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Research Article

Impacts of Ozone Treatment and Its Relationship with IGF-1 Levels After Injury of Soft Tissue: An Experimental Study in Rats Model

Sefer ÜSTEBAY [1] Ömür ÖZTÜRK [2] Ali BİLGE [3]
Döndü Ülker ÜSTEBAY [1] Aysu Hayriye TEZCAN [2]

[1] Kafkas University, Faculty of Medicine, Department of Pediatrics, TR-36300 Kars - TURKEY
[2] Kafkas University, Faculty of Medicine, Department of Anesthesiology and Reanimation, TR-36300 Kars - TURKEY
[3] Kafkas University, Faculty of Medicine, Department of Ortopedics and Traumatology, TR-36300 Kars - TURKEY

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Abstract

To investigate the effects of ozone treatment on soft tissue injury and to observe whether there is an alteration in serum IGF-1 levels after ozone treatment in an experimental rat model. Twenty-four adult Wistar albino 240-350 g male rats were randomly allocated into two groups. A standardized, experimental soft tissue injury was created on left hind limbs of animals. Group 1 underwent daily ozone treatment intraperitoneally (20 µg/mL), while Group 2 received only nutrition and routine care. All rats were evaluated regarding body weight and sensory and motor function on 5th and 15th days after experimental trauma. Blood samples were drawn from intracardiac in group 1 and group 2 serum levels of IGF-1 level were measured at the day of 15th. During follow-up period after formation of soft tissue injury, three rats in Group 2 had cutaneous infection that responded well to topical tetracycline treatment. Two groups displayed similar results regarding sensory and motor functions on 5th and 15th days. Serum IGF-1 level in Group 1 was significantly higher than that of Group 1 (P=0.03). Serum IGF-1 level was correlated with motor function on day 15 in Group 1 (P=0.04) and with motor function on day 5 in Group 2 (P=0.011). Ozone treatment may have favorable impacts on healing and regeneration process in connective and muscle tissues and these beneficial effects may be mediated by IGF-1. Further research is warranted to elucidate the role of IGF-1 in repair process and to provide additional new insights to the treatment strategies.

Keywords: *Ozone, Wound healing, Injury, Muscle, Rat, IGF-1*

Yumuşak Doku Yaralanması Sonrası Ozon Tedavisinin Etkileri ve IGF-1 Seviyesiyle Olan İlişkisi: Rat Modelinde Deneysel Bir Çalışma

Özet

Ozon tedavisinin yumuşak doku hasarı üzerindeki etkilerini araştırmak ve deneysel bir rat modelinde ozon tedavisinden sonra serum IGF-1 düzeylerinde bir değişikliğin olup olmadığını gözlemlemek amaçlandı. Yirmidört yetişkin Wistar albino 240-350 g ağırlığında erkek rat randomize olarak iki gruba ayrıldı. Her iki gruptaki ratların sol arka ekstremitelerine standartlaştırılmış deneysel yumuşak doku yaralanması oluşturuldu. Grup 1'e intraperitoneal olarak (20 µg/mL) günlük ozon tedavisi uygulandı. Grup 2'ye ise sadece beslenme ve rutin bakım yapıldı. Deneysel travmanın 5 ve 15. günlerinde tüm ratlar vücut ağırlığı, duyusal ve motor fonksiyon açısından değerlendirildi. Kan örnekleri Grup 1 ve Grup 2'de intrakardiak alındı ve serum IGF-1 seviyeleri 15. günde ölçüldü. Yumuşak doku hasarının oluşumundan sonra takip süresi boyunca Grup 2'deki üç rat topikal tetrasiklin tedavisine olumlu cevap veren kutanöz enfeksiyona sahipti. Her iki grupta, 5. ve 15. günlerde duyusal ve motor fonksiyonlarla ilgili benzer sonuçlar görüldü. Grup I'deki serum IGF-1 düzeyi grup 2'e göre anlamlı derecede yüksekti (P=0.03). Serum IGF-1 düzeyi, Grup 1'de 15. günde (P=0.04) motor fonksiyonu ile Grup 2'de 5. gündeki (P=0.011) motor fonksiyonlarıyla korele edildi. Ozon tedavisinin bağ dokusu ve kas dokusunda iyileşme ve rejenerasyon süreci üzerinde olumlu etkileri olabilir ve bu faydalı etkilere IGF-1 aracılı olabilir. IGF-1'in onarım sürecindeki rolünü aydınlatmak ve tedavi stratejilerine ek yeni anlayışlar sağlamak için daha fazla araştırma yapılması gereklidir.

