

Original

Effectiveness of Ozone Therapy on Tendon Healing: An Experimental Study in Generated Achilles Tendon Injury Model in Rats

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Abstract: The aim of this experimental study was to investigate the effects of ozone therapy on tendon healing in rats. A total of 60 adult male, 1-year old Wistar albino rats weighing 450-500 g were randomly assigned to the ozone group (Group 1, n:30) or the control group (Group 2, n:30). In both groups, the right and left Achilles tendons were cut transversally and then sutured. Group 1 underwent ozone treatment rectally (40 µg/mL) four times a week, while Group 2 received only nutrition and routine care. Ten rats from each group were sacrificed and evaluated in respect of histopathological and biomechanical properties at the end of the 2nd, 4th and 6th weeks. There were statistically significant differences between the groups in respect of fibroblastic proliferation ($p=0.042$) and inflammation ($p=0.001$) in the 2nd week. Fibroblastic proliferation was higher in the ozone group, and inflammation in the control group was found to be higher. Remodeling and fibroblastic proliferation were significantly increased in the ozone group in the 4th week ($p=0.007$ and $p=0.003$, respectively). In the 6th week, high remodeling and high fibroblast proliferation were observed in the ozone group compared with the control group ($p=0.020$, $p=0.004$, respectively). The biomechanical results revealed that the ozone group had significantly higher breaking load and breaking tensile stress values than the control group in the 6th week ($p=0.007$, $p=0.003$, respectively). The histopathological and biomechanical findings indicated that the ozone therapy had beneficial effects on Achilles tendon rupture healing.

Key words: Achilles tendon, Healing, Ozone, Rats

Introduction

Soft tissue trauma is commonly encountered in clinical practice. Soft tissue traumas are generally defined as injuries or disorders of the musculoskeletal system (bursae, muscle, ligament, tendon) other than bone. These injuries are often seen as a group in all of the sports injuries. Soft tissue injuries can result in swelling, limitation of movement and pain^{1,2)}.

Tendon disorders are common and lead to significant disability and pain. The Achilles tendon is one of the strong and thick tendons in the body. Being subject to more stress it is most frequently injured and torn tendon³⁾. Therapy options include surgical and non-surgical alternatives aiming early recovery and return to daily life. The optimal treatment for Achilles tendon ruptures and chronic Achilles tendinopathies remains clinically challenging, although several treatment approaches are used, such as splints, braces, cold packs, compression, elevation, non-steroidal anti-inflammatory drugs, analgesic drugs, electrotherapy modalities, local injection therapies, antioxidants and rehabilitation protocols⁴⁾. However, these treatment approaches are not always successful in the treatment of tendon injury and this has led to the development of new treatment modalities⁵⁾. In recent years, the application of regenerative therapies (platelet-rich plasma, prolotherapy, stem cell therapy, ozone

therapy etc.) and treatment has become widespread in the treatment of soft tissue trauma including muscle and tendon injuries^{6,7)}.

The ozone molecule is formed of three oxygen atoms. In ozone therapy, an oxygen/ozone (O_2/O_3) mixture with defined ratio is given into periarticular tissue, body cavities or the circulatory system. Ozone activates and decreases antioxidant levels in plasma. Hydrogen peroxide forms and it is the driving molecule for the biological and therapeutic effects. Antioxidant levels decrease and hydrogen peroxide stimulates a shock like effect on the tissues. This effect results in the stimulation of a variety of defense systems, including primarily antioxidant enzyme expression, leading to increased resistance to the oxidative processes⁸⁾. The first effect of hydrogen peroxide is on the hemoglobin-oxygen dissociation curve which is shifted to the right. With the increase in 2,3-diphosphoglycerate levels in the erythrocytes, oxygen is released to the tissues much easily. This biochemical mechanism explains the increased tissue partial oxygen pressure during the ozone therapy⁹⁾. Thus, it can be considered that ozone could provide new perspectives on the treatment of tendon disorders. The aim of this experimental study was to demonstrate the effectiveness of ozone therapy on a rat model of Achilles tendon injury.

Materials and Methods

A total of 60 adult male, 1-year old Wistar albino rats, each weighing 450-500 g were used in this experimental study. The rats were divid-

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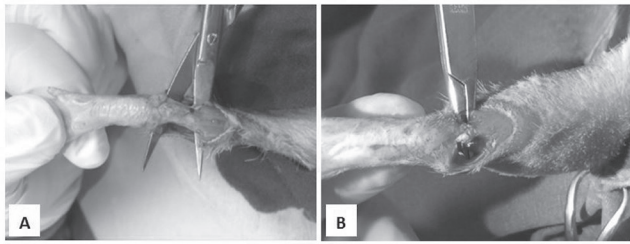


Figure 1. (A) Exploration of the Achilles tendon. (B) Tendon repair following injury.

