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# Therapeutic Effects of Ozone Therapy on Experimental Fracture Healing in the Rabbit Model

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## Abstract

Fracture repair is a complex biological process that requires the cooperation of various types of cells and materials. Today, various techniques such as low-density pulse ultrasonography and electrical stimulation are used to accelerate bone healing. The therapeutic effects of ozone on bone repair have been considered in recent years. The purpose of this study was to evaluate the effect of ozone gas on the speed of full-thickness bone defect healing. We selected 30 male weight equal rabbits and, in aseptic surgery, resected a 3-mm-thick piece of full-thickness of bone. Then we divided randomly them into two equal groups, the control, and the recipient. In the first, second, third, and sixth weeks of the radiographed bones were obtained. Also, in the third, sixth, and eighth weeks of each group, 5 rabbits were euthanized and their bones were histopathologically evaluated. Results from the second to sixth weeks of the study showed a significant difference between the treatment group and the control group. This difference was indicative of an increase in the rate of bone healing in the treatment group. Ozone therapy can therefore be considered effective in bone healing.

**Keywords**: Ozone therapy, Bone healing, Rabbit, Radius

## Introduction

Bones are an important part of the skeletal system of animals, which play an important role in providing the shape and structure of the body, mechanical activity, and protection of the body. In conditions such as trauma, pressure, bone diseases, and tumors, the tolerance of this tissue is reduced and it can break (Bocci & Di Paolo. 2009). Bone fracture repair is necessary for the survival of the injured animal and is also a complex biological process that requires the cooperation of various types of cells and materials (Bocci et al. 1998). The important point in bone healing is that it should be done with minimal scar tissue formation and maximum possible speed. In the last few decades, a lot of research has been done on speeding up the healing of bone

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fractures. Acceleration of bone healing is used to manage fractures, non-unions, osteomyelitis, bone tumor resection, joint fusion, and also in joint prosthetics. Today, to speed up bone healing, various methods such as pulsed ultrasound with low density and electrical stimulation are used. We want to investigate the effect of ozone gas on the speed of bone healing.

Ozone is a colorless and unstable gas that consists of three oxygen atoms and the reason for its instability is its mesomeric structure (Gist et al. 2009). In the past, this gas was known as a protector of the earth against ultraviolet rays in the stratosphere, and it was considered harmful and toxic in the troposphere. The therapeutic use of this molecule was not considered for a long time, but its therapeutic effects in combination with oxygen have been noticed in recent years (Andreula. 2011, et al. 2005 Monte, Sagai & Bocci. 2011). In the past few years, the effect of this gas on therapeutic processes has been almost determined, and the effective concentration of this gas has been carefully determined to use for therapeutic purposes. Therefore, the risk of its poisoning has been minimized and the techniques of using it in different tissues have made significant progress (Bocci.2011, Bocci.2013, et al.2005 Monte, Sagai & Bocci.2011). Ozone therapy can induce some biological responses such as activating the neuroprotective system, increasing blood circulation and oxygenation in ischemic tissue, regulating the function of cellular antioxidant enzymes and improving growth factors (Sagai & Bocci, 2011). The positive effects of ozone on cellular energy, oxygen metabolism, antioxidant defense system, and tissue blood circulation improve the wound healing process and modulate the immune system, but since its effects on bone healing are still unclear, it is necessary to conduct studies in this field (Oryan et al. 2011, Verhaar & Lems. 2010, Vos et al. 2012). The process of bone fracture repair involves several complex molecular mechanisms that are controlled by angiogenesis and other growth factors. Acceleration of bone healing is used for the management of fractures, nonunions, osteomyelitis, removal of bone tumors, joint fusion, and in joint prosthetics. Past research shows the positive effects of ozone therapy on the bone healing process in animals such as rats, which include: preventing bleeding in the transplant area, inducing angiogenesis, speeding up the process of cell proliferation, inducing the production of cell-matrix through Effect on the activity of osteoblasts (Laçin et al. 2018), improving the process of cortex formation and bone marrow organization (Duman et al. 2017). A study conducted about the use of Ozone as a rectal enema in mice showed that this substance caused a thicker periosteum, increased the process of vascularization, and overall improved the process of bone repair (Irban et al. 2015). A study conducted on the bone grafting process of rat skull also showed results based on strengthening bone formation and increasing the number of osteoblasts (Garcia et al. 2013).

