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Original Research Article

The effect of ozone therapy on experimental bone fracture healing in rats

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Ozone has been used as a therapeutical agent for a long time. In the literature, many beneficial effects are attributed ozone; it balances the oxidant-antioxidant system, leads to delivery of super enriched oxygen at a cellular level and optimizes cell function, etc. Previously, beneficial effect of ozone on cutaneous wound healing was shown in experimental model in mice.The aim of this study was to evaluate if there is a positive impact of ozone therapy on bone healing in an experimental fracture model in rats. After approval of experimental animal ethic committee, an open femoral fracture (ffx) was performed in 48 Spraque-Dawley male rats. They were randomly divided into two groups: in Group O, medical ozone/oxygen mixture (500mcg/kg) and in Group C, medical air in volume of 4 mL were given rectally for 3-consequtive days. At days 4, 7 and 13, eight rats from each group were euthanized, and histomorphometric and immunohistochemical bone tissue evaluation was undertaken from the femur sampling. Histomorphometrically, periost thickness was thicker, trabecular areas were larger, cartilaginous and new bone areas were smaller ($p<0.05$ for each) and TGF-β expressions were more intense in Group O($p<0.001$ for all times). Immunohistochemically, a significant increase in VEGF expressions in day 7 and 13 ($p<0.001$ for each) and higher β-catenin expressions were observed in Group O at day 7 and 13 ($p<0.001$ for each).The current data indicate that ozone therapy could have positive impacts on bone healing process.

Key words: Ozone therapy, bone healing, experimental fracture, rat

INTRODUCTION

Ozone is a natural chemical compound, consisting of three oxygen atoms. Joseph Lloyd Martin discovered and obtained medical ozone in a gas form in 1850 (Bocci et al., 1993; Bocci, 1994). In human body, ozone behaves differently according to the ph of tissue; in the acidic tissue; ozone application results in peroxidation and lipid degradation especially in obese people with atherosclerosis. At alkalosis (pH ≥8); after ozone injection, the number of OH^- is increased; they interact with cellular membrane's phospholipid. Hydrogen peroxide enters into

cell and effects intracellular metabolism. In erythrocytes, adenosine three posphate (ATP) and 2,3 diphosphoglycerate (DPG) levels increase and immune-competent cells, Nuclear Factor kappa B cells (NF-KB) and cytokines are released (Bocci, 1994; Rilling et al., 1987). Ozone has bactericidal, fungicide, and anti-inflammatory effects (Bocci, 1994). Systemic application of ozone leads to delivery of super enriched oxygen at a cellular level and optimizes cell function. This is achieved by activating the red blood cell, immune-competent cells and the enzymatic antioxidants and radical scavengers at a cellular level (Coppola et al., 1992; Bocci et al., 1993; Bocci, 1994; Iliakis et al., 2001).

Endochondral bone repair, shown at non-stabilized bone fracture healing, is characterized by the initial synthesis of cartilage followed by the endochondral sequence of bone formation. Different cell types and tissues take part in the endochondral bone healing process such as mesenchymal cells, osteoblasts and osteoclasts. Moreover, the endochondral bone repairs process is mediated by the periosteal layers, especially the inner layer and bone marrow tissue (Maes et al., 2006; Shapiro, 2008; Azuma et al., 2011). The first few days after the fracture are characterized by an inflammatory phase and the proliferation and differentiation of mesenchymal cells (MSCs) into cartilage and then into blood clot formation, angiogenesis, formation of cartilage, calcification of cartilage, cartilaginous transformation into the bone and remodeling (Dimitriou et al., 2005; Shapiro, 2008; Claes et al., 2013; Garcia et al., 2013).