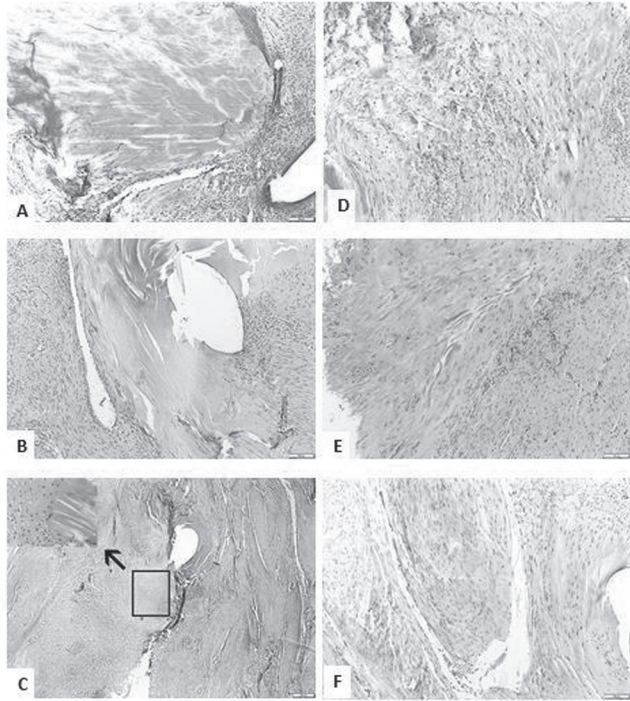


Figure 2. Histopathological evaluation of control group at week 2 (A), at week 4 (B), at week 6 (C) and ozone group at week 2 (D), at week 4 (E), and at week 6 (F). (Hematoxyline and Eosin, scale bar = 100 μ m).

ed into two groups of 30 rats in each using the 'simple random sampling' method: Group 1 (ozone group) and Group 2 (control group). Initially, an experimental tendon injury was performed in each group, with all rats in both groups receiving the same surgical hemi-transection injury to both Achilles tendons, followed by repair (Fig. 1). The surgical procedures were applied under general anesthesia with an intraperitoneal injection of a mixture of 50 mg/kg ketamine and 10 mg/kg xylazine. The ozone procedure was performed in Group 1, and Group 2 served as control subjects. At the end of the 2nd, 4th and 6th weeks, samples were taken from 10 rats randomly in each group, and results were compared at the different time points.

The study protocol was approved by the Ethics Committee for Animal Experiments of Mustafa Kemal University Medical School (decision no: 2015/10-3, dated 30.12.2015). The animals were supplied by Mustafa Kemal University, Veterinary Faculty - Department of Experimental Animals, and were kept in the same laboratory conditions during the study. The rats were fed with commercial diet and tap water.

Table 1. Biomechanical testing of the groups

	Ozone group	Control group	p value
2nd week			
Basal load (kN)	12.10	8.90	0.226
Load at break (kN)	10.60	10.40	0.940
Basal tensile stress (MPa)	12.80	8.20	0.082
Tensile stress at break (MPa)	9.80	11.20	0.597
4th week			
Basal load (kN)	12.90	8.10	0.070
Load at break (kN)	12.20	8.80	0.199
Basal tensile stress (MPa)	11.90	9.10	0.290
Tensile stress at break (MPa)	12.20	8.80	0.199
6th week			
Basal load (kN)	8.90	12.10	0.226
Load at break (kN)	14.10	6.90	0.007
Basal tensile stress (MPa)	9.50	11.50	0.450
Tensile stress at break (MPa)	14.40	6.60	0.003

Mann-Whitney U test was used.

Bold values show statistically significant P-values ($p < 0.05$).

Ozone Application

The ozone was administered by rectal insufflation four times per week. At each session, the administered dose of ozone was a concentration of 40 μ g/ml. Ozone (O₃) was generated by an ozone generator (Bizonix GmbH, Munich, Germany), allowing control of the gas flow rate and ozone concentration in real time through a built-in UV spectrometer. The ozone flow rate was kept constant at 3 l/min representing a concentration of 60 mg/ml and gas mixture of 97% oxygen + 3% O₃.

Sampling

After anesthesia, rats from the ozone therapy group and the control group were sacrificed and tissue was taken from each group of 10 rats at the end of the 2nd, 4th and 6th weeks. Tissue specimens were excised and placed in 10% buffered formaldehyde for histopathological analysis. The right leg of each rat in both groups was evaluated histopathologically, and each left leg was evaluated biomechanically.