Anahtar sözcükler: *Ozon, Yara iyileşmesi, Yaralanma, Kas, Rat, IGF-1*

İletişim (Correspondence)
+90 536 4326293
ustabay_dr@hotmail.com

INTRODUCTION

Insulin-like growth factors (IGFs) have critical roles in growth, development, cellular regulation and metabolism. IGF-1 is a 70 amino acid polypeptide hormone synthesized in the liver and released into the extracellular fluids. Although the major source of IGF-1 is the liver, synthesis has been shown to take place in other organs including kidneys, ovaries, testes, placenta, pancreas, skin and lungs. Low levels of circulating IGF-1 are seen in patients with chronic liver dysfunction, renal failure, malnutrition and other diseases. The principal role of the IGF-system is to integrate growth and metabolism [1].

In-vitro administration of IGF-1 to cultured rat skeletal muscle and myoblasts resulted in activation of cellular proliferation and differentiation [2]. These properties of IGF-1 indicate its important role in the regulation of myoblast cell proliferation and differentiation. IGF-1 also induces cell proliferation and controls cell differentiation in the initial stage of muscle regeneration. Once a sufficient amount of cells has been attained, cell differentiation is initiated [2]. It has also been shown that freezing can induce regeneration of cow skeletal muscle, and culture of those cells has demonstrated strong positivity for IGF-1 in myoblasts and myotubes. These results suggest that IGF-1 stimulates the proliferation and differentiation of myoblasts during the initial and middle periods of muscle regeneration [3].

Ozone is a powerful oxidant which gained popularity recently owing to its clinically proven therapeutic effects [4]. A high-frequency current field separates the oxygen molecule into the highly reactive monatomic oxygen that combines with another oxygen molecule and forms ozone. In the oral cavity, ozone is shown to penetrate the mucus membrane and effectively destroy bacteria. Ozone displays its multi-dimensional effects using promotion of oxygenation in addition to antibacterial, antifungal and antiviral properties. Furthermore, it elicits the immune response through production of cytokines such as interleukin-2 and interferon [5]. Ozone has been used empirically as a clinical therapeutic agent for chronic wounds, ischemic ulcers and diabetic wounds [6-8]. The beneficial effects of ozone on wound healing may be linked with decreased bacterial infection, attenuation of impaired dermal wound healing and increased oxygen tension due to ozone exposure in the wound area [6-8]. Ozone has a regulatory effect on inflammation and wound healing process, and it may display these effects using growth factors [6,9-12].

Surgical interventions are associated with injury of the cutaneous, connective and muscle tissues. Healing of these injuries may be incomplete and severe sequelae may occur eventually. In this purpose, additional surgical procedures, steroids, anti-inflammatory medications and antioxidants constitute the therapeutic foci for repair of skin and connective tissue. Beyond its effects associated with protein and glucose metabolism, recent studies focussed on the roles of IGF-1 on myogenesis, cellular differentiation, trauma and metabolic diseases [13,14]. Levels of IGF-1 may increase in response to trauma to the connective tissue including soft tissue and muscle [14]. In this experimental study, we investigated the effects of ozone treatment on the soft tissue injury and to observe whether there is an alteration of serum levels of IGF-1 in response to ozone treatment.

MATERIAL and METHODS

Study Design

The study was approved from the Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK/2015-094). Twenty-four adult Wistar albino 240-350 g male rats were maintained at the same center and housed in individual cages with free access to water and animal chow. The animals were maintained in a constant 12 h light/12 h dark cycle at constant room temperature of 21°C and humidity of 60%. After induction of anesthesia by ketamine hydrochloride (80 mg/kg), animals were placed at left lateral decubitus position. The left hind limb was shaved and sterilely prepared. An incision was made at the level of left femoral neck of every rat and cutaneous, subcutaneous and muscular layers were dissected. A standardized soft tissue injury was formed using a jeweler's microforceps for 30 sec. After suturing and closure of the wound, the rats recovered from anesthesia, and they were placed back to the cages. Animals were randomly allocated into two groups (n=12 for each).

Group 1 received daily ozone treatment intraperitoneally (20 µg/mL), while Group 2 received no additional interventions other than nutrition and routine care. All rats were evaluated in terms of body weight and sensory and motor function. At the end of 2 weeks, blood samples were drawn from intracardiac of Group 1 and Group 2 animals following anesthesia with 80 mg/kg ketamine hydrochloride. Serum levels of IGF-1 were measured. Animals were sacrificed by cervical dislocation. IGF-1 protein levels in the serum were measured using commercially available ELISA kits specific for rat IGF-1 (Boster Immunoleader, Wuhan, China), with assay sensitivity < 5 pg/mL and a range of 62.5-4000 pg/mL, in compliance with the manufacturer's instructions.

