RESEARCH ARTICLE / *Araştırma Makalesi*

Intra-articular Ozone Treatment of Infection in a Knee Prosthesis Model

Enfekte Diz Protez Modelinde İntraartiküler Ozon Tedavisi

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Abdtract

Objective	To investigate effect of a combination of ozone and antibiotics on the treatment of an infected knee prosthesis in a rabbit model (**Sakarya Med J 2019, 9(3):528-535**).
Materials and Methods	Twenty-four New Zealand rabbits underwent implantation of a polyethylene washer and screw into the lateral condyle of the right femur and intraarticular inoculation with Staphylococcus aureus. Four groups were examined: control, antibiotic (14 days), ozone (thrice weekly for two weeks), ozone+antibiotic. After the 7th day of surgery, all animals were clinically examined and their knees were irrigated with an NaCl solution. In all groups, two animals were chosen randomly and assessed for infection. All animals were sacrificed at the end of the 3rd week. Knee samples were taken from all animals, cultured, and evaluated via the histologic-histochemical Salter scoring system.
Results	There was a significant difference between the control group and all other groups in histologic-histochemical scores, but no other group-wise differences. Although they did not reach statistical significance, the histological-histochemical scores were lowest in the ozone+antibiotic group and the results of culturing most pronounced in the antibiotic group.
Conclusion	Ozone therapy may be considered to be an alternative option for the prevention of joint degeneration cause by infection in individuals with an intraarticular implant.
Keywords	ozone; prostheses and implants; knee, infection; ant-bacterial agents

Öz

Amaç	*Tavşanda enfekte diz protezi modelinde ozon ve antibiotik kombinasyonunun etkisini araştırmak..* ***(Sakarya Tıp Dergisi 2019, 9(3):528-535)***
Gereç ve Yöntemler	*24 Yeni Zelanda tavşanın sağ femoral kondiline polietilen pul ve vida yerleştirildi ve intraartiküler Staphylococcus aureus inoküle edildi. Dört grup incelendi: kontrol, antibiotik (14 gün), ozon (iki hafta boyunca haftada üç kez), ozon+antibiotik. Cerrahi sonrası yedinci günde tüm tavşanlar klinik olarak incelendi ve dizleri NaCI solüsyonu ile irrige edildi. Her gruptan 2 hayvan rastgele seçildi ve infeksiyon belirlendi. Tüm hayvanlar 3. hafta sonunda sakrifiye edildi. Tüm hayvanlardan diz örnekleri alındı, kültür ekimi yapıldı ve histolojik ve histokimyasal olarak Salter scorlama sistemi ile değerlendirildi.*
Bulgular	*Kontrol grubu ile diğer gruplar arasında histolojik-histokimyasal skor ve mikrobiolojik olarak anlamlı fark bulundu fakat diğer gruplar arasınsa fark yoktu. İstatistiki olarak önemli olmasada histolojik-histokimyasal sonuç ozon+antibiotik grubunda en düşüktü ve kültür sonucu en iyi antibiotik grubundaydı.*
Sonuç	*Ozon tedavisi, eklem içi implant bulunan kişilerde enfeksiyonun neden olduğu eklem dejenerasyonunun önlenmesi için alternatif bir seçenek olarak düşünülebilir.*
Anahtar Kelimeler	*ozon; protez ve implant; enfeksiyon; antibakterial ajanlar*

INTRODUCTION

Prosthetic infections after arthroplasty are one of the most important factors to negatively affect prosthesis surgery. Although primary prosthetic infections are rare, these negatively affect quality of life and even threaten life in some instances. These infections are associated with high treatment costs and often necessitate repeated operations and/or the prolonged use of antibiotics.[1]

There are numerous methods for the treatment of prosthetic infections. Among these are antibiotics alone, debridement with antibiotics, single- or multiple-stage prosthesis replacement, resection arthroplasty, arthrodesis, and amputation. Factors which much be considered before deciding on a treatment plan include the timeline of the infection symptoms/signs, the patient's age and general health status, and the virulence of the infecting microorganism.[2,3] Antibiotics play an important role in the treatment of prosthesis infections. Antibiotic therapy and debridement without prosthesis replacement may be successful in the treatment of some early prosthetic infections.[4] Broad spectrum antibiotics are preferred in the early postoperative period when treating microorganisms with unknown origin and suspected antibiotic susceptibility. Antimicrobial treatment can be further tailored after identification of the infecting microorganism(s) and determination of antibiotic susceptibility and continued until clinical and laboratory findings are resolved.[5]