Ozone therapy increases the amount of glycolysis of red blood cells, which by stimulating diphosphoglycerate leads to an increase in the amount of oxygen released to the tissues. Ozone activates the Krebs cycle by increasing the oxidative decarboxylation of pyruvate and stimulating the production of ATP. It also significantly reduces NADH and helps to oxidize cytochrome C. Stimulating the production of enzymes such as glutathione peroxidase, catalase, superoxide dismutase, and prostacyclin, which act as free radical scavengers and cell wall protectors, is another property of ozone that plays a significant role in bone repair (Hubert & Elvis. 2012).

Considering the importance of the subject, this study aimed to investigate the effect of intraperitoneal Ozone therapy in repairing full-thickness bone defects in rabbits over a period of 56 days (Bigham & Dehghani. 2008).

**Method and Materials**

To carry out this research, ozonated saline was used. This product has recently become commercially available, in which sterile saline is combined with ozone gas at a concentration of 20 µg/ml. This product is commercially sold in 250 ml bottles that contain 5 mg of ozone gas. Ozonated saline is a sensitive product that should be kept away from direct light and kept at refrigerator temperature. In addition, after each use, the bottle cap should be tightly closed. In this study, ozonated saline was purchased as a commercial product from an ozone therapy center in Najaf-Abad, Isfahan, Iran.

30 rabbits were selected for this study. To accustom the rabbits to new conditions, they were placed in a special cage for 15 days and fed with rabbit food. Rabbits received the anti-parasitic drug (ivermectin) subcutaneously at a dose of 0.2 mg/kg to clean external and internal parasites. The studied rabbits were randomly divided into two equal groups of control and treatment (ozone receiver) to conduct the research. They were sedated by intramuscular administration of Acepromazine (0.02 mg/kg) and anesthetized by intramuscular administration of ketamine (4 mg/kg), xylazine (5 mg/kg), and diazepam (0.4 mg/kg). After shaving the hair of the right hand and preparing for surgery, an incision was made on the skin of the craniomedial part of the right forearm. Then, the subcutaneous layer and deep fascia were pushed aside and cut between the flexor and extensor muscles, and a wide view of the radius was provided. After exposing the bone, right in the middle region of the diaphysis of the bone, a 3 mm wide bone defect was created transversely in the cross-section of the bone using an electric bone saw. After the surgery, the muscles were sutured with a 4-0 Vicryl suture and the skin with a Nylon 3-0 suture. Due to the lack of use of implants in the surgical site, a heavy bandage of the surgical area was used to immobilize the site. Then, complete care was taken until the rabbits fully recovered from anesthesia. After surgery, in the control group, sterile normal saline was used for intraperitoneal injection (the same volume as ozonated saline solution) for 3 consecutive days and then every other day until the 14 days after surgery, and in the treatment group, sterile ozonated saline injected intraperitoneally for 3 consecutive days (at a dose of 4 mg/kg), and then the injection was continued every other day until the 14 days after surgery. To prevent postoperative infection, the enrofloxacin 5% (with a dose of 5 mg/kg for 3 days, once a day intramuscularly) was injected into the rabbits. Ketoprofen 10% (with a dose of 1 mg/kg for 5 days) was used to reduce pain in the surgical site. Also, the bandage was changed daily to check the position and prevent infection.

All the rabbits were examined daily and when changing the bandage, in terms of any injury, wound, inflammation, or non-healing of the surgical wound.

