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RESEARCH ARTICLE

Preventive and Curative Effects of Medical Ozone in Rats Exposed to Experimental Osteomyelitis

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ABSTRACT

Investigation of preventive and therapeutic effects of medical ozone in rats with acute osteomyelitis was aimed in this study. *Staphylococcus aureus* solution was injected intramedullary into right femurs of each animal to form acute osteomyelitis. Preventive Osteomyelitis Group (POG) was taken an ozone session by rectal insufflation daily at a dose of 500 µg/kg for 15 days with an ozone generator starting a week before microorganism injections, whereas applied nothing to Untreated Osteomyelitis Group (UOG). Treatment Osteomyelitis Group (TOG) was administered the same ozone therapy sessions as POG. But this application was started just after 4 days following the microorganism injection. At the end of the ozone-therapy, two femurs of each animal in 3 groups were excised and examined radiographically, microbiologically and histopathologically. It was found that radiographical differences between UOG and other two osteomyelitis groups were significant, but those between POG and TOG were insignificant. There was not any microbiological reproduction in conventional culture methods. Histopathologically; mild, moderate and severe inflammation findings in the POG, TOG and UOG were observed respectively. Histopathological healing was significant in TOG compared to UOG, and it was significant in POG compared to TOG. Conclusively, it was thought that preventive and curative effects of medical ozone in rats exposed to experimental osteomyelitis had been found.

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INTRODUCTION

Ozone (O_3) is a triatomic molecule consisting of three oxygen atoms and an unstable gas that cannot be stored. It is the third most potent oxidant after fluorine and per-sulfate; although not being a radical molecule, and is naturally produced by the photo dissociation of molecular oxygen (O_2) into activated oxygen atoms, which then reacts with further oxygen molecules (Ozler *et al.*, 2009; Bocci *et al.*, 2011a; Saini, 2011). Initial years after being discovered, it was used for disinfection; however, studies conducted have come into questions for medical usage of ozone. Hydrogen peroxide (H_2O_2) produced by oxidative stress and lipid oxidations mediates the biological effects of ozone-therapy by acting as a second messenger. Repetition of ozone administration creates a resistance against oxidative stress via inducing antioxidative system. Moreover, levels of several cytokines are increased depending on the fatty acid oxidation in cell membranes (Bocci *et al.*, 2011a; Ijazi *et al*., 2016; Zubair *et al*., 2016).

The ozone improves blood circulation, tissue oxygenation and general metabolism. It upregulates the cellular antioxidant enzymes and modulates the response of immunity cells like a cytokine by improving phagocytosis and diapedesis of phagocytes. It stimulates the release of staminal cells in the patient's bone marrow, and also stimulates angiogenesis and fibroblasts formation together. It enhances the release of growth factors from platelets and accelerates the exchange of protons and electrons after dissolving in plasma, reactivating the metabolism all over the body (Ozler *et al*., 2009; Bocci *et al*., 2011b; Agrillo *et al*., 2012).

Osteomyelitis is an acute or chronic inflammatory process of the bone and its related structures secondary to an infection with pyogenic organisms. The most common pathogen isolated from patients with osteomyelitis is *Staphylococcus aureus*. Because of the variety in disease presentations and pathophysiology of osteomyelitis, it is very difficult to evaluate the disease in clinical studies. it requires prolonged antibiotic and surgical treatments, and

is associated with chronic morbidity (Shirtliff *et al.*, 2001; Patel *et al.*, 2009; Jacqueline *et al.*, 2010).

In present study, it was aimed to investigate the preventive and therapeutic effects of medical ozone in rats with acute osteomyelitis highly difficult to treat medically and surgically. This study was expected that the ozone could have potentials to prevent the infection and to speed up the healing, and also it could be a good alternative option against osteomyelitis.

MATERIALS AND METHODS

Experimental animals: This study was approved by the Ethics Committee and performed according to the guidelines on the Experimental Unit of Animals of Mustafa Kemal University (http://www.mku.edu.tr). For the study, 24 male Wistar rats aged approximately 5 months and weighed 300-500 g were used. The animals were placed individually in separate cages with *ad libitum* access to food and water. All the animals were randomized into 3 equal groups named the UOG, POG and TOG including right femurs. All left femurs of these groups without osteomyelitis were evaluated as control group (CG).