Ozone (O3) therapy is an adjuvant treatment used to treat infection. O3 is an unstable, non-stored gas which consists of three circular atoms.[6] When O3 interacts with a biomolecule, it oxidizes it and alters the microenvironment. Ozone has been proven to be toxic to many bacteria and reacts and oxidizes antioxidants such as polyunsaturated fatty acids, ascorbic acid and uric acid, thiol compounds containing –SH groups such as cysteine, reduced glutathione, albumin carbohydrates, enzymes, RNA, and DNA.[7]

The typical reaction between ozone and an organic biomolecule can be summarized as follows:

$$R\text{-}CH{=}CH\text{-}R' + O_3 + H_2O \rightarrow R\text{-}CH{=}O + R'\text{-}CH{=}O + H_2O_2$$

A polyunsaturated fatty acid reacts with the ozone to yields lipid oxidation products and hydrogen peroxide (H_2O_2). The double bonds among carbon atoms are affected in this reaction between ozone and fats, while the functional side chain bonds containing polyamino acid in proteins are most influenced.[8] In fact, H_2O_2 which is not an oxygen radical, mediates many of the biological and therapeutic effects of ozone as a secondary messenger.[9] Additionally, acute phase reactants cause a local increase in interleukins and cytokines.[10]

It has been reported that ozone serves as a disinfectant at high concentrations (approximately 80 to 100 μg/mL) and wound healer that increases epithelialization and granulation at lower concentrations (approximately 10 to 40 μg/mL).[11] Medical ozone, specifically, is always a mixture of pure ozone and pure oxygen at a concentration of 1 to 100 μg/mL (0.05% - 5% O_3).

Intraarticularly applied ozone is dissolved in water contained in the synovial fluid, stimulating the synthesis of matrix proteins such as collagen and glycosaminoglycan. Chondrocyte and matrix proliferation stimulated by H_2O_2 further lead to increased synthesis of articular cartilage and thus local site healing.[12]

The use of ozone in the treatment of prosthesis infection has not previously been assessed in an experimental animal model. The objective of this study was thus to investigate the effect of using a combination of ozone and antibiotics to treat an infected knee prosthesis model in rabbits.

MATERIALS and METHODS

All animal experiment protocols were approved by the Animal Research Committee at Pamukkale University Medical Faculty (12/12/2012 dated and approval number

B.30.2.PAÜ.0.20.05.07/51). Experimental, microbiological, and histopathological stages of the study were respectively conducted in microbiology, histology, and pathology laboratories of the Pamukkale University, Experimental and Clinical Research Center. Additionally, all experiments were performed in accordance with the 1995 Helsinki Declaration and the Ethics in Experimentation Animals, respectively. This study performed on February 2013- May 2013. This is a prospective experimental study.

Experimental Animal Modeling: The study was conducted using female, white New Zealand rabbits, weighing between 1800 and 2600 g (mean 2000 g). All surgical procedures were performed under general anesthesia [35 mg/kg i.m. Ketamine HCl (Pfizer) and 5 mg/kg i.m. Xylazine HCl (Bayer)]. After the induction of anesthesia, the animals' right knees were shaved and sterilized. A 2-cm incision was then made lateral to the knee joint and the joint capsule was opened. Next, 0.1 mm of bone cement was applied to the tunnel from the lateral femoral condyles that was created utilizing a drill. A sterile screw [2.7 x 14 mm (TST, Istanbul, Turkey)] and a sterile polyethylene washer (thickness of 1.5 mm and diameter of 6 mm with a 3 mm hole in the middle) was implanted in the lateral femoral condyle (Figure 1a, 1b). The joint capsule was the sutured closed with 4/0 monofilament polydioxanone absorbable sutures and the skin with 3/0 synthetic non-absorbable sutures (Figure 1c).

Infected Prosthesis Modelling: S. Aureus (Staphylococcus Aureus) ATCC (American Type Culture Collection) 25923 strain was used to induce infections (Figure 1d). The virulence of this S Aureus strain was tested in a preliminary study (data not shown). A culture antibiogram susceptibility test revealed susceptibility of the strain to cefazolin sodium. After a 24-hour incubation of the strain in 5% sheep blood agar, a bacterial suspension of 2×105 cfu/mL was prepared following dilution with 0.9% physiological saline solution using the grown colonies. The tubes containing the microorganism was kept at +4°C in the freezer to prevent bacterial proliferation and retain the organism number prior to inoculation. The prepared suspensions were injected into the right knee joint.