Transforming growth factor (TGF)-β, a regulator factor and signaling pathway, effects bone healing process via regulation of osteoblast differentiation and its reduction in osteoblasts cause higher level of bone mineral content and bone mass (Shapiro, 2008). Bone is a well-vascularized tissue and organ. Vascular endothelial growth factor (VEGF), an angiogenesis promoter factor, effects stimulation of neovascularization and regulation of fracture healing, and these effect the arrangement of osteoclast and osteoblast activity (Street et al., 2002; Gruber et al., 2006; Shapiro, 2008). Angiogenesis plays an important role at bone formation during fracture healing (Maes et al., 2006; Holstein et al., 2011). β-catenin, which belongs to the WNT/canonical signaling pathway, has a crucial effect on osteoblast differentiation and function (Khosla et al., 2008; Chen and Alman, 2009; Agholme and Aspenberg, 2011).

Previously, Valacchi et al. (2011) researched the effect of ozonated sesame oil on cutaneous wound healing in mice. Their results show that ozonenated sesame oil leaded to an earlier and higher response of cells involved in wound repair, a higher angiogenesis, as well as enhanced VEGF expression.

We know that cellular functions, angiogenesis and VEGF are also important issues for bone fracture healing. However, systemic application of ozone leads to delivery of super enriched oxygen at a cellular level and optimizes cell functions. We decided to investigate if ozone will demonstrate the same positive effect that was shown at cutaneous wound healing, in bone fracture healing also.

The aim of this study was to evaluate the effect of ozone therapy on bone healing in an experimental fracture model in rats.

MATERIALS AND METHODS

Animals and experimental design

After obtaining, the experimental animals ethics committee's approval, 48 Spraque-Dawley male rats, weighing 250-280gr were included in the study.

Animals were anesthetized with injection of xylazine hydrochloride (50 mg/kg, Bayer United German Pharmaceutical Factories, Istanbul, Turkey) and ketamine hydrochloride (50 mg/kg, Parke-Davis, Istanbul, Turkey) intraperitoneally (i.p.). After shaving the right legs, surgical fields were disinfected with 10% povidone iodine and then draped under sterile conditions. According to the open femoral fracture technique, (Le et al., 2001), a vertical lateral incision at the femur was followed by muscular blunt dissection. The femur bone was fractured transversally with a manual bone cutter at the level of diaphysis. The bone was not fixated. Then, edge of wound was closed with a non-absorbable suture.

After performing femoral fracture (ffx), radiographic examinations were done in order to standardize ffx, while rats were still under the effects of anesthetic drugs. Radiographic analysis was performed by Siemens syngofast view (2011, Germany). The X-ray images were taken bothat the prone position and at the lateral position while their right legs were abducted (Figure 1).

After radiological imaging approved standard ffx; rats were randomly allocated into one of two groups by using sealed enveloped technique choosed by the physician. In Group O (n=24), ozone/oxygen mixture (according to dose (500 mcg/kg), ozone's concentration was ranged 30-35 mcg/mL in order to be given in a fixed volume) and in Group C (n=24), medical air in volumes of 4 mL was given rectally for 3-consequtive days. Days 4, 7 and 13 are the milestones of the fracture healing process (Le et al., 2001), therefore in order to see how these processes are affected the eight rats from each group were euthanized with decapitation and afterwards the right femurs were removed. Healing at the fracture site was evaluated histomorphometrically and immunohistologically.

Femur sample analysis

Histomorphometrical analysis

For every sample, 5 serial sections were obtained and a minimum of five adjacent fields in each section was

Figure 1. Radiographic image of open femoral fracture in rat

quantified at a magnification of × 40 objective lens. The thicknesses of periosteum, the number and areas of trabecules and the area of cartilaginous, new bone and trabecules were evaluated histomorphometrically with a semi-automatic image analysis system, The University Of Texas Health Science Center At Santorino (UTHSCSA) image tool for Windows version 1,28 program (Aktug et al., 2006; Ignatius et al., 2011).

