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Comparison of the Effects of Low-Level Laser Therapy and Ozone Therapy on Bone Healing

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Abstract: This study aims to compare the effect of low-level laser therapy (LLLT) and ozone therapy on the bone healing. Thirty-six adult male Wistar albino rats were used for this study. Monocortical defects were shaped in right femur of all rats. Defects were filled with nano-hydroxyapatite graft. The animals were divided into 3 groups and each group was than divided into 2 subgroups. Then, LLLT with a diode laser was applied to the first group (G1), ozone therapy was applied to the second group (G2), and no treatment was applied to the third group as a control group (G3). Animals were sacrificed after 4th and 8th weeks and the sections were examined to evaluate the density of the inflammation, the formation of connective tissue, the osteogenic potential, and osteocalcin activity. As a result, there were no significant differences among the groups of 4 weeks in terms of new bone formation. In the immunohistochemical assessment, the number of osteocalcin-positive cells was higher in the laser group compared to the other group of 4 weeks; this difference was statistically significant in the LLLT and ozone groups ($P < 0.05$). Histomorphometric assessment showed that the new bone areas were higher in the LLLT and ozone groups; furthermore, there was a statistically significant difference in the LLLT in comparison with the control group at 8th week ($P < 0.05$). At the same time immunohistochemical assessment showed that osteocalcin-positive cells were considerably higher in G2 than G1 at 8th week ($P < 0.05$). The findings of this study may be the result of differences in the number of treatment sessions. Further studies are therefore needed to determine the optimal treatment modality.

Key Words: Bone healing, low-level laser therapy, ozone, rat

Bone loss can result from various surgical procedures, traumas, or pathologies, and the process of bone healing has been broadly studied.[1] Bone regeneration is an extremely complex

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period, which comprises multiple biological reactions, such as the synthesis of DNA and the action of a great number of cells and proteins.[2] To enhance bone repair, various practices have been recommended, including the use of various types of grafts, fibrins, membranes, ozone, and bisphosphonates; low-level laser therapy (LLLT); hyperbaric oxygen therapy; and ultrasound.[3–5]

The biological effects of lasers were first studied in 1967 by Inyushin; Mester then applied lasers to accelerate the healing of chronic ulcers, ushering in the laser therapy model in 1971.[6] Contrary to other light sources, lasers produce monochromatic, harmonic, and collimated electromagnetic radiation. These features provide lasers with unique applications.[7] Light is first absorbed by photoreceptors in the cells when a laser is applied to the tissue. When absorbed, biochemical reactions can be regulated and mitochondrial respiration can be stimulated, producing molecular oxygen and ATP.[8] The synthesis of DNA, RNA, and cell-cycle regulatory proteins can be enhanced by these reactions, thus promoting cell proliferation.[9] There have been several studies describing the use of LLLT to stimulate wound healing,[10,11] nerve regeneration,[12] and collagen synthesis.[13,14]

Ozone (O3) is a triatomic molecule, consisting of 3 oxygen atoms; whereas it usually presents as a gas, it has been used in both gaseous and aqueous forms for medical purposes and can be dissolved in either water or oil.[15] The use of ozone medically is owed to its antimicrobial, disinfectant, and healing properties. Ozone is commonly used in restorative dentistry, endodontic procedures, oral surgery, and periodontology.[16] It can also be used during various clinical procedures, such as the management of early carious lesions, ulcerations, and herpetic lesions of the oral mucosa; the sterilization of cavities, root canals, and periodontal pockets; and the cleaning of dentures.[17]

Many investigations have attempted to quantitatively evaluate the influence of LLLT and ozone on bone constitution, but comparisons of both treatments are rare. The objective of the present investigation was to compare the influence of ozone and LLLT on grafted bone healing at 2 different times postsurgery.

MATERIALS AND METHODS

Ethical Statement

This study's experimental protocols were approved by the Experimental Animal Ethics Committee of Inonu University School of Medicine (2013/A14).

Experimental Animals

In total, 36 adult male Wistar albino rats were used. Their body weights ranged from 240 to 260 g at the beginning of the experiments. 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. All experimental procedures followed the guidelines of the Animal Care and Use Committee of the Inonu University Faculty of Medicine Experimental Animal Center.