Recovery of motor and sensory function were evaluated on 5th and 15th days following experimental cutaneous and muscular injury. Analysis of motor and sensory functions were assessed via free walking pattern and foot reflex withdrawal test, respectively.

Motor Function Test

For comparison, the motor activity of the rats was assessed by the modified Tarlov system which we had used previously in the monkeys, 4 as follows: Grade 0: complete paralysis of legs; Grade 1: flicker of movement; Grade 2: good movement at all joints but without walking or weight-bearing; Grade 3: walking and weight-bearing, but not normally; Grade 4: normal [15,16].

Sensory Function Test

The sensory function was assessed by means of the foot reflex withdrawal test as described by De Koning et al.[17]. The rat was gently immobilized by hand and the sole of the foot was directed towards the examiner. An electric current was applied to the foot sole by means of two stimulation electrodes. Six current strengths ranging from 0.1 to 0.6 mA were tested. Rats with an intact innervation will instantaneously retract their paw upon skin contact with the electrodes. Rats exposed to sciatic nerve crush initially fail for this response. Failure to withdraw the foot on stimulation at 0.6 mA indicated no recovery. Rats reacting to a 0.1 mA current were considered to be completely recovered.

Outcome Parameters

This experimental study was focused on comparison of 2 groups in terms of serum IGF-1 levels on 15th day. Moreover, sensory and motor functions on 5th and 15th days were noted. Sensory function was recorded as all or none response, while motor function was graded in accordance with modified Tarlov system as described above [15,16].

Statistical Analysis

Analysis of data was made with IBM Statistical Package for Social Sciences (SPSS) software version 20.0 for Windows (SPSS Inc., Chicago, IL, USA). Comparison of 2 independent groups in terms of quantitative variables was performed by Mann-Whitney U test. Pearson Chi-Square and Fisher's Exact tests were used for evaluation of categorical variables. Correlation between variables was tested with Spearman's rho test. Quantitative variables are demonstrated as median-interquartile range (minimum-maximum). Confidence interval was 95% and differences associated with a P value less than 0.05 were considered as statistically significant.

RESULTS

An overview of measurements in 2 groups is displayed in *Table 1*. Two groups did not differ with respect to body weights. During follow-up period after formation of soft tissue injury, 3 rats in Group 2 had cutaneous infection that responded well to administration of topical tetracycline ointment (Imex®, Assoss Pharmaceuticals, Istanbul, Turkey). In Group 1, no infections were detected. Two groups displayed similar results in terms of sensory and motor functions on 5th and 15th days. *Table 2* presents a comparative overview of these aforementioned variables in 2 groups. Serum IGF-1 level in Group 1 was significantly higher than that of group 1 (P=0.03). No remarkable differences were observed between groups in terms of motor functions on day 5 (P=0.55) and day 15 (P=0.32) or

Table 1. *An average overview of serum IGF-1 levels, motor and sensory functions on days 5 and 15 in Groups 1 and 2*

Group	Motor Function		Sensory Function		Serum IGF-1 Level (pg/mg protein)
	Day 5	Day 15	Day 5	Day 15	
1	3.33±0.654	3.92±0.29	0.92±0.291	1	11.90±2.44
2	3.08±0.792	3.67±0.493	0.67±0.490	1	10.35±0.48

Table 2. *Comparison of serum IGF-1 levels and motor and sensory functions on 5th and 15th days in 2 groups*

Variable	Day	Tarlov Score	Group 1 n (%)	Group 2 n (%)	*P* Value
Motor function	5	2	1 (8.3)	3 (25)	0.55
		3	6 (50)	5 (41.7)	
		4	5 (41.7)	4 (33.3)	
	15	3	1 (8.3)	4 (33.3)	0.32
		4	11 (91.7)	8 (66.7)	
Sensory function	5	0	1 (8.3)	4 (33.3)	0.32
		1	11 (91.7)	8 (66.7)	
	15	0	0	0	-
		1	12 (100)	12 (100)	
Serum IGF-1 level (pg/mg protein)			10.10-0.88	11.65-1.70	0.003*