Histological analysis

Bone samples were fixed in 10% neutral buffered formalin for 24 hours and then decalcified in decalcifier solution (Shandon TBD-2 Decalcifier, UK) at room temperature for 5 days. Each specimen was dehydrated using graded ethanol series and later cleared in xylene, before being embedded in paraffin. The paraffin blocks were cut into 5µm and the sections were mounted on glass slides. The sections were stained with hematoxylen-eosin for evaluating bone tissue and Safranin O for evaluating callus tissue, cartilaginous and new bone area (Aktug et al., 2006; Ignatius et al., 2011).

Immunohistochemical analysis

Tissue sections of 5µm were taken from the paraffin blocks. The tissue blocks were chosen carefully after histological assessment of sections stained with hematoxylen and eosin (hematoxylen acc. to Gill III, Cat. no. 1.05174; eosin solution in 0.5% alcohol, Cat. no. 1.02439, Merck, Darmstadt, Germany). For immunohistochemical staining, sections were incubated overnight at 60˚C and then immersed in xylene and rehydrated through a series of ethanol solutions. Sections were washed with both distilled water and phosphate-buffered saline solution (PBS) (P4417; Sigma-Aldrich, St Louis, MO) for 10 minutes and then treated with citrate buffer (ph:7.6) for 5 minutes at pressure cooker. Following washing with PBS, sections were delineated with a Dako Pap pen (S2002, Dako, Glostrup, Denmark) and incubated in a solution of 3% hydrogen peroxide for 5 minutes to inhibit endogenous peroxidase activity. After washing in PBS, sections were incubated with primary antibodies 1 hour at 40˚C in a humidity chamber. Sections were washed 3 times for 5 minutes each with PBS; sections were incubated with secondary antibody using the NovoLink polymer detection system (Novocastra, RE715K). After washing with distilled water, sections were counterstained with Mayer's hematoxylen and washed again with distilled water. All sections were mounted with mounting medium (Shandon EZ-Mount, Mounting Medium, USA) and immunoreactive cells were evaluated by an Olympus BX51 bright-field microscope (Olympus, America Inc., USA). The presence of a brown precipitate indicated a positive reaction for primary antibodies. To determine the immunoreactivity of sections, a series of semi-quantitative analyses were performed in tissue materials. HSCORE was calculated using the following equation: HSCORE= $\sum$Pi (i+1); where i is the intensity of labeling with a value of 1, 2 or 3, (weak, moderate, or strong, respectively) and Pi is the percentage of labelled cells for each intensity, varying from 0 to 100% (Uslu et al., 2007).

Statistical analysis

For statistical analysis, Nonparametric ANOVA test, Kruskall Wallis test and Dunn's test as a post-hoc test were used ($p<0.05$). For this analysis the GraphPad Instat program was applied.

RESULTS

Histomorphometrical analysis

Data and expressions related to histomorphometrical analysis were given in Table 1 and Figure 2.

Table 1. Histomorphometrical analysis

	Day 4 Control	Ozone	Day 7 Control	Ozone	Day 13 Control	Ozone
Periosteum Thickness	33.8±6.3	43.8±4.3*				
Trabecular Number			15.7±4.5	19.3±3.5	19.3±4.7	23.6±6.2
Trabecular Area			1120±220	1641±330*	782.91±150	1566.24±315*
Cartilaginous Area			21134±2825	12367±707*	91885±1393	65638±1200*
New bone Area			68899±3809	48179±2216*	136196±2013	112690±1939**