Study Design and Experimental Procedures

The experiment was designed with 36 rats. Monocortical defects were made in the right femurs of all rats. The defects were filled with nanohydroxyapatite (Bego oss s inject, Bremen, Germany). The animals were divided into 3 groups, and each group was then divided into 2 subgroups. LLLT with a diode laser was used in the first group (G1), ozone therapy was used in the second group (G2), and no treatment was used in the third group (control, G3). Animals were sacrificed after the 4th and 8th weeks.

Surgical Procedures

To prevent the postoperative infection, all rats were given intramuscular amoxicillin (50 mg/kg), every 24 h for 4 days, starting 1 day before surgery. They were also given an analgesic, every 24 h for 3 days, starting immediately after surgery.

All surgical procedures were performed under sterile conditions. All animals were anesthetized preoperatively with an intramuscular injection of ketamine (Eczacibasi Ilac Sanayi, Istanbul, Turkey; 40 mg/kg body weight). The right femoral region of the animals was shaved and the cutaneous surface was disinfected with a topical povidone–iodine solution before the operation. An incision, approximately 3 cm in length, was made on the skin surface and muscle. A monocortical defect, $3 \times 6\,cm^2$ in size, was made on the superior lateral side of the femur with a trephine bur used at low speed under irrigation with saline. After preparing the defect, graft material (nanohydroxyapatite) was implanted into the bone defect. The soft tissues were then repositioned and sutured with 4–0 polyglycolic acid sutures. The animals remained under observation until their full recovery.

LLLT with a Diode Laser

LLLT (CHEESE Dental Laser System, DEN4A) was applied immediately after operation and was repeated 3 times per week (every other day) during the experimental time of 4 weeks (12 sessions). The gallium–aluminum–arsenide diode laser device operates in the near-infrared spectrum at a continuous wavelength of 810 nm and a power output of 0.3 w. A dose of $12\,J/cm^2$ was applied around the defect per session yielding a total treatment dose of $144\,J/cm^2$.

Ozone Treatment

The ozone application device was a portable ozone delivery system (OzoneDTA generator, APOZA, Taiwan). A topical ozone application protocol was performed using an ozone generator (OzoneDTA, Taiwan) on the day of surgery at 80% concentration (4th grade) via a 90-degree probe for a duration of 30 s (as suggested by the manufacturer).

Specimen Preparation

After a 4 to 8-week healing period, rats were sacrificed by an overdose of general anesthetic. A surgical burr was used to harvest the bone containing the experimental area from the animal's femur postmortem.

The samples, including the surgical sites, were fixed in 10% neutral-buffered formalin, decalcified in ethylenediaminetetraacetic acid and embedded in paraffin. Five micron-thick sections were stained with hematoxylin and eosin.

For immunohistochemical analysis, thick sections were placed on polylysine-coated slides. After rehydration, the samples were transferred into citrate buffer (pH 7.6) and heated in a microwave oven for 20 min. After cooling for 20 min to room temperature, the sections were washed with phosphate-buffered saline (PBS). Then sections were placed in 0.3% H_2O_2 for 7 min and then washed with PBS. Sections were incubated with primary rabbit polyclonal anti-osteocalcine (Thermo, USA) antibody for 30 min, rinsed in PBS, and incubated with biotinylated goat anti-polyvalent antibody for 10 min and streptavidin peroxidase for 10 min at room temperature. Staining was terminated with the addition of chromogen plus substrate for 15 min; slides were then counterstained with Mayer hematoxylin for 1 min, rinse in tap water, and dehydrated.