** Statistically significant*

Table 3. Correlation analysis between serum IGF-1 levels and sensory and motor functions				
Group	**Variable**		***P* Value**	**r Value**
1	Serum IGF-1 level	Motor function on day 15	0.04*	0.590
	Motor function on day 5	Motor function on day 15	0.004*	0.764
2	Serum IGF-1 level	Motor function on day 5	0.011*	0.700
	Motor function on day 5	Sensory function on day 5	0.002*	0.032
** Statistically significant*				

sensory functions on day 5 (P=0.32) and day 15. Correlation analysis indicated that serum IGF-1 level was correlated with motor function on day 15 in Group 1 (P=0.04) and with motor function on day 5 in Group 2 (P=0.011). Motor function on day 5 was correlated with motor function on day 15 (P=0.004) in Group 1 and with sensory function on day 5 in Group 2 (p=0.002) *(Table 3)*.

DISCUSSION

The aim of the present study was to assess the effects of ozone treatment in surgically induced soft tissue injury and to investigate whether there may be an association with IGF-1 levels. Our results demonstrated that IGF-1 levels are higher in the group receiving ozone treatment and there is a correlation between serum IGF-1 levels and motor function.

Impact of ozone treatment on wound healing has been studied in current literature [18,19]. However, the effects of ozone on a soft tissue injury model and its relationship with IGF-1 levels had not been reported in an experimental rat model. Ozone may result in increased angiogenesis and facilitate re-establishment of vascularization in the connective tissue. Thereby, improvement of oxygen and nutrient supply will enhance wound healing and repair of injury at the site of injury [18,19]. The precise mechanisms underlying these activities may be linked with anti-microbial property of ozone as well as amplification of the immune response through release of growth factors, interferons and interleukins [20]. Ozone may constitute a saf and effective therapeutic option by means of influencing oxygen metabolism, cell energy, anti-oxidant defense mechanisms, immunomodulation and vascular system [21].

In accordance with these data, our results support that ozone treatment can be a promising option for improvement of healing after soft tissue injury. We came across with 3 cases with infection at the site of traumatic injury in the experimental group that did not receive ozone treatment, whereas no infection was detected in the group that underwent ozone treatment. Even though the level of evidence is not very high, this observation reminds the anti infectious effects of ozone. Ozone can be administered through many routes depending on the site of lesion and clinical picture [22].

Adjunctive treatment alternatives must be preferred only after the failure of comprehensive wound management strategies. Further scientific evidence is necessary for supporting the use of intralesional ozone injection in treatment of chronic wounds. Notably, ozone therapy is not recommended for deep, severely infected or necrotic wounds. Moreover, since ozone treatments are frequently applied at non-academic health institutions, negative results and complications may be underestimated [23].

In addition to the inflammatory parameters, growth factors can exhibit a pivotal role in the wound-healing process. Insulin-like growth factor (IGF) not only promotes the migration of keratinocytes, but wounds with decreased levels of IGF may have less healing capacity [24]. Non-healing wounds in diabetic patients had low IGF-1 levels and exogenous application of IGF-1 promoted wound healing [25]. Remarkably, the correlation of IGF-1 with healing enhancement is a process with synergistic action with other growth factors [26].

In our study, we have focussed particularly on the injury of the connective and muscular tissue. Among the well-known factors, only IGF-1 is known to promote the hypertrophy, regeneration, proliferation and differentiation of skeletal muscles. The local production of autocrine/paracrine IGF-1 is particularly important in the skeletal muscle regeneration process. The impacts of IGF-1 on skeletal muscle cells is important for understanding the wound healing and repair mechanisms and regeneration [27].

Wound healing is a multi-dimensional and complex process that occurs with the interaction of many factors. Therefore, outcomes of the current study are prone to be influenced by multiple parameters. We could not detect a significant difference between sensory and motor functions of 2 groups. However, the occurrence of infection in the group that did not receive ozone is noteworthy. Moreover, a significant increase in serum IGF-1 levels after administration of ozone should be noted. Beneficial of effects of ozone on wound healing that may be mediated through IGF needs to be investigated in further trials. Correlation of serum IGF-1 level with a motor function on day 15 in ozone group reminds that this growth factor may be linked with late term regeneration of muscle tissue after injury.

Main limitations of the present study include experimental design, lack of a sham group and possible impacts of metabolic, environmental and technical factors which may interfere with the outcome parameters of this study.

To conclude, the current outcomes imply that ozone treatment may have favorable impacts on healing and regeneration process in connective and muscle tissues. These beneficial effects may be mediated by IGF-1. Further experimental and clinical research are warranted to elucidate the role of IGF-1 in the repair process and to provide additional new insights to the treatment strategies.

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