When compared with same day Control Group values, *p < 0.05 and **p < 0.01

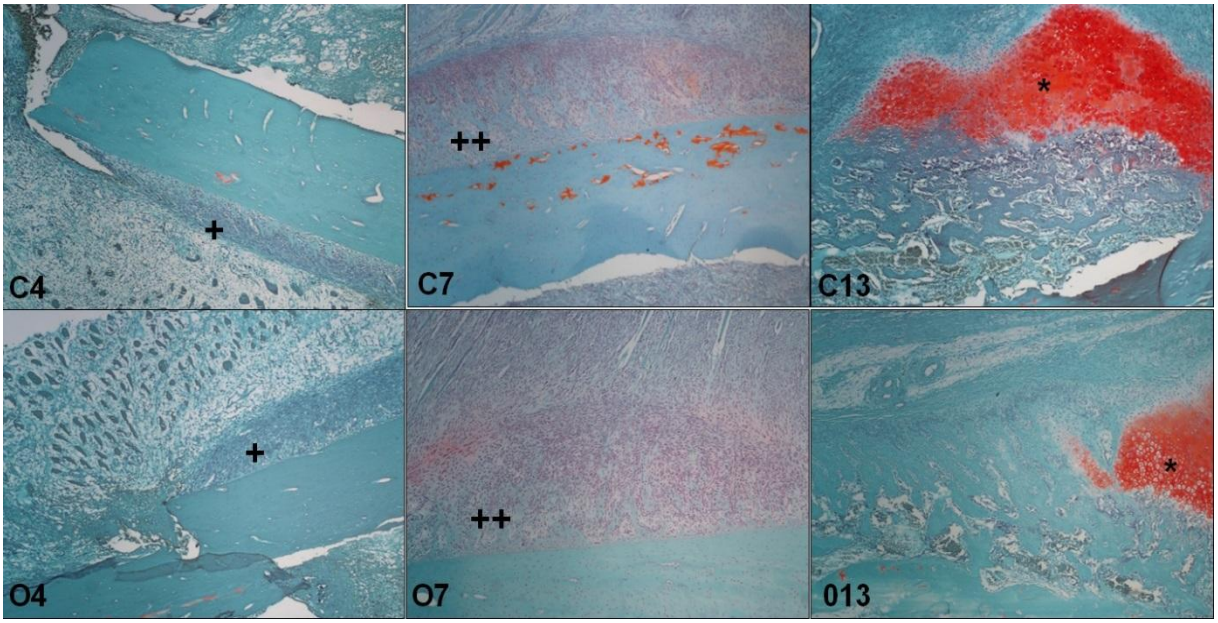

Figure 2: Evaluation of histomorphometricalfeatures: periosteal thickness (+), new trabecular area (++), cartilaginousarea (*) in Days 4,7 and 13.C: Control Group, O:Ozone Group. Safranin O staining (×40)

Periosteal thickness as a sign of stem cell activation: On day 4, when compared with Control Group there was a significant increase in the periosteum thickness in Ozone Group (p<0.05).

Trabecular number as an indicator of of bone healing at the early period: When compared with the Control Group, trabecular numbers per area in Ozone Group were higher both on day 7 and 13; but these differences were not statistically significant (p>0.05).

Trabecular area as a sign of bone healing at the early period: When compared with Control Group, trabecula areas were found to be larger in Ozone Group both on day 7 and 13 (p<0.05 for each).

Cartilagenous Area: When compared with Control Group, there were significant decreases in Ozone Group in day 7 and 13 (p<0.05 for each).

New bone area: New bone areas were found to be smaller in Ozone Group in Day 7 and 13 (p<0.01 for each).

Immunohistochemical analysis

Expressions and scores related to immunohistochemical analysis were given in Figure 3 and 4.

H-SCORE values of TGF-β, VEGF and β-catenin immunoreactivity in fracture healing were demonstrated. These were increased in TGF-β in day 4, 7 and 13 in Ozone Group compared with Control Group (p<0.001)(Figure 4.1).