Histologic and Histomorphometric Analysis

The sections were examined using a Leica DFC 280 light microscope by a histopathologist blinded to the status of the

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animals. Four qualitative variables were examined during histological analyses:

1. The density of inflammation around the defect area was assessed. Grade 0 (no inflammation), grade 1 (mild; presence of < 25% of inflammatory cells in the area), grade 2 (moderate; presence of 26%–50% inflammatory cells in the area), and grade 3 (severe; presence of >51–100% inflammatory cells in the area).
2. The formation of connective tissue in the defect area was investigated. Grade 0 (no connective tissue), grade 1 (mild; presence of <25% connective tissue deposition in the area), grade 2 (moderate; presence of 26%–50% connective tissue deposition in the area), and grade 3 (severe; presence of >51% connective tissue deposition in the area).
3. The osteogenic potential, that is, the ratio of the area of new bone formation to the area of the total defect, was calculated as a percentage.
4. An estimation of osteocalcin activity in the defect was made. Qsteocalcin-positive cells (osteocalcin is produced by osteoblasts and is often used as a marker for the bone formation process) were counted in the area of the defect. The mean number of osteocalcin-immunopositive cells was determined.

All analyses were performed using a Leica Q Win Image Analysis System (Leica Micros Imaging Solution Ltd, Cambridge, UK). Histological assessments were performed in 5 randomly selected sites for each specimen at × 40 magnification.

Statistical Analysis

Statistical analysis was carried out using the SPSS for Windows version 13.0 (SPSS Inc., Chicago, IL, USA) statistical program. All data are expressed as the median (min–max). Normality for continued variables in groups was determined by the Shapiro-Wilk test. The variables did not show a normal distribution ($P < 0.05$). Kruskal-Wallis and Mann–Whitney U tests were used for comparison of variables among the studied groups. A $P < 0.05$ was regarded significant.

RESULTS

Histomorphometric and Immunohistochemical Assessment

At the end of 4 weeks:

G1: the lowest amount of new bone was found in the laser group but the differences were not statistically significant. The graft material was observed as a spherical, calcified structure in various forms and sizes in the defect area. Moreover, the graft material was surrounded by connective tissue (Fig. 1A). Osteocalcin-positive cells were observed in the connective tissue and on the surface of bone particles (Fig. 1B). The number of osteocalcin-positive cells was higher in this group than in any other groups after 4 weeks.

G2: although the amount of new bone formation was greater than that in the laser group, the differences were not statistically significant ($P > 0.05$; Fig. 1C). The localization of osteocalcin-positive cells in G2 was similar to that in G1 (Fig. 1D).

G3: the greatest amount of new bone was found in this group after 4 weeks, but the differences were not statistically significant ($P > 0.05$). However, bone marrow and graft material were observed among the bone fragments. The histology of the graft material was completely different from the existing bone, so it was easily differentiated within the defect area (Fig. 1E). Osteocalcin-positive cells were seen in the bone marrow (Fig. 1F).

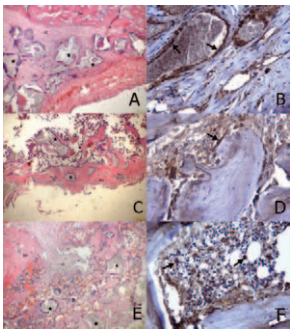


FIGURE 1. At the end of the 4th week: (A,B) laser group; (C,D) ozone group; (E,F) control group. bn:bone, ct: connective tissue, star: graft material, arrows: osteocalcin-positive cells. A,C,D: H-E X4; B,D,F: osteocalcin immune staining X40.

At the end of 8 weeks:

G1: the greatest amount of new bone formation was found in the laser group after 8 weeks. A great number of adipocytes and megakaryocytes in the bone marrow were observed (Fig. 2A). Moreover, hyaline cartilage and connective tissue were identified between the bone formation areas. Osteocalcin-positive cells were found in the bone marrow rather than on the surface of bone spicules (Fig. 2B).

G2: bone formation was similar to that observed in the laser group (Fig. 2C). In addition, statistically significant differences were not found in terms of connective tissue deposition or intensity of inflammation between the ozone and laser groups ($P > 0.05$). Osteocalcin-positive cells were higher in the ozone group than in the laser group ($P < 0.05$; Fig. 2D).

G3: the lowest amount of new bone formation was found in this group after 8 weeks (Fig. 2E). Many megakaryocytes were observed in the bone marrow. Osteocalcin-positive cells were identified on both the surfaces of bone spicules and in connective tissue (Fig. 2F).

Comparisons of new bone area among all groups are shown in Table 1. The intensity of inflammation and connective tissue deposition, and the number of osteocalcin-positive cells are shown in Table 2.

