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Effect of ozone therapy on autogenous bone graft healing in calvarial defects: a histologic and histometric study in rats

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Aim: The purpose of this study was to analyze histologically the effect of ozone therapy in combination with autogenous bone graft on bone healing in rat calvaria.

Methods: Critical size defects were created in calvaria of 27 male Wistar rats. The animals were divided into three groups of nine animals each: autogenous bone graft group ($n = 9$); autogenous bone graft with ozone therapy group (80%, 30 s 3 d for 2 wk, $n = 9$); non-treatment (control) group ($n = 9$). Animals were killed after 8 wk. Histomorphometric assessments, using image analysis software, and histological analyses were performed. Primary outcome was total bone area. Secondary outcomes (osteoblast number, new bone formation) were also measured.

Results: Histomorphometrically, the total bone area in the autogenous bone graft with ozone therapy group (9.3 ± 2.2) were significantly higher than that of the autogenous bone graft group (5.1 ± 1.8) ($p < 0.05$). Also, the ozone therapy group significantly increased the percentage of total bone area compared to the autogenous bone graft group ($p < 0.05$). The osteoblast number significantly increased in the autogenous bone graft with the ozone therapy group (58 ± 12.3) compared to the autogenous bone graft group (9.3 ± 3.5) ($p < 0.05$). Also, it was observed that autogenous bone graft with ozone therapy group showed significant new bone formation when compared to the autogenous bone graft group ($p < 0.05$).

Conclusion: Ozone therapy enhances new bone formation by autogenous bone graft in the rat calvarial defect model.

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The goal of bone augmentation procedures is to stimulate or at least facilitate the growth of new bone into the augmented site (1). Various types of graft materials have been used for alveolar bone augmentation procedures (2). Autogenous grafts are considered the gold standard due to their osteogenic, osteoinductive and osteoconductive properties. In spite of potential osteogenic properties of autogenous bone grafts (AG), a tendency toward unpredictable resorption and morbidity at the donor site represents obstacles to this procedure (3). Also, long-term studies indicate that buccal tissue volume was reduced over time after autogenous

block bone grafting (4,5). It was also demonstrated that the surgically augmented height with an autogenous block graft decreased to 60% after 10 mo.(3) While the exact mechanism of this phenomenon is not fully understood, physiologic stress applied to graft, embryological origin and graft-to-recipient bone contact have been suggested as factors that affect resorption in bone graft healing. Also, during the healing process, angiogenesis and neovascularization of the graft are crucial for integration (3).

Ozone is normally present as a gas made of three atoms of oxygen with a cyclic structure, which has been used in a gaseous or aqueous form (6). Ozone therapy can induce several biological responses such as improve blood circulation and oxygen delivery in ischemic tissue, enhance general metabolism by improving oxygen delivery, upregulate cellular antioxidant enzymes, and induce a mild activation of the immune system and enhance the release of growth factors (7).

Most of the published articles considering the use of ozone in dentistry have been in relation to its antimicrobial effects (8–11). Also, there is insufficient evidence in the application of ozone in oral and maxillofacial surgery (12). As ozone has a therapeutic effect that facilitates wound healing and improves the supply of blood, we have hypothesized that ozone therapy could enhance the stability and predictability of AGs. Therefore, the aim of the present study was to examine the validity of this hypothesis in a critical size defect model experimentally created in rat calvaria and analyze the bone formation when ozone therapy in conjunction with AGs.

Materials and methods

Experimental protocols were approved by the Animal Ethics Committee of Cumhuriyet University School of Medicine. In total, 27 Wistar male rats were used. Their body weight ranged from 300 to 330 g at the beginning of the experiment. The animals were kept in individual cages in a room with 12 h day/night cycles, an ambient temperature of 21°C and *ad libitum* access to water and a standard laboratory pellet diet.

Surgical procedures

Animals were anesthetized preoperatively with an intramuscular injection of ketamine (Eczacıbasi Ilac Sanayi, İstanbul, Turkey) (40 mg/kg body weight). The surgical site was shaved and disinfected. An incision was made in the scalp in the sagittal plane across 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 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. Animals were randomly divided into three groups as follows:

- Control group (n = 9), receiving no bone graft material or ozone therapy.
- AG group (n = 9), receiving only autograft without ozone therapy.
- AG with ozone therapy (AG + ozone) group (n = 9), receiving graft with ozone therapy (30 s, three times a week for 2 wk).

