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Original

Effects of Gaseous Ozone Treatment on Bone Regeneration in Femoral Defect Model in Rats

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Abstract: The purpose of this study was to investigate the effect of ozone therapy in rat femoral fractures. Following the right open femoral fractures and intramedullary Kirschner wire fixation of the fracture in 60 male Wistar albino rats, the animals were divided into two groups of 30 animals each: ozone therapy group (n=30); non-treatment (control) group (n=30). Ozone was administered by rectal insufflation. After the rats were sacrified fracture healing was assessed by biomechanical and histopathologic evaluation. Histological and mechanical findings indicated that the ozone therapy had a significantly positive effect on bone healing.

Key words: Ozone therapy, Fracture healing, Rat femur

Introduction

Fracture healing is a complex process effected by many extrinsic and intrinsic factors. In spite of numerous previous studies, fracture healing is one of the most interesting fields of orthopedics and traumatology, but not yet fully defined. Fracture healing occurs as a result of multitude cellular and biochemical processes. In addition to bony tissue, the periosteum, subperiosteal layer external soft tissues, and bone marrow play active roles[1]. Despite the regenerative capacity of skeletal tissue, this biological process sometimes fails and fracture healing may delay or even develop pseudoarthrosis or nonunions[2]. The use of animal models have made it possible to investigate fracture healing from all perspectives such as biomechanics, and histology[3].

Ozone is normally present as a gas made of three atoms of oxygen with a cyclic structure. It can be administered with great flexibility via intramuscular, subcutaneous, intraperitoneal, intrapleural, intraarticular, periarticular, myofascial, intradiscal, intraforaminal, intralesional, topical or locoregional, nasal, tubal, auricular, oral, vaginal, urethral and intrabladder, rectal, cutaneous, dental route but it should not be injected intravenously as a gas because of the risk of oxygen embolism, given the fact that the gas mixture contains always no less than 95% oxygen[4]. Ozone therapy can induce several biological responses such as activates neuroprotective systems, 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[5]. When oxygen-ozone gas mixture comes in contact with either a moist human skin[6] or the rat colorectal mucosa, ozone dissolves immediately in the water overlaying the epithelium and reacts with either sebum, or mucoproteins, and any other biomolecules present in the liquid film generating hydrogen peroxide and lipid ozonation products. Only lipid ozonation products are absorbed by lymphatics and venous capillaries, then enter into the general circulation[7]. In the light of the knowlege given above we have hypothesized that ozone therapy will improve the fracture healing

The purpose of our study was to investigate the effect of ozone therapy in femoral rat fractures by mechanical and histologic evaluation.

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Materials and Methods

Animals

Experimental protocols were approved by the local Animal Ethics Committee. Eighty four male Wistar albino rats, 4–4.5 months old and weighing 350–400 g, were enrolled in this study. The animals were housed in a cage (floor area 900 cm2 and height 20 cm) with free access to tap water and standard laboratory rodent diet (with 1.1 % calcium, 0.8% phosphorus and 1500IU/kg vitamin D3) in a 12 h/12 h light-dark cycle.

Experimental protocol

The animals were anesthetized with combination intramuscular

injections of ketamine HCL (50 mg/kg, Ketalar; Eczacibasi, Istanbul, Turkey) and xylazine (10mg/kg, Rompun; Bayer, Istanbul, Turkey). All phases were performed under sterile conditions. The right hind limb was shaved, a 2-cm lateral incision was made, and soft tissues displaced medially and laterally to expose the mid-femoral diaphysis of the right hind limb. Animals were subjected to a standardized right open femoral fracture with bone cutting forceps. After manual reduction, the fractured femur was fixed with intramedullary Kirschner wires. The wounds were irrigated and closed using 3-0 nylon sutures. To prevent postoperative infection, ceftriaxone was given to the animals as intramuscular injections preoperatively and once daily postoperatively for 2 days(30 mg/kg) as well as carprofen (4 mg/kg) for pain relief pre- and postoperatively. The fractures were left without further immobilization. All rats resumed full weightbearing of the fractured limb within 1 week as confirmed by the absence of a visible limp.

The ozone delivery system used was the Ozonix Ozone Generator (Biozonix GmbH, Munich, Germany). O3 obtained from medical grade oxygen was used immediately and it repre sented only about 3% of the gas (O2 + O3) mix ture. The O3 concentration isme asured by using an UV spec trophotomete r at 254 nm and is very precise. The ozone dose is the product of the O3 concentration (expressed as mg/l) by the gas (O2 + O3) volume (l). By knowing the body weight of the rat the O3 dose is calculated as 1 mg/kg[8].