DISCUSSION

The present study examined and compared the impact of ozone and LLLT on bone tissue throughout the period of bone healing both histomorphologically and immunohistochemically. In this experimental work, we found that although the amount of new bone formation was greater in G2 than in G1, the difference was not statistically significant at early stages of regeneration (4 weeks after

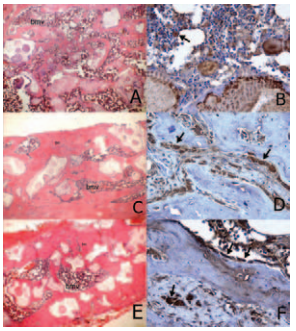


FIGURE 2. At the end of the 8th week: (A,B) laser group; (C,D) ozone group; (E,F) control group. bn:bone, ct: connective tissue, star: graft material, arrows: osteocalcin-positive cells. A,C,D: H-E X4; B,D,F: osteocalcin immune staining X40.

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TABLE 1. Comparison of New Bone Area Among in all Groups (as a Percentage)

Groups/Time	New Bone Area	$P < 0.05$
4th week		
1. G1	14.7 (5.1–30.1)	(4) (5) (6)
2. G2	20.0 (17.4–25.7)	(4) (5)
3. G3	21.7 (17.0–30.0)	(4) (5)
8th week		
4. G1	37.0 (30.8–50.6)	(1) (2) (3) (6)
5. G2	34.6 (18.7–49.6)	(1) (2) (3)
6. G3	27.7 (0.0–36.6)	(1) (4)

surgery; $P > 0.05$). However, the amount of new bone formation was greater in both G1 and G2 compared with that in G3, but the only statistically significant difference was among G1 and G3 after 8 weeks with regard to new bone formation.

In this study, the Wistar rat experimental model was used because the animals are easily lodged and fed, they exhibit a rapid healing period, are a cost-effective model, and have been routinely utilized in other experimental studies[18] of bone regeneration.

Several types of bone graft materials exist to fill bone defects. Although autogenous grafts are the "criterion standard," they require substantial surgical time and are associated with donor site morbidity, which consists of hematoma formation, infection, and pain. Additionally, these grafts are frequently resorbed in large defects before osteogenesis is completed,[19] which has led to the development of various alternative graft materials.[20] Hydroxyapatite is the most investigated biomaterial both clinically and histologically, with regard to the process of bone healing.[21] Furthermore, nanohydroxyapatite bone grafts have been studied for the last 10 years. We thus chose a nanohydroxyapatite graft material to fill the defect areas.

Osteocalcin is an extracellular matrix protein synthesized and secreted via the process of osteoblastic differentiation and mineralization.[18] In our study, the number of osteocalcin-positive cells was higher in the ozone group than in the laser group at the end of 8 weeks.

Laser therapy is an effective, noninvasive stimulator of osteogenesis and osseointegration of graft materials, and may improve bone growth and functional recovery.[22] Results of our study are in line with those of other studies that have investigated bone regeneration in animals.[19,23] Ribeiro et al[24] used a gallium arsenide (GA–AS) laser (735 nm, 16J/cm^2) on grafted bones in the tibias of rats, observing improvements in bone repair with LLLT in the irradiated animals. However, other studies did not find any effects of LLLT on the repair of mineralized tissues.[22]

TABLE 2. Comparison of Intensity of Inflammation and Connective Tissue Deposition and Number of Osteocalcin-Positive Cells in all Groups

Groups/ Time	Intensity of Inflammation	Connective Tissue Deposition	Number of Osteocalcin
4th week			
1. G1	0.0 (0.0–2.0)	1.0 (0.0–3.0)	8.0 (3.0–17.0)
2. G2	1.2 (0.0–1.2)	0.0 (0.0–3.0)	5.7 (2.0–12.0)
3. G3	0.0 (0.0–2.0)	0.1 (0.0–3.0)	7.0 (2.0–25.0)
8th week			
4. G1	0.0 (0.0–1.0)	0.0 (0.0–3.0)	5.3 (0.0–12.0)
5. G2	0.0 (0.0–1.0)	0.0 (0.0–3.0)	9.0 (2.0–54.0)
6. G3	1.0 (0.0–3.0)	1.0 (0.0–3.0)	7.0 (1.0–26.0)
$P < 0.05$	2–3, 3–6, 2–5, 2–4	1–2, 1–3, 1–4, 1–5, 2–6, 4–6, 5–6	1–2, 1–4, 2–5, 4–5

The conflicting results of LLLT on biostimulation are primarily related to variations in radiation parameters.