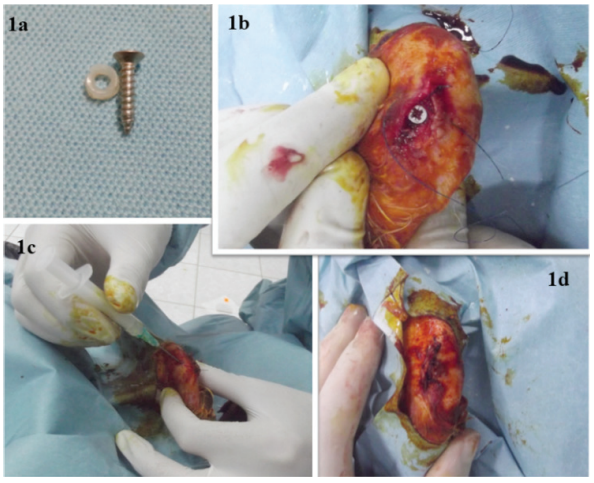

Figure 1: A sterile screw and a sterile polyethylene were implanted into the lateral femoral condyle (a, b) and the joint capsule was sutured (c). Staphylococcus Aureus was used to induce infection (d).

Treatment and Follow-up: Daily dressing changes were carried out in all rabbits. No treatment except daily wound site cleaning, was used during the first seven days of the study. To clinically confirm the development of septic arthritis, culture specimens were collected from the injection site via aspiration of 1 cc of physiological saline to the right knee joint under anesthesia and sterile conditions. The collected samples were cultivated in 5% sheep blood agar and allowed to incubate at 35oC. Following incubation, isolated bacteria were confirmed to be S. aureus.

Twenty-four rabbits were divided into four groups. Each group contains 6 rabbits: First group received no therapy (Tx-0), second group received antibiotics alone (Tx-Ab), third group received ozone alone (Tx- O_3), and fourth group received antibiotics plus ozone (Tx-Ab+O_3).

Antibiotics were administered via an intramuscular injection of a single dose of 250 mg of cefazolin for 2 weeks.

The ozone group was administered 1 cc of 10 to 20 µg/mL intraarticular ozone every other day during 2 weeks. An ozone generator (PZ-85, Cold Plasma Ozone Generator 110V-50 Hz, 110 V) was used to administer ozone treatment (Figure 2a).

Histology and Microbiology

Culture samples were obtained from the rabbits' knees under sterile conditions via injection and aspiration using 1 cc physiological saline at the end of the third week. The collected culture samples were cultivated with 5% sheep blood agar and kept to incubate at 35oC. Following incubation, the isolated bacteria were confirmed to be S. aureus.

After anesthesia was applied and samples were collected for culture, the rabbits were sacrificed via intracardiac administration of 5 mL 7.5% potassium chloride. Following dissection of the right knees of the samples, the distal femur was cut with a microsaw (CONMED, USA) in the supracondylar region (Figure 2b, 2c).

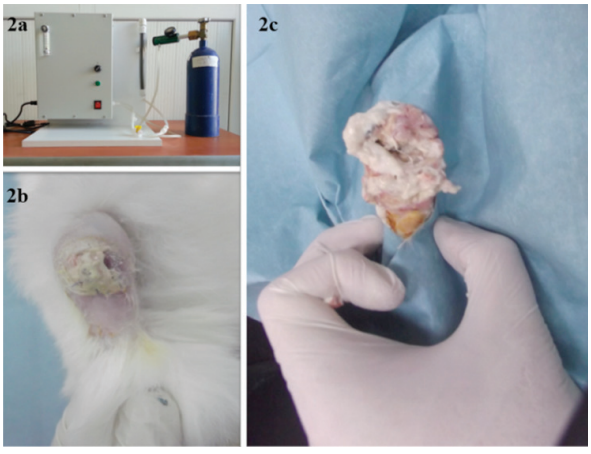

Figure 2: Cold Plasma Ozone Generator (a). Views of the infected knee before and after arthrotomy (b, c).

The medial condyle was separated with a preserved cartilage surface and kept in 10% formalin for histopathologic examination. The tissue samples were further subjected to decalcification in 10% nitric oxide for 28 hours. The samples were then washed with water for 30 minutes. Tissue monitoring was performed using a closed system full automated tissue monitoring device (Leica ASP 300). Sections with a 5-µm thickness were made via a microtome (Leica, USA) from paraffin-embedded tissue blocks. These sections were then stained with hematoxylin & eosin (H&E) and examined under a microscope (Olympus, USA). A modified scoring system developed by Salter et al. was used to assess histological-histochemical changes in the articular infection (Table 1).[13]

Table 1: Salter scoring system to assess cartilage changes after articular infection