Experimental groups

The animals were randomly divided into two groups: one control group and treatment group (ozone therapy group) with the same body weight, 30 rats per group. Treatment began immediately after an experimental femoral fracture. The ozone were administered by rectal insufflation two times in a week. According to the protocol subsets of animals from each ozone therapy group and control group were sacrificed after 2, 4 and 6 weeks after surgery and the samples were collected. After the right femurs had been dissected from the surrounding soft tissues, the previously placed intramedullary Kirschner wires were removed. The site of the fracture was compared with biomechanical testing and histologic analysis.

Biomechanical Testing

Half of the right femurs for each subgroups were tested by the three-point bending method using a mechanical testing machine (Zwick/Roell, 1446, Germany). The femur was placed, facing its anterior surface down, on the two lower support bars (12 mm apart) with loading bar positioned at the fracture site, or middle femur. Load was applied until breakage and ultimate load was determined by a connected computer. All specimens were consequently loaded to failure point at a static rate of 20 mm/min and force versus displacement data was recorded. Biomechanical data were studied using the Mann–Whitney U test. A P value of less than 0.05 was considered statistically significant for mechanical results.

Histologic analysis

Histologic evaluation of fracture healing is composed of four phases in rats; first five days hematoma; days 5-14, chondrogenesis; days 14-21, endochondral ossification; and days 21 and over, remodeling[8]. Bone regions including the defective sites were decalcified in a 20% reduced formic acid solution for a period of 48 hours following the 10% formaldehyde fixation for three days. After tensile testing, tissues were dehydrated by a series of increasing ethyl alcohol, and embedded into paraffin. The longitudinal sections obtained from these blocks by microtome were stained by hematoxylin-eosine and then analyzed in microscope. Histopathologic evaluation of the fracture healing was performed according to the Lane and Sandhu[9] modified by Heiple et al.[10]. This scoring system evaluates union, spongious bone formation, cortical bone formation, and bone marrow organization.

Statistical analysis

The analysis was undertaken using the SPSS software package, version 21.0 for Windows (SPSS Inc. Chicago, IL, USA). Biomechanical and histopathologic scores were analyzed using Mann–Whitney U test. A P value of $P<0.05$ was considered to be statistically significant.

Results

Macroscopic analysis of bones at postoperative 2, 4 and 6 weeks showed that union was achieved in the fracture areas. Callus was adequate and there was no fracture line movement.

Results of histologic analysis

According to the histologic scoring system of Lane and Sandhu, as modified by Heiple et al. there was statistically significant difference between the total histological scores of the control and treatment group with respect to union, cortex formation, and bone marrow organization (Table 1).

At 2 weeks histopathological evaluation showed that union and bone marrow formation have significantly higher scores in treatment group (Table 1). In the treatment group there was osteochondral union in all cases (Fig. 1). Bone marrow was also detected in all cases, and the marrow spaces in one of the sample was occupied more than half, whereas others occasionally had fibrinous material in the bone marrow. In the control group there was no bone marrow, but there was fibrous union in all samples. Evaluation of spongious bone formation showed mostly active new bone formation in both groups. Cortical formation had started

Table 1. Median (minimum-maximum) values of the histologic score by groups

Parameter	Week 2	Week 4	Week 6	Total
Control group (n=13)				
Union	1(1-1)	2(1-3)	2(2-4)	2(1-4)
Spongious bone formation	2(2-2)	2(2-3)	2.5(2-4)	2(2-4)
Cortical bone formation	0(0-0)	0(0-1)	0.5(0-3)	0(0-3)
Bone marrow organization	0(0-0)	1(1-2)	2(1-3)	1(0-3)
Treatment group (n=15)				
Union	2(2-2)	2(2-3)	3(2-4)	2(2-4)
Spongious bone formation	2(2-2)	2(2-3)	3(3-4)	2(2-4)
Cortical bone formation	0(0-2)	0(0-3)	3(2-3)	2(0-3)
Bone marrow organization	1(1-2)	1(1-3)	3(1-3)	2(1-3)
P values of the histologic evaluation				
Union	0.008	0.343	0.214	0.013
Spongious bone formation	1.000	1.000	0.333	0.251
Cortical bone formation	0.317	0.368	0.067	0.027
Bone marrow organization	0.011	0.439	0.018	0.016

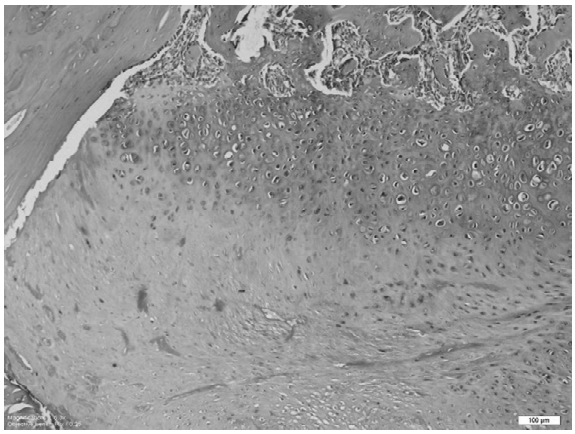


Figure 1: Histology results showing osteochondral union between the fracture sites. Scale bar=100 μm, H&E.