AG was harvested from the left side of calvarium with a standard trephine bur (Mıs Implant Tech, Shlomi, Israel; 5 mm diameter). After preparing the recipient site, bone graft was ground with a manual bone crusher, and implanted in to the bone defect. The soft tissues were then repositioned and sutured to achieve primary closure (4-0 silk; Dogsan Sanayi, İstanbul, Turkey). To prevent postoperative infection, ceftriaxone was given to the animals as intramuscular injections for 3 d (30 mg/kg). They were also given an intramuscular analgesic, 4 mg/kg carprofen (Rimadyl; Pfizer, New York, NY, USA), every 24 h for 3 d, starting immediately after the operation. Healing progressed uneventfully in all animals and no postoperative complications were noticed. All surgical procedures were performed by the same operator (H. Ozdemir).

The ozone delivery system used was the Ozonix Ozone Generator (Biozonix GmbH, Munich, Germany). According to information given by the manufacturers, Ozonix is a device that produces ozone at a fixed concentration of 2100 p.p.m. through a connected handpiece. The ozone generator conforms to all European Union legislation covering medical devices [CE, 93/42/EWG (EEC)]. A sterile, specially formed perio-tip, attached to the handpiece, was hand-guided over the whole defect area analogous to clinical procedure. It was applied with (80% oxygen) for 30 s per d, 3 d a wk for 2 wk.

Histologic evaluation

At the end of the 8 wk follow-up, animals were killed by a lethal anesthetic dose of barbiturate. 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 (6 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 E 600, 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. Osteoblasts were defined as cuboidal cells immediately adjacent to osteoid. Histological evaluation of new bone formation (13) was scored as follows: no bone formation as 0; mild visible bone formation as 1; moderate visible bone formation as 2; and dense visible bone formation as 3.

Histomorphometric evaluation

Histometric analysis was performed by another examiner blinded with respect to the treatment rendered. The images

of the histologic sections in all groups were captured by a digital camera connected to a light microscope with an original magnification (× 4). The digital images were saved on a computer. The Clemex Vision-Lite 5.0 software (Clemex Technologies, Quebec, Canada) was used for the histomorphometric analysis. The total bone area analyzed corresponded to the entire area of the original surgical defect. The total bone area (mm^2) was measured, including all tissues within the boundaries of newly formed bone. Also, the total bone area was considered 100% of the area to be analyzed and the percentage of the total bone area was calculated.

Statistical analysis

After evaluation of normality with Kolmogorov–Smirnov test, data were analyzed with ANOVA followed by the Tukey test for pairwise comparisons. p values less than 0.05 were considered statistically significant. All statistical analyses were performed using a commercial computer program (SPSS system version 14; SPSS Inc., Chicago, IL, USA). The total bone area and osteoblast number was determined as the expected primary and secondary outcomes of the study, respectively.

Results

The progression of healing was uneventful in all animals and no postoperative complications were noticed such as inflammatory tissue responses, exposure of graft material, or allergic reaction. Histological evaluation demonstrated that there was no damage to the dura mater due to the creation of calvarial defects in any of the specimens. In the control group, healing was characterized by thin fibrous connective tissue in which there was a large amount of collagen fibers and fibroblasts oriented parallel to the wound surface, and some blood vessels filling the defects with no sign of regenerative bone formation (Fig. 1).

In the AG group, the center part of the bone defect was filled by connective tissue and remaining bone grafts.

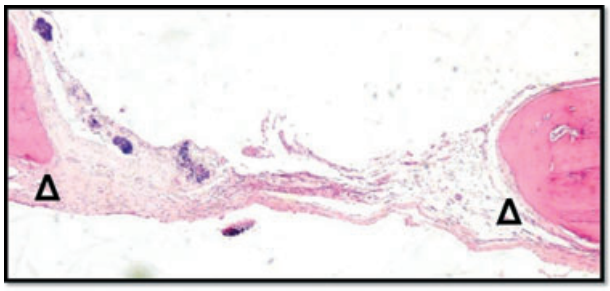

Fig. 1. A typical histologic picture of the control group (hematoxylin and eosin, × 50).