Histopathologic findings	1 point	2 points	3 points	4 points
Loss of cartilage cells	Normal	< %10	%10-%25	> %25
Erosion and matrix loss	Normal	< %10	%10-%25	> 25
Aggregation of chondrocytes	Normal	< %10	%10-%25	> 25
Occurrence of adherences or pannus	No adherence or pannus	Only on the cartilaginous sides	Covering less than 50% of the surface	Covering more than 50% of the surface

Statistical Analyses: Microbiological results were evaluated with a X2 (Chi-square) test. Histological and microbiological results are expressed as numbers and percentiles. Histopathologic values were compared using the Kruskal Wallis H test (or Dunn test for multiple comparisons). Histopathologic values are given as interquartile ranges (IQRs) with median values. P-values <0.05 were considered to be statistically significant. Statistical analyses were performed using IBM SPSS Statistics software (Version 23.0. Armonk, NY: IBM Corp.).

RESULTS

Increased temperature and swelling at the infection site and susceptibility to articular motion started within the first 48 h (hours) after bacterial inoculation. These findings became even more evident by 72 h. Materials obtained after the first injection and aspiration had a purulent quality. Spontaneously drained fistulae were observed in the knees

of four rabbits in the control group and two rabbits in the ozone group.

Four different histological parameters were assessed, including loss of cartilage cells, erosion and matrix loss, aggregation of chondrocytes, and the occurrence of adherences or pannus. Histological chemical and microbiological outcomes were further recorded for each subject (Table 2). Infection, as was microbiologically indicated, continued in the cultures collected at the end of the third week in untreated group. Cultures were positive in 2 rabbits at the ozone therapy group and 1 rabbit at the ozone and antibiotic therapy group. No cultere positiveness was observed at the antibiotic group (Table 2).

Table 2: Histopathological and microbiological findings, this table shows effectiveness of ozone therapy

		Loss of cartilage cells (point)	Erosion and matrix loss (point)	Aggregation of chondrocytes (point)	Occurrence of adherences or pannus (point)	Microbiology
Group 1: Tx-0	1	2	2	3	2	+
	2	3	3	2	3	+
	3	4	4	4	4	+
	4	3	2	2	2	+
	5	4	3	3	4	+
	6	2	2	3	3	+
Group 2: TxAb	7	1	1	1	1	-
	8	2	1	2	2	-
	9	1	1	1	1	-
	10	1	2	2	2	-
	11	2	2	2	1	-
	12	1	1	1	2	
Group 3: Tx- O3 Group	13	2	2	3	2	+
	14	3	4	3	3	+
	15	2	1	2	2	-
	16	1	2	2	1	-
	17	1	1	1	1	-
	18	1	1	1	1	-
4: Tx-Ab+O3	19	1	1	1	1	-
	20	1	1	1	1	-
	21	1	1	1	1	-
	22	1	1	1	2	-
	23	2	3	3	2	+
	24	1	1	1	1	-

Histological and microbiological outcomes were statistically evaluated across groups. These were significantly more common in the Tx-0 group than in the other groups ($p<0.05$), and no statistically significant differences were found between the other groups ($p>0.05$) (Table 3). Regardless of treatment method, the responses of the subjects to any treatment were superior to those in the Tx-0 group.

Table 3: Group-wise comparison of histological and microbiological results, this table shows that ozone therapy prevent chondrocytes erosion

		Tx-0	Tx-Ab	Tx- O3	Tx-Ab+O3	p
Loss of cartilage cells		4 [2-6]	3 [2-4]	1 [1-2]	1.5 [1-2]	**0.007** *
Erosion and matrix loss		4 [2-6]	2.5 [2-3]	1 [1-2]	1.5 [1-2]	**0.033** *
Aggregation of chondrocytes		4 [2-6]	3 [2-3]	1.5 [1-2]	2 [1-3]	**0.024** *
Occurrence of adherences or pannus		4 [2-6]	3 [2-4]	1.5 [1-2]	1.5 [1-2]	**0.016** *
Microbiology	Negative	0 (0)	6 (100)	4 (66.7)	5 (83.3)	**0,002** **
	Pozitive	6 (100)	0 (0)	2 (33.3)	1 (16.7)	

Data were shown as median [IQR] or n (%).
*: According to Dunn's Multiple Comparison test results, Tx-0 group was found to be statically different than all other groups ($p<0.05$), whereas there was no significant difference among the treament group ($p>0.05$).
**: According to Chi-square binary comparison test with Bonferroni correction Tx-0 group was found to be statistically significantly different than all other groups ($p <0.05$), and there was no statistically significant difference among the treatment groups ($p> 0.05$).