Microorganism injections: Following the general anesthesia with 5 mg/kg xylazine (Alfazine®, Alfasan) and 30 mg/kg ketamine (Alfamine®, Alfasan) intramuscularly, a 14-gauge needle was inserted through the lateral aspect of the right femoral condyls into the cavity after cutaneous and subcutaneous incises. Then, 0.2 ml of the solution containing *S. aureus* ($3x10^5$ cfu) modified from Patel *et al*. (2009) was injected with an insulin syringe intramedullary into right femur of each animal in three groups to form acute osteomyelitis after withdrawing the needle sequentially. *S. aureus* strain ATCC 25923 obtained from Microbiology Laboratory of the university was grown on nutrient agar and suspended in sterile PBS. The suspension was measured in Den-1 device (McFarland Densitometer, Biosan) and set to Mac-Farland 0.5. Samples were also confirmed in PCR.

Ozone-therapy: POG was taken an ozone session by rectal insufflation daily at a concentration of 40 µg/ml and a dose of 500 µg/kg for 15 days (Barber *et al.*, 1999) with an ozone generator (Ozone Helps EOG 100, Bio-Med) starting a week before microorganism injections, whereas nothing was applied to UOG, TOG was administered the same ozone therapy sessions as POG. But this application was started just after 4 days following the microorganism injection.

Radiography: At the end of medical ozone applications, all cases were killed under deep anesthesia with 10 mg/kg xylazine and 100 mg/kg ketamine intramuscularly, sacrificed and done radiographical examination of relevant right and left femurs on digital radiography. Radiographical parameters evaluated (Table 1) were modified substantially from those described by Shirtliff *et al*. (2001). Following the cutting the all femurs supracondilarly, intramedullar samples from all right femurs were taken for microbiological examinations.

Histopathology: Bone samples were initially fixed in 10% neutral buffered formalin overnight at 4ºC then thrown into the phosphate buffered saline. After that, tissues were placed in 10% formic acid solution at room temperature for one week for decalcification. Sections were processed routinely and embedded in paraffins after fixations. Bone samples were processed, sectioned at 5 µm and stained with hematoxylin-eosin (Luna, 1968). All sections were examined on a light microscope and microphotographed (Olympus Cx31, DP12).

Statistical analysis: Non-parametric analyses obtained from radiographical and histopathological findings were undertaken statistically using the SPSS software package, version 23.0 for Windows (SPSS Inc. Chicago, IL, USA). Each median was initially created for all subjects within groups. Then, "Kruskal-Wallis Test" for comparison between three groups and "Mann Whitney U-Test" between for couple groups were used, and intergroup medians (P) were obtained. The value of $P<0.05$ was statistically considered as significant.

RESULTS

Microbiological aspect: As there was not any micro-biological reproduction in conventional culture methods in first 24 hours, incubation time was extended to 48 and 72 hours, but bacterial growth was again not performed.

Radiographical findings: The most severe osteomyelitis findings were identified in UOG. In addition, it was seen that the bone shaft had grown totally and become rectangular when compared to healthy left femur. Impairments of right femurs were moderate in terms of the integrity changing in bones with the lesion and spreading of osteomyelitis when compared to left femurs. Patellar destructions were mild and other radiographical findings were determined to be severe (Fig.1). Total score for this group was found to be 95. The findings in TOG were mild to moderate without severe lesions. Patellar disintegration was close to negative and the change in bone integrity was mild when compared to left femurs. Extents of osteomyelitis and focal radiolucent areas were also mild, whereas other radiogram findings were moderate compared to UOG. The group's score was counted as 51 and calculated to be 46.3% less than UOG score. Although not encountered to severe radiographical findings in POG, the rate of disintegration of the patella was close to negative as TOG. Changes in bone integrity and extents of osteomyelitis in the group were found to be mild, whereas other findings were moderate compared to UOG. The score of this group was 45 and the reduction ratio was 52.6% compared to UOG (Tab.1). Findings in UOG compared to other two groups were statistically significant ($P<0.05$). However, TOG and POG were found insignificant when compared to each other.