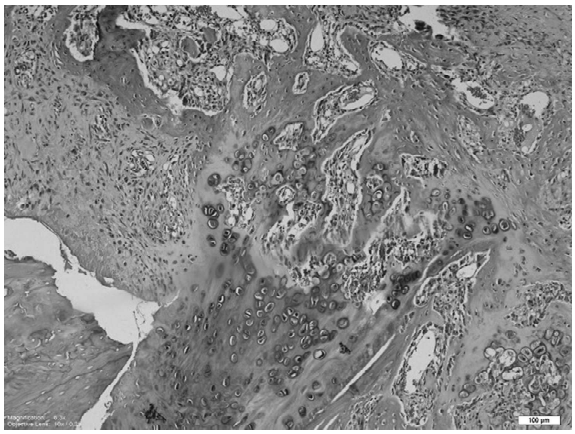


Figure 2: Spongious bone formation. Mostly active new bone formation. Scale bar=100 μm, H&E.

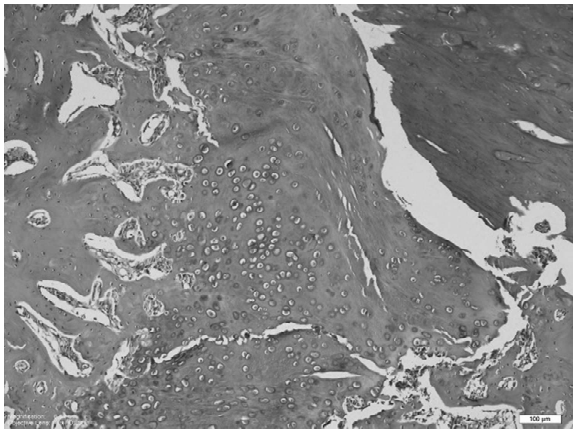


Figure 3: Bone marrow formation. The specimen was fully occupied by the red bone marrow. Scale bar=00 μm, H&E.

in one speciemen in control group. None of the other cases in either group showed formation of the cortex.

At weeks 4 there was no statically significant difference between the treatment and control group respect to union, cortex formation, bone marrow organization, and spongious bone formation (Table 1). Both groups showed similar results for the cortical healing. Cortex was detected in one case only. Bone marrow was detected in all cases, and the marrow spaces in most of the cases had fibrinous material. Spongious bone showed mostly active new bone formation in both groups (Fig. 2). In both groups there was at least fibrous union. There was no case of nonunion in either group. Most of the speciemens demonsrated osteochondral union.

At weeks 6 histopathological examination showed that only bone marrow formation have significantly higher scores in treatment group. In the control group bone marrow was detected

Table 2. Median (minimum-maximum) and Pvalues of the biomechanical analysis

	Week 2	Week 4	Week 6
Control group (n=13)	18.5(4-35)	30.5(26-38)	30(15-53)
Treatment group (n=13)	19.5(8-36)	50.5(22-85)	50(50-70)
P value	0.468	0.248	0.045

in all cases, and the marrow spaces in one of specimen was fully occupied and there was fibrinous material in one sample. In the others the marrow spaces was occupied more than half. The treatment group was consist of six specimens and all of them were fully occupied by the red bone marrow (Fig. 3). Union, spongious bone and cortical bone formation showed high scores, and some of the samples demonsrated highest score being 4 according to the histologic scoring system of Lane and Sandhu, as modified by Heiple et al.

Results of biomechanical analysis

Load was applied until breakage and ultimate load was determined. After 2 and 4 weeks there was some difference between two groups. The treatment group showed higher ultimate load but this was not statically significant. After 6 weeks biomechanical evaluation showed that the treatment group has higher scores than control group and the difference was statically significant (Table 2).