DISCUSSION

While debridement is often performed, the first line therapy for treatment of early prosthetic infections is often antibiotic therapy alone.[14] In the experimental model employed here, we compared results from different medical treatments without any surgical displacement. From this, we assessed the effects of ozone therapy for the treatment of infection following intraarticular implantation were studied. To evaluate changes to cartilage after intraarticular infections, we followed the classification paradigm designed by Salter et al.[13] In the present study, joint infection was directly induced following the implantation of a false intraarticular-like prosthesis and consequential changes to the surrounding cartilage were observed.

Intraarticular infections are known to cause severe, permanent damage to the infected joint.[15] In the experimen-

tal model employed here, we observed advanced histopathological degenerative changes to the joint cartilage of untreated animals ($p<0.05$). Additionally, there were no significant histopathological differences between the treatment groups (Tx-Ab, Tx- O_3, and Tx-Ab+O_3) ($p>0.05$).

Destruction occurs to the joint cartilage after an intraarticular Staphylococci infection.[15] Because S. aureus is the most common causal agent of infections developing after knee prosthesis surgery, we used a strain of S. aureus in the intraarticular infection model employed here.[15] While infection was detected in the Tx-0 group, infection was comparatively minimal in all of the treated groups assessed here, regardless of treatment method (Tx-Ab, Tx- O_3, and Tx-Ab+O_3) ($p<0.005$).

Recent studies have shown that ozone therapy efficiently combats many microorganisms including S. aureus, Streptococci spp, Escherichia coli, Enterococcus faecalis, and P. aeruginosa. Studies also have shown that ozone therapy can disinfect against S. aureus and MRSA strains in vitro.[16] In vivo studies have further suggested that ozone therapy is safe and exhibits antibacterial effects in the treatment of peritonitis. Notably, ozone can also sterilize both gram positive bacteria and gram-negative bacteria.[17] Furthermore, ozone offers other advantages such as improved wound healing, enhanced immune function, and serves as an environmentally-friendly and high efficacy treatment modality.[6]

Greatly enhancing its clinical accessibility, ozone gas is now available in an oxygen solution.[18] In the last decades, many orthopedic centers in Europe have begun to treat knee osteoarthritis (OA) patients with intra-articular or peri-articular ozone insufflation as there is evidence supporting the role of ozone injections in the management of knee OA symptoms.[19] Treatment with an oxygen-ozone solution can improve tissue oxygenation and inhibit inflammatory mediators via down-regulation of the pro-inflammatory cytokines TNFα and TNFR2. Ozone therapy can also induce moderate-intensity oxidative stress and inhibit local inflammatory responses.[18] Bocci at al. and Musumeci at al. further demonstrated that the efficacy of ozone therapy for OA may be due to its stimulation of cartilage synthesis.[12] Specifically, Intraarticular injection of O3 has been suggested for the treatment of osteoarthritis and has yielded positive results. Mishra at al. performed a cross-over study with two therapeutic methods in 46 patients with knee OA where participants were treated with a single injection of methyl-prednisolone or three, monthly ozone injections. After 6 months, the researchers observed an 80% response rate in the ozone group versus a 60% rate in the steroid group.[20] Similarly, in the present study, we note positive histopathological and micropathological changes with ozone therapy after induced infection of an intraarticular implant ($p<0.05$).

While it offers some significant and intriguing results, the present study has several limitations which warrant discussion. First, although our results may suggest that antibiotics and ozone therapy were equally effective, as there were no significant difference between the groups, and that there was no advantage of the combined use of antibiotics with ozone therapy as might have been expected, we were unable to directly measure this due to the present study's limited sample size. Furthermore, while we might assume that the intraarticular implant model used in the present study is sufficient to model human knee prostheses, there is no clear prior evidence of this. Although we observed histopathological and microbiological effects here, it is not possible to determine that our methods were sufficient to recapitulate human surgical or post-operative healing conditions. We further contend that a greater sample size might have improved our ability to detect more features of

the model which might have lent it greater validity.

The present study indicates that ozone therapy may act similarly to antibiotic therapy in the treatment of an intraarticular prosthesis infection. Although antibiotic therapy is the gold standard for prosthesis cases complicated by infection, the use of combined treatments may be beneficial. Further scientific studies are needed to determine the most effective and appropriate dose of ozone therapy alone and/or in combination with antibiotics for this and other infection applications. Furthermore, ozone therapy may serve as an alternative option for the prevention of joint degeneration after intraarticular implantation.

Limitations

This study involved small number of subject.

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