Radiographs of the rabbits' hands were taken on the 7th, 14th, 21st, and 42nd days after surgery in lateral view. The distance of the film from the X-ray source was about 70 cm and the radiography machine was set at 45 kV and 20 mA/s. The modified grading system of Lane and Sandhu (Table 1) was used to evaluate and grade the prepared radiographs (Bigham et al. 2008). In this way, the minimum score is zero and the maximum score of a bone is ten. On the 21st, 42nd, and 56th days and in each stage, 5 rabbits from the control group and 5 rabbits from the treatment group were humanely euthanized by injecting a large dose of phenobarbital drug in the form of intracardiac injection. Then the radius bone of each rabbit was separated and all the soft tissues around it were separated from the bone. The bones were placed in 10% formalin for histopathological evaluation. For better penetration of formalin, 24 hours later, the samples were placed in freshly prepared 10% formalin. Then the samples were placed in 10% nitric acid for one week

to decalcify. Then the samples were washed with water and processed by an Auto-Technicon machine. The slices were stained with hematoxylin and eosin (H&E) and their microscopic slides were prepared using a light microscope and Dino-lite digital lens and Dino capture software. The samples were histopathologically evaluated based on Heiple's evaluation system (Table 2) (Karimi et al. 2013). In this system, ossification was evaluated with four criteria of union, cancellous bone formation, cortical bone formation, and bone marrow formation (scores zero to four).

## Results

### Radiographic evaluation

Radiographs were taken from both control and treatment groups on days 7, 14, 21, and 42 after surgery. The obtained results were analyzed using SPSS software and the 2-independent-samples test (Mann-Whitney U test). According to forms 1 and 2 of the radiographs (pages 9 and 10) and table 3 (page 10) in terms of bone formation, rearrangement, and union between the two control and treatment groups, except for the first week ($p=0.382$), there is a significant difference ($p<0.001$) was observed in all weeks, which means that the control group performed weaker than the treatment group.

### Histopathological evaluation

By performing the 2-Independent-samples test (Mann-Whitney U test) analysis for histopathological data, there was a significant difference between the treatment and control groups in terms of bone cortex formation in the third and sixth weeks ($p=0.006$ and $p=0.011$), but in the week Eighth ($p=0.393$) no significant difference was observed. In terms of bone marrow formation, a significant difference was observed in the third ($p=0.024$) and sixth ($p=0.042$) weeks, but no significant difference was observed between the two groups in the eighth week ($p=0.279$). In terms of cancellous bone formation in the third ($p<0.001$) and sixth ($p=0.018$) weeks, there was a significant difference, but there was no significant difference in the eighth week ($p=0.089$) and finally, in terms of the union in the third weeks ($p=0.006$) and in the sixth ($p=0.005$) there was a significant difference, while in the eighth week ($p=0.177$) such a difference could not be observed. In all comparisons where the p-value was less than 0.05, the results were considered significant (Table 4 and Figures 3, 4, and 5).

**Table 1: modified Lane and Sandhu scoring system**

| | |
|---|---|
| Bone formation | |
| No evidence of bone formation | 0 |
| Bone formation occupies 25% of defect | 1 |
| Bone formation occupies 50% of defect | 2 |
| Bone formation occupies 75% of defect | 3 |
| Bone formation occupies 100% of defect | 4 |
| Remodeling | |
| No evidence of remodeling | 0 |
| Remodeling of the medullary canal | 1 |
| Full remodeling of the cortex | 2 |
| Union (proximal and distal evaluated separately) | |
| Nonunion | 0 |
| Possible union | 1 |
| Radiographic union | 2 |
| Total point possible per category | |
| Bone formation | 4 |
| Proximal union | 2 |
| Distal union | 2 |
| Remodeling | 2 |
| Maximum Score | 10 |

**Table 2: Radiographic results of numbers in the form of the median (minimum-maximum) in the studied groups in different weeks. P value<0.05 = significant different**

| time | P value | treatment | control |
|---|---|---|---|
| First week | 0.382 | 2(1-3) | 2(1-3) |
| Second week | <0.001 | 4(4-6)* | 3(2-4) |
| Third week | <0.001 | 8(6-9)* | 3(2-5) |
| Fourth week | <0.001 | 9(8-10)* | 6(5-9) |