Discussion

It is well known that ozone has a therapeutic effect that facilitates wound healing and improves the supply of blood, and it may be applied as a gas or dissolved in water for medical purposes. The aim of bone augmentation procedures is to induce or facilitate the healing of the bone. Studies using of ozone on bone healing in orthopaedicsare necessary because its efficacy is still unknown. Ozone positively influences cell energy, oxygen metabolism, antioxidant defense system, microcirculation in tissues. It improves wound healing, modulates immune system, and acts as an antibacterial agent[11-13]. Femoral defect model has been regarded as an appropriate experimental model of bone regeneration. In femoral defect model, surgical procedures can be relatively simple to accomplish, preperation of tissue speciemen is easy, observations can be focused on healing process of bone[14-18]. For enhancing bone regeneration and healing performance, several treatment modalities and more recently ozone were applied. Immunomodulator effects of ozone can increase oxygene in tissues and improve the healing phase in fracture site. In the present study, we investigated the effects of gaseous ozone treatment on bone regeneration in femoral defect model in rats. We demonstrated that bone regeneration are improved in the treatment group. Some authors recommend that ozone therapy may be beneficial in the treatment of bisphosphonate-induced bone necrosis or in extractive sites during and after in oral surgery because it might stimulate cell proliferation and soft tissue healing[19]. Recently, ozone has been used in several study on bone healing due to its immunomodulatory effects. Ozdemir et al analyzed the effect of ozone therapy in combination with autogenous bone grafts on bone healing in critical size defectin rat calvaria. Ozone application was performed with a ozone genarator. They reported that ozone therapy increased osteoblast numbers and total bone area and bone formation in autogenous bone grafts in the rat calvarial defect model[20]. Erdemci et al[21] evaluated effect of the systemic and topical ozone administration on alveolar bone healing following tooth extraction in rats. Long-term preoperative and postoperative systemic ozone application create more trabecular bone formation comparing to only the preoperative systemic ozone application. Also, Kazancioglu et al[22] compared low level laser therapy and ozone therapy on bone regeneration. Critical size defect were created in all rats and defects were treated with biphasic calcium phosphate graft material. Then ozone was applied. At the end of the 4 weeks, in ozone group more new bone area was occured compare with other groups. Ozone application has been found at least as effective as low level laser application. In an another study by Kan et al the effects of hyperbaric oxygen and systemic ozone therapy, on calvarial defect in rats was evaluated. Histomorphometric analysis showed more new bone formation in all experimental groups compared with the control group. Similarly, microtomographicanalysis showed larger volumes of new bone in all experimental groups compared with the control group. The use of hyperbaric oxygen and ozone, were shown to be effective in increasing bone healing[23]. Buyuk et al investigated the of effects of different concentrations of ozone therapy on bone regeneration in response to an expansion of the inter-premaxillary suture in rats. Fibrotic area, new bone area, osteoclast and osteoblast numbers, and the amount of vascularity were significantly higher in treatment groups compared with the control group[24]. Alan et al analyzed the effect low-level laser therapy and ozone therapy on bone healing results 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 low-level laser therapy[25]. Puritiy and dose performed during ozone therapy is an important advantage of ozone for obtaining good results. In our study, ozone was applied

gaseous form with an ozone genarator. Gaseous ozone administration did not cause any side effects in rats. Although some protocols defined by the manufacturer, there is not a particular gaseous ozone application technique for bone regeneration in literature. Additionally, there are many ozone generators on the market. The amount of ozone generated by these devices are different. For this reason, studies performed with ozone devices have been achieved different results. Administration of O3 in rats could be established neither by inhalation, due to its toxicity[26-27] nor by ozonated autohaemotherapy for technical difficulties. however the colorectal tract, although somewhat empirical, is easy, nontoxic and has the rationale that ozonated products reach the liver via portal circulation[-28]. It is reported that the correct dose of ozone is 1mg/kg in the rat and 0.25 mg/kg in humans, respectively[8].

It is well known that ozone therapy improves blood circulation and oxygen delivery, enhances general metabolism, induces the release of growth factors[29-30] and we also know that vasculature, tissue oxygenation has a central role in fracture healing. Growth factors; IGF-1, PDGF, TGF-β, FGF, VEGF stimulate osteoblast proliferation and differantiation, migration of progenitor cells and osteoblasts, progenitor cell proliferation, angiogenesis, chemotactic for macrophages and endothelial cells and vasodilatation[31-32]. Our study has shown that ozone therapy significantly improves fracture healing. Ýn our opinion this occurs by the above-mentioned effects of ozone. however further studies are required to elucidate the exact hole mechanism.

In conclusion, fracture healing in rat bone fracture model was significantly increased by rectal insufflation of ozone as shown by biomechanical and histological evaluation

Conflict of Interest

The authors have declared that no COI exists.

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