Histopathologic Evaluation

At the end of weeks 2, 4 and 6, a total of 10 rats from each group were sacrificed under anesthesia. Thus, after 2, 4 and 6 weeks of ozone administration following injury, the tissues were evaluated and scored by an expert pathologist. Tissues were fixed in 10% buffered formaldehyde. All of the tissues were sampled. After routine alcohol, xylene and paraffin tissue processing, tissues were embedded in paraffin blocks. Five-micron thickness slides were prepared and stained with hematoxylin and eosin. Evaluation was performed under the light microscope (Olympus BX50-F4, Tokyo, Japan) with the scoring system below: Histopathological scoring¹⁰⁾:

- Remodeling: 0=absent, 1=partial, 2=complete but immature, 3=complete and mature
- Fibroblastic Proliferation: 0=absent, 1=partial, 2=complete but immature, 3=complete and mature
- Collagen deposition: 0=absent, 1=partial, 2=complete but immature, 3=complete and mature
- Inflammation: 0=none; 1=scant; 2=moderate; 3=abundant

Biomechanical Testing

All Wistar rats were sacrificed at the end of 2, 4, or 6 weeks of tendon injury with an overdose injection of ketamine/xylazine combina-

Table 2. Histological analysis of the groups

	Ozone group (n=30)			Control group (n=30)		
	2 nd week (n=10)	4 th week (n=10)	6 th week (n=10)	2 nd week (n=10)	4 th week (n=10)	6 th week (n=10)
Remodeling						
0	8	3	4	10	9	9
1	2	6	4	-	1	1
2	-	1	2	-	0	-
3	-	-	-	-	-	-
Fibroblastic proliferation						
0	1	-	-	6	5	3
1	7	2	2	3	3	5
2	2	5	4	1	2	2
3	-	3	4	-	-	-
Collagen deposition						
0	-	-	-	-	-	1
1	-	-	2	1	5	3
2	2	5	4	4	2	3
3	8	5	4	5	3	3
Inflammation						
0	-	-	-	-	-	-
1	-	-	1	6	3	-
2	5	9	6	4	4	6
3	5	1	3	-	3	4

Chi-square test was used.

Remodeling: 0=absent, 1=partially, 2=complete, but immature, 3=complete and mature Fibroblastic

Proliferation: 0=absent, 1=partially, 2=complete, but immature, 3=complete and mature

Collagen deposition: 0=absent, 1=partially, 2=complete, but immature, 3=complete and mature

Inflammation: 0=none; 1=scant; 2=moderate; 3=abundant

Table 3. Comparison of the histological findings of the groups

Ozone versus Control	
Remodeling	
2 nd week	p = 0.146
4 th week	p = 0.007
6 th week	p = 0.020
Fibroblastic proliferation	
2 nd week	p = 0.042
4 th week	p = 0.003
6 th week	p = 0.004
Collagen deposition	
2 nd week	p = 0.148
4 th week	p = 0.070
6 th week	p = 0.381
Inflammation	
2 nd week	p = 0.001
4 th week	p = 0.754
6 th week	p = 0.483

Chi-square test was used.

Bold values show statistically significant P-values (p<0.05).

tion. Mechanical testing was applied using a materials testing Instron universal machine (Instron LX 600 model). The tendon tissues were fixed between two metal clamps and tension was applied at a constant speed of 1 mm/s until failure. The device on which the biomechanical evaluations were made consisted of two sections. The attachment point of the Achilles tendon to the calcaneus tubercle of the rat was placed on the lower section of the device and the proximal section of the Achilles tendon was placed on the upper section with the aid of a holding apparatus. The initial load value on the tendon attached to the machine and the tensile stress value were measured and recorded by the machine. By stretching the tendon, the load and tensile stress were increased. The

load and tensile stress values causing breakage of the tendon were recorded separately for each tendon.

Statistically analysis

Statistical analysis was performed using the IBM-SPSS version 23.0 software (IBM Corp., Armonk, NY, USA). Data were presented as count (percentage) or mean $\pm$ standard deviation. Normality was tested by using Kolmogorov-Smirnov test. The Mann Whitney U-test was used for the analysis of data. A *p* value of <0.05 was considered statistically significant.

Results

In the histopathological evaluation of the Achilles tendons of both groups, there were statistically significant differences between the groups in respect of fibroblastic proliferation (p=0.042) and inflammation (p=0.001) at the end of the 2nd week. The fibroblastic proliferation was higher in the ozone group, and inflammation was higher in the control group (Figs. 2A and D). Remodeling and fibroblastic proliferation were significantly increased in the ozone group in the 4th week (p = 0.007 and p=0.003, respectively). No statistically significant difference was determined between the groups in respect of inflammation and collagen deposition at 4 weeks, although the collagen deposition was higher in the ozone group (Figs. 2B and E). At 6 weeks, high remodeling and high fibroblast proliferation were observed in the ozone group compared with the control group (p=0.020, p=0.004, respectively; Figs. 2C and F). The biomechanical results revealed that the ozone group had significantly higher scores than the control group at 6 weeks in respect of both the breaking load and breaking tensile stress values (p = 0.007, p = 0.003, respectively; Table 1). The comparison of the histopathological findings of the groups is given in Table 2 and Table 3.