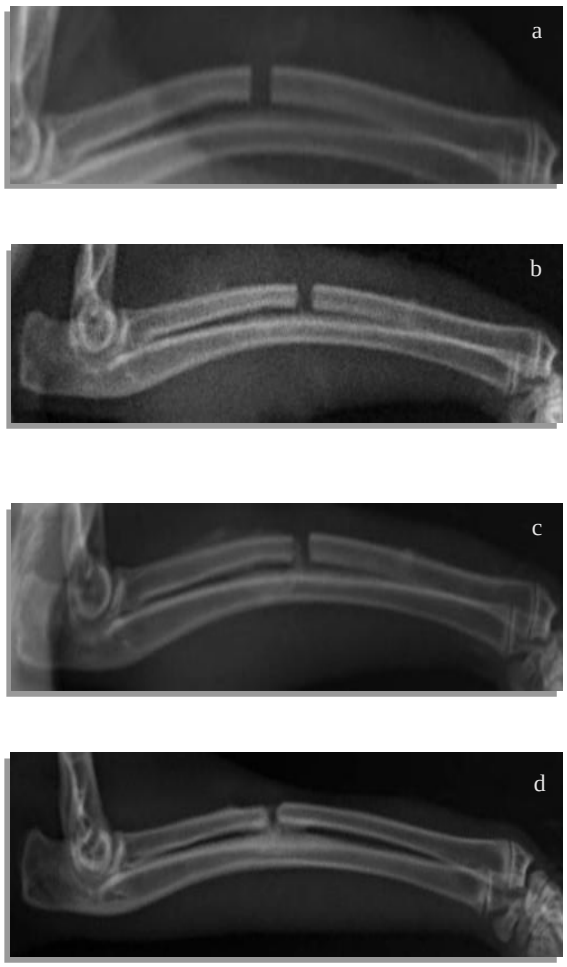


**Fig 1: control group radiographs**
**a-first week b-second week**
**c-third week d-sixth week**

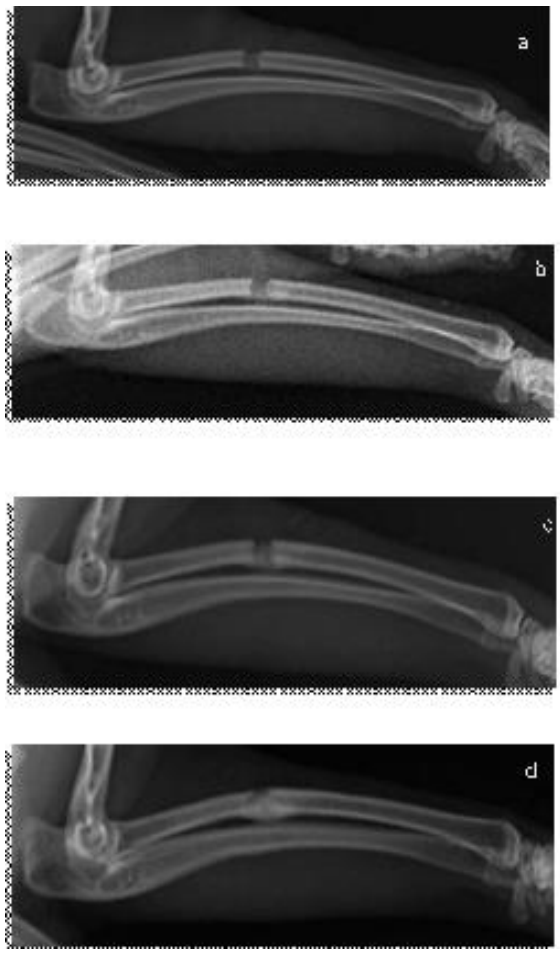


**Fig 2: treatment group radiographs**
**a-first week b-second week**
**c-third week d-sixth week**

**Table 3: Comparison of histopathological results of numbers in the median (min-max) form in the study groups in different weeks. P value<0.05 = significant different**

| Week | Groups & P value | Cortical bone | Bone marrow | Cancellous bone | Union |
|---|---|---|---|---|---|
| Third | Control | (3-1)2 | (2-1)1 | (2-1)1 | (2-1)1 |
| | treatment | (3-2)3 | (2-1)2 | (3-2)2 | (3-2)2 |
| | P value | 0.006* | 0.024* | 0.001>* | 0.006 |
| sixth | control | (3-2)3 | (2-1)2 | (3-2)2 | (3-2)2 |
| | treatment | (4-3)3 | (3-2)2 | (3-2)3 | (4-2)3 |
| | P value | 0.011* | 0.042* | 0.018* | 0.005* |
| eighth | control | (4-3)3 | (3-2)2 | (3-2)3 | (4-3)3 |
| | treatment | (4-3)4 | (4-2)2 | (4-3)3 | (4-3)4 |
| | P value | 0.398 | 0.279 | 0.089 | 0.177 |