Histopathological findings: Bone sections of CG showed a normal histological appearance. Neutrophil leukocyte proliferations, micro-abscess formations, necrosis, hemorrhages and slightly connective tissue growth were observed at different rates in UOG (Fig. 2a & 2b). When compared to POG and TOG; most severe symptoms,

especially severe inflammation, were observed in this group (Fig. 2c & 2d). Mild to moderate inflammation and insignificant infection findings were observed in TOG. The cell infiltration with neutrophilic leukocytes significantly was decreased (moderate), whereas being increased in connective tissue cells. On the other hand, neither hemorrhages nor necrotic bone areas were observed in TOG (Fig. 3). POG showed signs of too few inflammation (mild). In POG, it was observed that neutrophil leukocyte cell infiltration significantly was reduced, unlike connective tissue growth was increased (Fig. 4). As same as in the TOG, any hemorrhages and necrosis were not seen in POG. Histopathological findings of unused ozone group were found to be significant statistically compared to both ozone used groups ($P<0.05$). When compared the POG and TOG to each other, differences were also found to be significant statistically ($P<0.05$).

Table 1: Scoring of radiogram findings of cases in all groups

Radiogram Parameters	UOG (n=8)	TOG (n=8)	POG (n=8)
Symmetrical comparisons of femurs	15	06	03
Extent of focal radiolucent areas	21	08	13
Extent of focal opacities	17	12	10
Extent of Osteomyelitis in the bone	14	08	08
Destruction rate of bone cortex	20	13	09
Patellar destruction rates	08	04	02
TOTAL SCORES	95	51	45

Scores were evaluated as: Absent (0), mild (1), moderate (2) and severe (3).

DISCUSSION

S. aureus is definitely the most frequent pathogen responsible for osteomyelitis (Chen *et al.,* 2014) for up to 70-90% of confirmed cases (Castellazzi *et al.,* 2016). The emergence of bacterial strains resistant to antibiotics makes it imperative to address to new antibiotics and to focus the research for new molecules that are capable of adequately penetrating bones to overcome the problems (Castellazzi *et al.,* 2016). That was why this pathogen was preferred as a best choice in the study.

Osteomyelitis treatment requires a multidisciplinary approach. Although surgical debridement for 4-6 weeks and parenteral antibiotics are the main treatment options, the treatment takes a long time and possible to occur side effects. Inability to supply with blood of inflammation regions, regional tissue hypoxia and ischemia are the major causes of the failure in the treatment. Recently, polymethylmethacrylate pets releasing the antibiotic following the debridement and inhalation of hyperbaric oxygen were obtained good results, but osteomyelitis was still not completely been solved (Shirtliff *et al.,* 2001; Oguz *et al.,* 2011). So that, medical ozone was used as an alternative treatment option in this research. Moreover, Oguz *et al.* (2011) stated that medical ozone was more effective than hyperbaric oxygen and the use of the two together in the same patient created a synergistic effect in terms of adjuvant therapy.

Today, medical ozone has found a wide use of the opportunity with strong antibacterial, antiviral, antifungal, immunomodulatory, detoxification and oxygenation effects (Guven *et al.,* 2008; Shinozuka *et al.,* 2008). After application, the ozone is released and generates direct molecular level reactions in the medium. Then, it destroys bacteria indirectly by the production of free radicals via the oxidation starting the destruction of cell walls and cytoplasmic membranes of microorganisms. The permeability increases after membranes are damaged and ozone molecules can easily enter into the cells (Almaz and Sonmez, 2015). The ozone oxidizes all microorganisms and their toxins. Such features justify the current interest in its application in medicine, and have been indicated for the treatment of many different pathologies (Saini, 2011).

Ozone-therapy is used as an adjuvant therapeutic modality in pathophysiological conditions such as wound healing, ischemic and infectious disorders where severe inflammatory processes and immune activation are involved (Ozler *et al.,* 2009; Bocci *et al.,* 2011a; Tasdemir *et al.,* 2013). The ozone does not act on specific receptors; its main mechanism of action is indirect, that is, by the production of a controlled and moderate oxidative stress which induces subsequent adaptive responses, reacting rapidly with antioxidants and polyunsaturated fatty acids (Clavo *et al.,* 2015; Cakır *et al.,* 2016). Medical ozone enhances vascularization of the underlying bone and stimulates the formation of bone sequestrum and granulation tissues exposed to osteomyelitis consequently (Agrillo *et al.,* 2012). O_3 exposure is also associated with activation of transcription factor NFkB, which is important to regulate inflammatory responses and it increased levels of TGF-β that is a critical factor in tissue remodeling (Travalgi *et al.,* 2010). Steinhart *et al.* (1999) reported that the effects of O_3 in osteomyelitis are due not only to fibroblasts activation and neoangiogenesis but also to the bactericide effects against *S. aureus.*

When considered together these effects of medical ozone; it was convinced that these factors made with the synergistic effect, increased the defense system, good penetrated into inflamed tissues and reduced the infection. On the other hand, considering that the use of drugs with tons of antibacterial, antifungal, antiparasitic, antiviral, antiinflammatory and other supporting drug therapies in our country and the World; contribution of harmless and inexpensive ozone-therapy to the national economy could not be undeniable. The ozone used alone or as a complementary agent will contribute to the reduction of drug amounts and doses, and also reduce the side effects of them to a minimum.