Although, there was no significant increase of VEGF expressions in day 4 (p >0.05), VEGF expressions were more intense in day 7 and 13 in Ozone Group (p<0.001 for

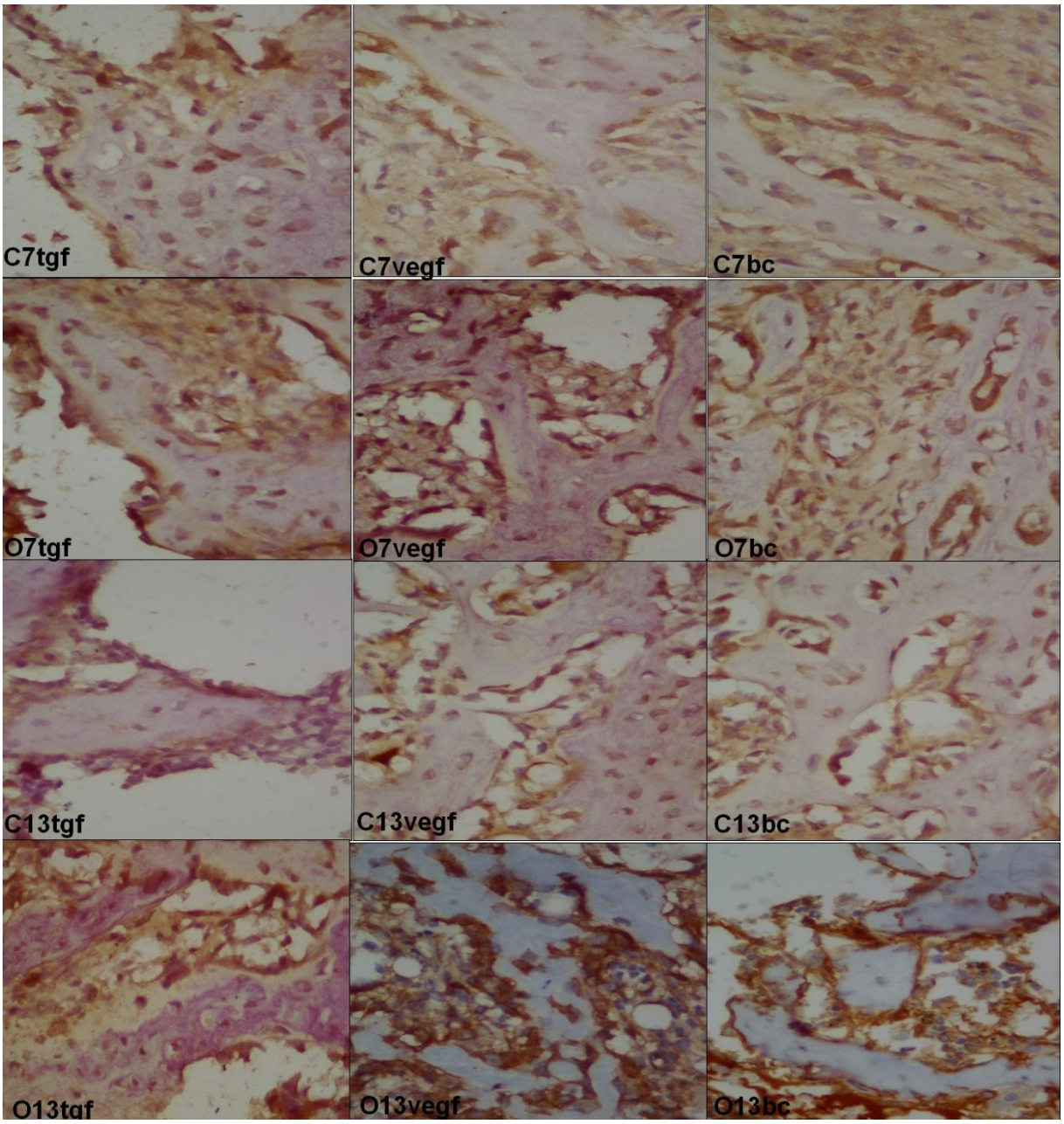

Figure 3: Microscopial appearances of expressions of TGF, VEGF and Beta-Catenin in Control, and Ozone Groups. (× 400). More intense expressions of TGF, VEGF and Beta-Catenin were detected in Ozone Group. Scores related to these expressions were given in Fig ure4.

each) (Figure 4.2).

