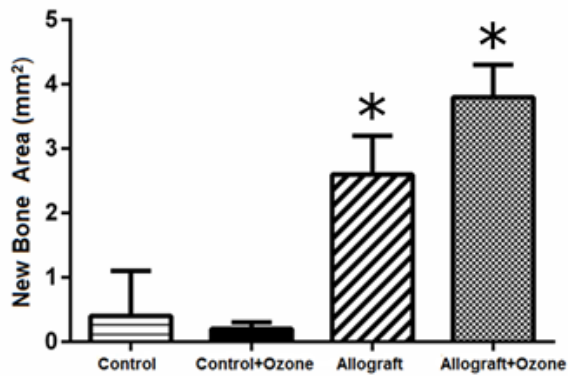


Figure 4. New bone area for all groups. * $p < 0.05$ vs. the other groups.

DISCUSSION

In this study, the issue of whether there is a contribution of the ozone gas and allograft material to new bone regeneration in critical defects in rat calvaria was determined. The rat calvarial defect model was preferred for this purpose. Because; (i) surgical procedures on the rat calvarial bone are relatively simple to perform; (ii) observations can be focused on the healing process of the bone, as there are no major nerves or blood vessels around the rat calvaria; (iii) the calvarial defect model has many similarities to the maxillofacial region, as anatomically the calvaria consists of two cortical plates with a region of intervening cancellous bone similar to the mandible; (iv) preparation of tissue specimens is easy; and (v) spontaneous healing would not occur at the control defect (critical size defect).¹⁷⁻¹⁹ Therefore, 5-mm diameter, spontaneously non-healing, critical sized calvarial defects were created in our study.²⁰⁻²³ Furthermore,

in the control group, the healing was characterized with the collagen fibers and high amounts of fibroblasts in the connective tissue. Bone formation was observed only in the edges of defect.

By using bone grafting in the bone defects, the expected healing was to see regeneration and new bone formation.²⁴ However, in previous studies, it has been reported that FDBA and DFDBA type allograft were observed in new periodontal ligament, cement and alveolar bone formation as a percentage of 50-60% and 80 %, respectively.²⁵⁻²⁸ Also, several studies suggested that allograft (Puros®) was superior than the other graft materials, and that it was almost as effective as autogenous graft.²⁹⁻³¹

Ozone can be applied in different ways such as gaseous, oil or water. In our study, we used the ozone generator that produced gaseous ozone. Gaseous ozone application did not cause any side effects in rats. However, there are many ozone generators on the market while there was no protocol recommendations for bone healing or bone regeneration. Also, the amount of ozone produced by these devices are different. In animal studies performed with ozone devices have been obtained different results.

While ozone gas is preferred as an antibacterial agent in dentistry, it also has properties of activating the blood components (erythrocytes, leucocytes, platelets, endothelial cells), and this may have an important role in healing wounds due to its positive effect on key processes such as microcirculation, antioxidant defense system, oxygen metabolism.³² Nogales et al.³³ showed that ozone application must be included in the dentistry practice, and that it could even be an alternative to antibiotic treatment in alveolitis treatment.

Low level laser, hyperbaric oxygen, platelet rich plasma and platelet rich fibrin and other treatment regimens were tried in order to increase of the bone graft healing in the literature and different results were reported. The effects of ozone on bone healing have been histologically examined in recent studies.³⁴⁻³⁶ Kazancıoğlu et al. demonstrated that both ozone and laser therapies had a positive effect on bone formation in rat calvarial defect compared with the control group; also, ozone therapy was more effective than low level laser treatment.³⁵ Another study by the same authors³⁶ suggested that ozone therapy was useful for the reduction of postoperative pain and increased quality of life after third molar surgery. However, it had no effect on postoperative swelling or trismus. In our study, ozone increased osteoblast numbers and total bone area in allograft+ozone group comparing to allograft group at 8 th weeks.

In addition to the studies that dealt with the beneficial effects of ozone, there are also reports of adverse reactions associated with its use.³⁷ Bocci et al.³⁸ reported that long term inhalation of ozone induced to health problems while low-dose ozone could be calibrated well and showed positive effects and triggered the antioxidant capacity of the blood .

CONCLUSION

Within the limitations of the present study, it was concluded that the ozone therapy increased bone formation with allograft in rat calvarial defect model. This study is a first in terms of results and analyzed studies related to ozone and allograft. However, additional studies are required to clarify the effects of different ozone applications on new bone formation.

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