A layer of newly formed bone was presented on the surface of some remaining bone grafts. A fibrous connective tissue surrounded the remaining bone grafts (Fig. 2C). This tissue contained a large amount of collagen fibers with a moderate amount of fibroblasts and numerous blood vessels.

Histopathologically, foci of active bone resorption on AG particles were rarely observed in the AG + ozone group wounds. Ozone therapy resulted in significantly more new bone formation compared to the AG group ($p < 0.05$) (Fig. 2A). AG placement resulted in a significant increase in new bone formation compared to the control group (Fig. 2C) ($p < 0.05$). Also, in the control group, histologically, there was no detectable new bone formation.

There was no detectable osteoblastic activity in the control group. AG resulted in an increase in osteoblasts compared to the control group ($p < 0.05$) (Figs 2D and 3). Also, ozone therapy resulted in significantly higher osteoblast numbers compared to the AG alone ($p < 0.05$) (Fig. 2B).

Histomorphometric analyses demonstrated that there was no detectable bone formation in the control group as assessed by the area of new bone in the defect area (Fig. 4). In all the AG groups, the total bone area was significantly higher than that of the control group ($p < 0.05$). Also, AG+ozone group was significantly increased both the total bone area and percentage of total bone area compared to the AG group (Fig. 5) ($p < 0.05$).

Discussion

Studies addressing the use of ozone in bone regeneration in dentistry are necessary because its effectiveness is still unknown. Also, in this study, we have tested the hypothesis that ozone therapy will enhance osteoblastic activity and new bone formation by autogenous graft use in critical size defects in a rat calvaria model. The results demonstrate that ozone therapy augments new bone formation and leads to a simultaneous amplification of the osteoblasts.

AGs have been recommended by many authors for both periodontal defects and around dental implants (14–17). However, it was reported that AGs showed continuous bone volume reduction during healing and follow-up periods (14). During maxillary sinus augmentation, the volume of autogenous grafts was reduced to 49.5% of the initial volume after 6 mo (18). Therefore, several treatment modalities such as bisphosphonates, low-level laser therapy and hyperbaric oxygen therapy have been researched in enhancing bone healing (19–21). Jan *et al.* aimed to evaluate the effect of hyperbaric oxygen on the repair of critical-sized defects in the presence and absence of a non-vascularized AG (19). Consequently, they suggested that hyperbaric oxygen enhances bony healing in ungrafted defects and may decrease residual graft volume in autogenous bone grafted defects. However, hyperbaric oxygen therapy is only palliative treatment because, after 2 h, hypoxia resumes in ischemic areas and the therapeutic effect is minimal and temporary. On the other hand, during ozone therapy, ozone triggers a series of biological mechanisms that lead to normalizing the delivery of oxygen for several days with consequent therapeutic effects (6). Also, in the present study, the

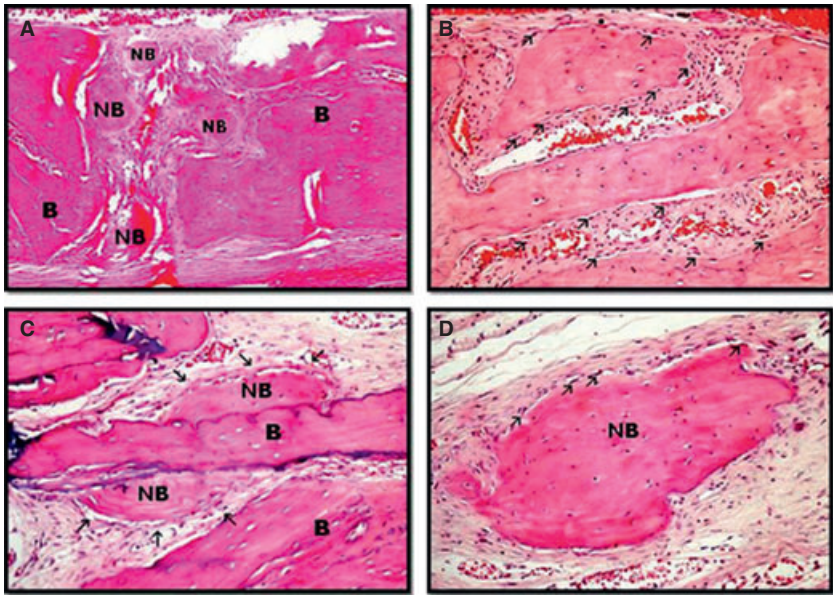

Fig. 2. (A) A typical histologic picture of new bone area in the autogenous bone graft + ozone group [hematoxylin and eosin (H&E), × 50]. (B) Photomicrograph of osteoblastic activity in the autogenous bone graft + ozone group (arrows) (H&E, × 50) (C) A typical histologic picture of new bone area in the autogenous graft group (arrows) (H&E, × 50). (D) Photomicrograph of osteoblastic activity in the autogenous bone graft group (arrows) (H&E, × 50). B, bone; NB, new bone area.