Discussion

Ozone therapy, is being used for the treatment of a wide range of diseases and seems to be promising in the tissue ischemia. The underlying mechanism is thought to be via formation of a mild, transient, and controlled oxidative stress. This oxidative medium facilitates the antioxidant system and modifies the immune system response¹⁰. Ozone positively influences cell energy, oxygen metabolism, antioxidant defense system, microcirculation in tissues. According to these mechanisms of action, it was hypothesized that ozone therapy could be useful in tendon repair. The healing effects of ozone have not been previously demonstrated in tendons or ligaments of animals. To the best of our knowledge, this study is the first report on the effects of rectal ozone on the biomechanical properties of Achilles tendon healing in rats. Therefore, the findings may have clinical implications for the application of ozone in the treatment of tendon injuries.

The results of this study, which considered the effects of ozone treatment after Achilles tendon rupture repair, demonstrated that ozone was effective in histopathological healing at week 2, and this effect was shown to continue at week 6. Moreover, the biomechanical effect (the load and tensile stress) on the injured Achilles tendons improved significantly with ozone treatment at 6 weeks post-injury when compared with the control group.

Type I collagen is the major component of normal tendon, greatly contributing to the strength of the tissue. Makita *Y et al*¹¹ investigated the effects of ozone on collagen type-I and inflammatory cytokine production in human gingival fibroblasts (HGFs). They reported that ozone significantly enhanced collagen type-I production by HGFs within 24 hour, and inhibited the pro-inflammatory cytokines including interleukin-6 and interleukin-8. Following the ozone administration, it causes a local reaction or inflammation. The fibroblasts which are attracted to the irritated area then begin to lay down the collagen which will form new ligament or tendon tissue. Because the inflammation produced is very important for the healing process. In our study, no statistically significant difference was determined between the groups in respect of inflammation and collagen deposition at 4 weeks, although the collagen deposition was higher in the ozone group.

There is a limited number of clinical studies in literature about ozone effectiveness in the management of musculoskeletal diseases¹². The common view in these studies is that the activation of the regenerative process is shown using local ozone therapy¹³. The changes in cytogram of wounds throughout the ozone therapy is as follows: Within 5-7 days neutrophils are reduced in number, and mononuclear cells (lymphocytes and macrophages) appear. Both mononuclear cells and neutrophils show signs of complete phagocytosis. In the second week, the inflammatory cells (lymphocytes and monocytes) also start to vanish, and remaining macrophages and emerging fibroblasts are the signs for the tissue repair. Moreover, ozone inhibits cyclooxygenase and stimulates histamine and monoamine oxidase, which reduces vasodilation and muscle hypertonicity, and results in an overall analgesic and anti-inflammatory effect^{13,14}. The mechanism of medical ozone treatment includes an increase in glycolysis, lipolysis and reduction in platelets. It also increases the blood concentration of 2,3-bisphosphoglycerate. The overall effects are eutrophic, anti-inflammatory and analgesic as well as greater flow of blood and oxygen to the tissues¹⁴. In accordance with these data, the results of the current study support that ozone treatment can be a promising option for improvement of healing after Achilles tendon rupture.

There are various current studies related to the acceleration of tendon healing and obtaining a strong tendon structure. Some of the meth-

ods used are bioactive grafts, gene therapy, and PRP¹⁵. However, erythropoietin, stem cell and bFGF administration and high-voltage pulsed current applications have been investigated and have not shown any benefit on tendon healing¹⁶.

In the current study, the focus was particularly on the injury of the tendon tissue. Philippou *A et al* reported that the local production of IGF-1 is particularly important in regeneration, hypertrophy, proliferation, and differentiation of skeletal muscles¹⁷. In another study by *Duman et al*, the effects of rectal ozone therapy on femur fracture in rats were evaluated¹⁸. In that study, biomechanical and histological evaluations showed that the healing in a rat bone fracture model was significantly increased by rectal insufflation of ozone in all experimental groups compared with the control group.

The main limitations of the present study were the experimental design, lack of a sham group and possible impacts of metabolic, environmental and technical factors which may have interfered with the outcome parameters.

In conclusion, in the light of the findings, the biomechanical and histological recovery of the repair of Achilles tendon rupture in a rat model suggests that the use of ozone therapy may be a good therapeutic adjuvant treatment in tendon healing.

Conflict of Interest

The authors have declared that no COI exists.

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