There were increases in day 7 and 13 in β-catenin expression in Ozone Group compared with Control Group ($p<0.001$) (Figure 4.3).

DISCUSSION

Endochondral ossification, related to periosteum and external soft tissues was demonstrated around fracture site. At the beginning of the process MSCs, converted into chondroblast, start to proliferate by day 3 after fracture. This proliferation process, from days 7 to 21, results in soft callus formation. If the periosteum is removed, the fracture callus development is diminished (Dimitriou et al., 2005; Azuma et al., 2011). In our study, it was shown that periosteum thicknesses of the Ozone Group were higher than the Control Group on day 4. In our study cartilaginous areas in the Ozone Group were smaller than the Control Group in days 7 and 13. Previous studies claimed that callus

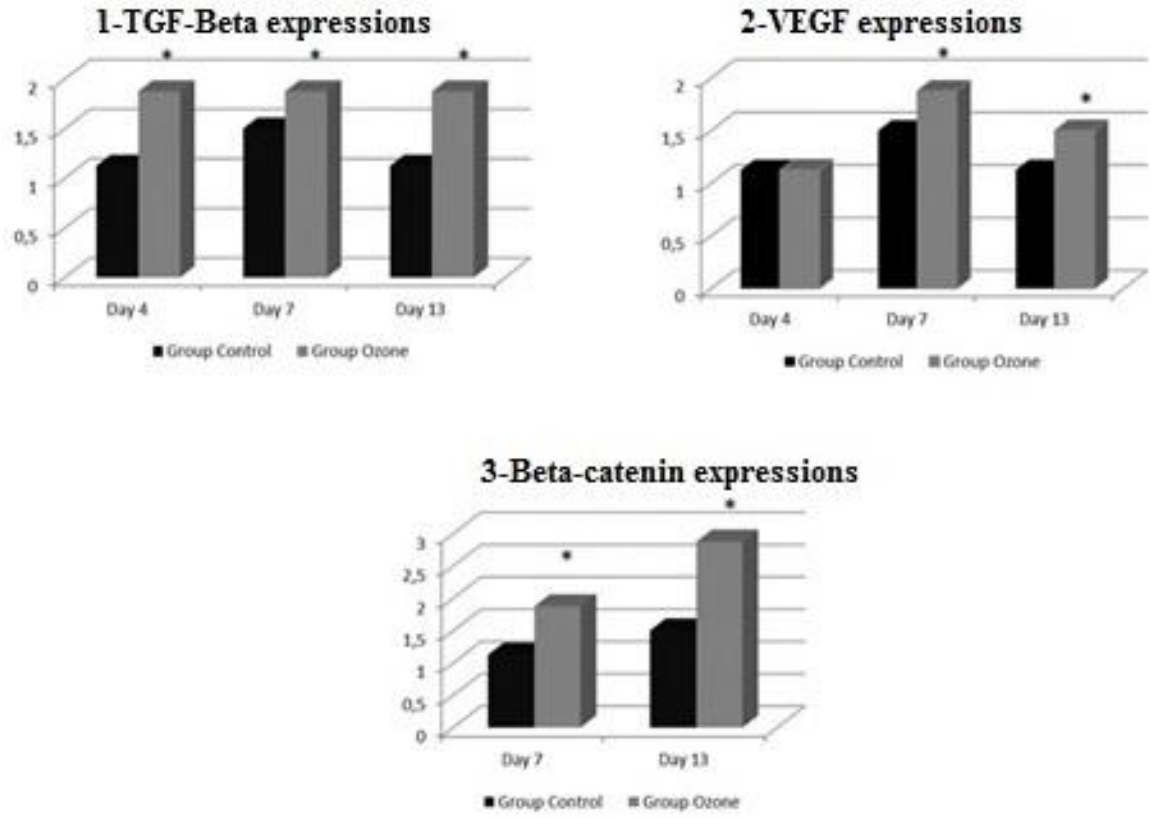

Figure 4: Scores of expression of TGF, VEGF and Beta-Catenin in Control, and Ozone Groups. *When compared with same day Control Group values, $p < 0.001$