To obtain excellent outcomes, the dose (frequency, dose, power, number of sessions, wavelength, and technique of applying) that is applied throughout the laser treatment is important.[20,25] There are controversial ideas about the ideal doses for LLLT: whereas some authors recommend a dose of 1 to 5 J/cm^2 to stimulate wound healing,[26] others argue that 16 J/cm^2 for each session more effectively increases the positive effects on bone metabolism.[27] In addition, others have suggested higher doses, so it is not yet possible to establish an ideal treatment protocol for lasers.[28,29] The LLLT dose for this study was chosen based on an earlier study regarding bone defect healing using an autogenous bone graft.[30]

Some studies were conducted daily for 1 to 2 weeks of LLLT. We chose to use 3 LLLT sessions/week to mimic the clinical treatment regimen. We can say that the dose for LLLT applied to the grafted bone in our protocol was sufficient to stimulate bone repair.

Systemic effects of LLLT have been defined by several authors.[31] Several growth factors and cytokines are released into the circulation after LLLT, which could affect the untreated side of an experimental animal.[19] We used different animals in the test and control groups to avoid bias owing to possible systemic effects of LLLT.

Ozone can have effects on oxygen metabolism, cell energy, antioxidant defenses, immunomodulation, and the vascular system.[32] As such, few authors have reported that ozone is beneficial for treating bone necrosis or areas of oral surgery in patients using drugs such as bisphosphonates, since they stimulate cell proliferation and soft tissue healing.[33]

Ozone can activate neutrophils and stimulate the synthesis of cytokines, leading to improvements in the immune system. In that way, ozone therapy positively affects the outcomes of some infectious diseases.

Ozone therapy can be applied via a range of methods, generally involving the mixing of ozone with a range of gases and liquids, and injecting these solutions into the body, including the vagina, rectum, muscle, and beneath the skin, or by autohemotherapy.[16] A few studies have applied ozone therapy during oral and maxillofacial surgery. Agrillo et al[33] cured 94 patients of bisphosphonate-related jaw osteonecrosis (BRONJ) and reported that if used twice weekly for 3 min, ozone therapy was effective. Petrucci et al[34] suggested that ozone therapy applied 15 days can successfully treat BRONJ. A separate study reported that ozone does not stimulate the osteoblastic activity of gingival cells during the regeneration of the periodontium around implants.[35] Ozone is known to have bactericidal and antioxidant characteristics, which might elucidate its positive effects on the healing process, enhancing wound healing and regulating the immune system. Ozdemir et al[5] investigated the impact of ozone therapy on bone defects treated with autogenous bone grafts in rats and reported improvements in bone regeneration and numbers of osteoblasts in autogenous bone. Kazancioglu et al[20] assessed the impact of LLLT and ozone on bone healing in rat calvaria. They formed critical size defects on 30 rat calvaria and separated the animals into 3 groups: a control group, an LLLT group, and an ozone therapy group. The authors sacrificed all the animals after 1 month. As a result of the study, they reported that the total area of new bone was significantly greater in the ozone group than in the control or LLLT groups. The differences between their study and our results may be because of the number of treatment sessions.

In conclusion, the present outcomes emphasize the stimulatory effects of laser and ozone therapies on the healing of grafted bone defects. However, there are no significant differences between ozone therapy and LLLT. The findings of this study may be the

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result of differences in the number of treatment sessions. Further studies are therefore needed to determine the optimal treatment modality.

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Split Cartilage Resection of Nasal Dome: A Solution to Ptotic Nasal Tips

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Objective: In ptotic noses, it is rather difficult to achieve the intended functional and cosmetic result without modifying the alar

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