Medical ozone was also studied in dogs (Han *et al.,* 2007), cattle (Ogata and Nagahata, 2000) and horses (Alvarenga *et al.,* 2009) rarely, and the results were generally positive. In the literature, studies in laboratory animals have grown like an avalanche especially at the beginning of the 2000s and in subsequent years. Clinical practices have also spread simultaneously with a great deal in human medicine. However, in the farm and pet animals clinical practice about ozone-therapy has not been studied enough until nowadays. So this study has demonstrated that scientific studies on which medical ozone was applied should be also increased in these animals and expanded further in veterinary practice. Furthermore, simple application methods like rectal insufflation adopted in the study, being well tolerated by patients, the absence of any side effect and low cost render the ozone-therapy appropriate, practical, safety, economical and effective if versatile and practically risk-free ozone are applied by skilled hands in clinical studies.

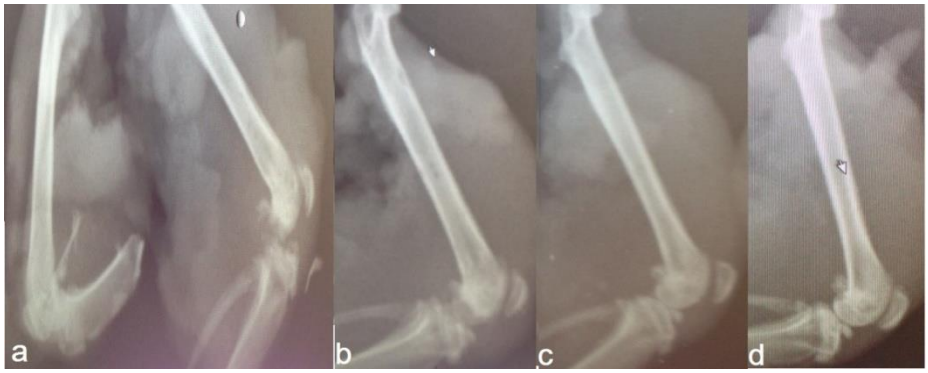

Fig. 1: A symmetrical appearance of left and right femurs of the same case (a), the bone with osteomtelitis was deformed and markedly thickened compared to the control. Radiogram examples of untreated (b), treated (c) and preventive (d) osteomyelitis groups showing osteomyelitis spread rates throughout the diaphysis. While covering all bone medulla and also available bone thickening in UOG (b), osteomyelitis lesions in other two groups remained almost limited in the distal one third of the medulla (c & d).

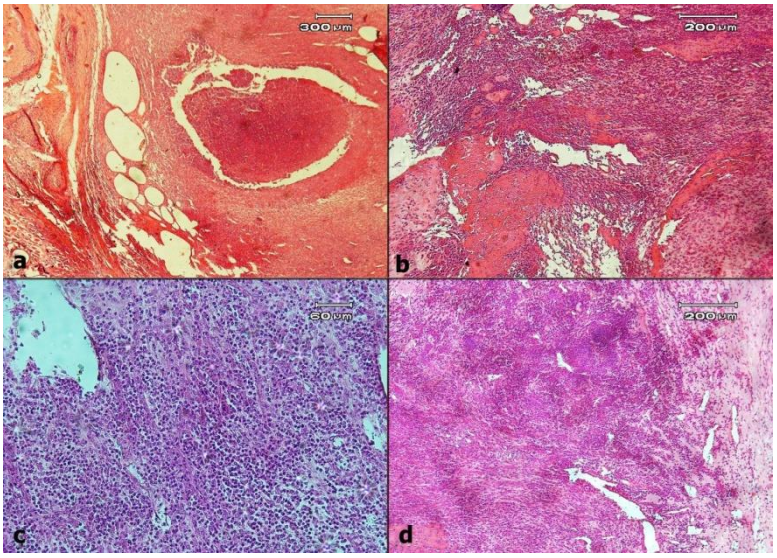

Fig. 2: a) UOG; wide hemorrhagic areas with necrosis and intramedullary abscesses, **b)** Intense neutrophil leukocyte cell infiltration with haemorrhagic areas, **c)** the close appearance of neutrophil leukocytes and **d)** Micro-abscesses with neutrophil leukocyte accumulations. H & E stain. a=x300, b & d=x200 and c=x60.