is replaced with bone on day 14 in the fracture site so that small cartilage regions are left. Diniz et al. (2008) suggested that continuation of a large cartilaginous callus, together with deficiency of trabecular bone, results in delayed healing and less mature callus formation in rodents. In our study, higher trabecular number per area and larger trabecular area in Ozone Group, were the signs of better healing process.Transforming Growth Factor β (TGF-β) is a potent mitogen for bone cells and controls proliferation of undifferentiated mesenchymal and osteoprogenitor cells, osteoblast and chondrocytes, expressed from early stages of fracture healing. Secretion of TGF-β begins in day 0 in the fracture site and secretion continues through to day 21 of healing process. MSC proliferation and angiogenesis begin in day 3, because of TGF-β expression. TGF-β expression starts at the beginning of endochondral ossification process in day 7 after the fracture and continues through healing process. Our results showed that, TGF-β expressions were higher in Ozone Group than in the control all times.

The bone fracture healing process contains several complex molecular mechanisms which are controlled angiogenesis and other growth factors. The expression of VEGF, which has an important role in the angiogenesis, starts on day 14 after the fracture, and goes through all of the fracture healing process. Bone healing is related to new blood vessel formation in the fracture area. During the fracture repair phase, both intraosseous and extraosseous arterial circulation increases. Blood supply reaches a peak above the pre-injury levels at 2-4 weeks post-fracture in rats, and decreases gradually thereafter (Nikolaou and Tsiridis, 2007; Wohl et al., 2009). Previous studies demonstrated that VEGF expression has been shown to stimulate endothelial cell proliferation and its expression significantly increases during endochondral ossification. Callus architecture is determined by VEGF expression, highly expressed in mesenchymal cells and osteoblast cells during the first week of healing, but decreases after eleven days (Geiger et al., 2007; Kanczler and Oreffo, 2008). In our study, VEGF expressions were found higher in the Ozone Group than the Control Group in both day 7 and 13.

Bodine (2008) showed that, β-catenin expression increases in bone fracture healing process both human and rodents when differentiation of MSCs to osteoblast and chondroblast in the earlier period of bone fractures healing. Betacatenin has a crucial effect in the differentiation of MSCs to osteoblast in the bone fracture healing. Moreover, β-catenin has multiple correlative effects with other bone growth factors such as BMPs and TGF-β (Kanczler and

Oreffo, 2008; Bodine, 2008). Our results showed that β-catenin expressions were higher in Ozone Group than Control Group in day 7 and 13.

Factors impairing fracture healing may include the limited capacity of the elderly to neutralize reactive oxygen species of the respiratory chain. Free radicals produced by the respiratory chain may cause oxidative damage to various cellular components which may affect cellular function, also involving cells of the osteogenic and chondrogenic pedigre (Day et al., 2005; Yavropoulou; Yovos, 2007). Antioxidants, vitamins C and E, and the enzymes superoxide dismutase, catalase and glutathione peroxidase may contribute to bone regeneration. Recent findings suggest that reactive oxygen species play a role in bone loss following ovariectomy (Carrington, 2005; Kloss et al., 2006). In animal models, vitamins C and E, antioxidants like ozone, had a positive effect on fracture healing (Lean et al., 2003).

CONCLUSIONS

Thicker periostium and better trabecular formation, together with more intense expressions of VEGF, TGF-β and β-catenin were all signs of better bone fracture healing. In the light of this study, we can speculate that ozone therapy has positive impacts on the early period of bone healing process. Also, it could have positive impacts on the later period of bone healing process.

Competing Interest

The authors declare that they have no competing interests.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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