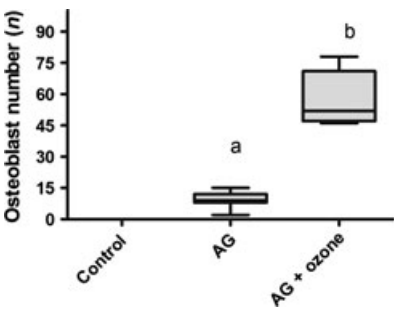

Fig. 3. Osteoblast numbers of all groups, $^{a}p < 0.05$ vs. control and AG + ozone groups, $^{b}p < 0.05$ vs. control and AG + ozone groups. AG, autogenous bone graft.

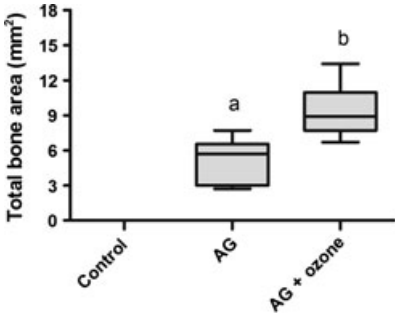

Fig. 4. Total bone area (mm^2) of the study groups and controls. $^{a}p < 0.05$ vs. control and AG + ozone groups, $^{b}p < 0.05$ vs. control and AG + ozone groups. AG, autogenous bone graft.

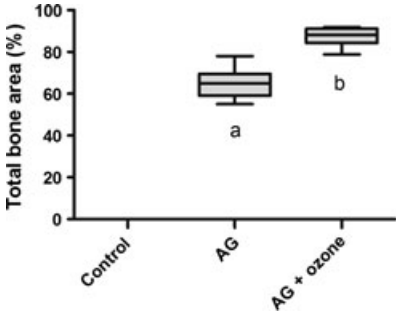

Fig. 5. Total bone area (%) of the study groups and controls. $^{a}p < 0.05$ vs. control group, $^{b}p < 0.05$ vs. control and AG + ozone groups. AG, autogenous bone graft.

histopathological results and histomorphometric analysis demonstrate that newly formed bone area are increased when graft material has been used with ozone therapy.

The clinical evidence for the application of ozone in dentistry is not extensive. Ozone can react with blood components (erythrocytes, leukoyctes, platelets, endothelial cells and vascular system) and positively affect oxygen metabolism, cell energy, the immunomodulator property, antioxidant defense system and microcirculation (12). Based on this finding, some authors suggest that ozone therapy in the management of bone necrosis or in extractive sites during and after oral surgery in patients treated with bisphosphonates may stimulate cell proliferation and soft tissue healing (22,23). Also, our histopathological results in the present study reported that ozone therapy increased osteoblast numbers.

In the experimental studies, the calvarial defect model has been regarded as the most selective experimental model of bone regeneration (2,24–27). We selected this model 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 (28) and physiologically, the cortical bone in the calvaria resembles an atrophic mandible (29); (iv) preparation of tissue specimens is easy; (v) parameters can be simply and accurately measured in each specimen (30); and (vi) spontaneous healing would not occur at the control defect (critical size defect) (31). In the present study, a 5 mm critical sized defect was created in the calvaria of rats because bony lesions above this critical size become scarred rather than regenerated (32–36). This was confirmed by the lack of bone regeneration at the control defect in our experiment.

Within the limitations of the present study, it is concluded that the ozone therapy increased bone formation in AG in rat calvarial defect model. Considering our data, it can be suggested that ozone therapy provides additional new insights into therapeutic strategies in improving bone regeneration in dentistry, but further experimental and clinical evaluations are needed.

Conflict of interest

The authors declare that they have no conflict of interests.

Source of funding

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