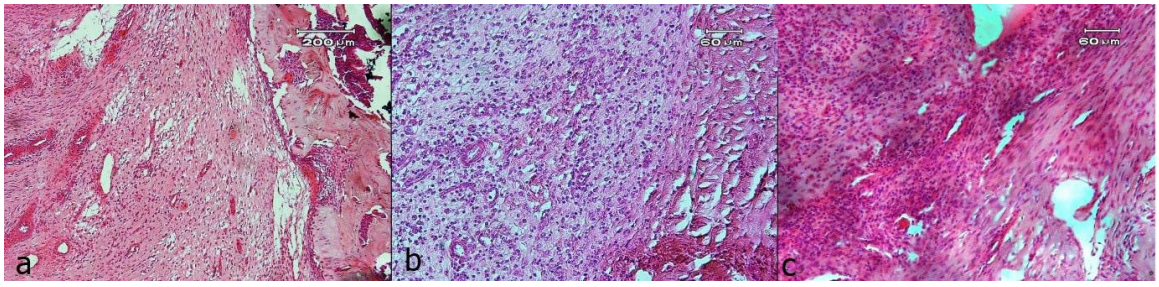

Fig. 3: a) TOG; haemorrhagia was decreased and hyperemic blood vessels were appeared, **b)** Inflammatory cells (neutrophil leukocytes) decreased and connective tissue cells increased and **c)** Connective tissue cells with a little inflammation. H & E stain. a=x200 and b & c=x60.

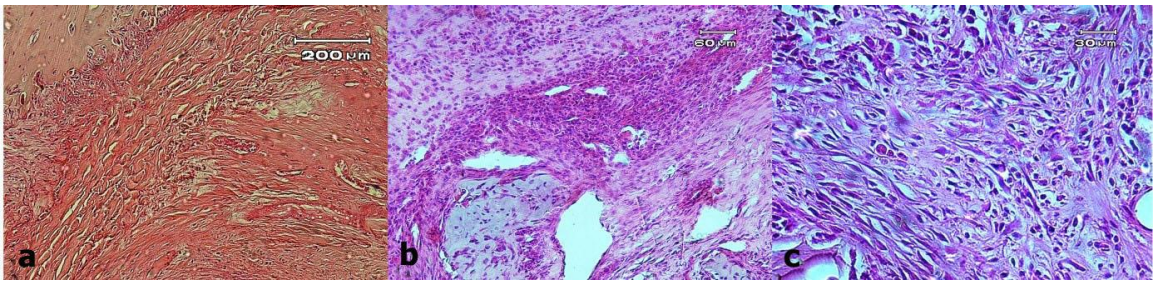

Fig. 4: a) POG; severe increase in fibroblastic growth and mature connective tissue cells (fibrocytes) appeared, **b)** Significant reduction at neutrophil leukocytes and **c)** Severe increase in fibroblastic growth and mature connective tissue cells (fibrocytes) appeared. H & E stain. a=x200, b=x60 and c=x30.

Thus, neutrophil leukocytes and other inflammatory cells that are the primary response to bacterial infections were decreased by medical ozone as reported in earlier studies (Martínez-Sánchez *et al.*, 2005; Muto *et al.*, 2008; Tasdemir *et al.*, 2013). Similar to previous studies, therapeutic and protective effects of medical ozone against infectious diseases were again proven by this study. Because the necrotic and hemorrhagic alterations were bottom out with medical ozone treatment, it was concluded that medical ozone could prevent the formation of reactive oxygen free radicals that were responsible for membrane permeability and cellular damage (Elvis and Ekta, 2011; Viebahn-Hänsler *et al.*, 2012), In short, ozone prevented the oxidative and histological damages. It has been seen that results were very remarkable. As well as the protective effect, the curative effect of it was highlight by disappearing of necrosis and even hemorrhages in all individuals.

Conclusions: It was concluded that the inflammation and the cellular damage can be prevented by medical ozone treatment at experimental osteomyelitis.

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Authors contribution: RG and MT conceived and designed the project and this study. They both executed the surgical experiment, ozone application and interpreted the radiograms. Bone samples are evaluated by SYO histopathologically. All authors critically revised the manuscript and approved the final version.

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