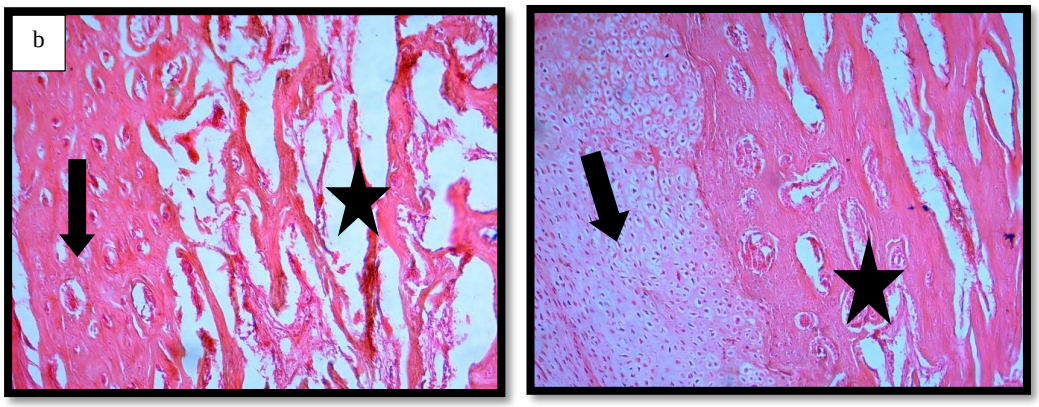


**Fig 3: Histopathological comparison of control and treatment groups in the third week**
**a: The formation of cancellous bone (arrow) with the presence of cartilage tissue (star) at the fracture site in the third week of the control group (H&E, X100)**
**b: the union of the bone by the primary cortical bone with the formation of cancellous bone (arrow) and the presence of bone marrow (star) in the third week of treatment (H&E, X100**

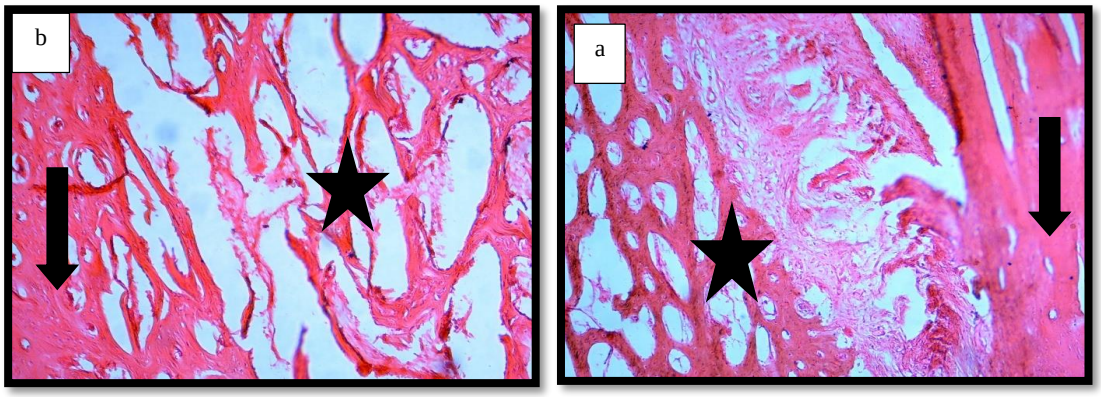


**Fig4: Histopathological comparison of control and treatment groups in the sixth week**
**a: union by the formation of cortical bone (arrow) and the presence of cancellous bone (star) in the middle of the fracture site in the sixth week of the control group (H&E, X100)**
**b: union by relatively rearranged cortical bone (arrow) with the removal of cancellous bone from the fracture space and the presence of large cavities containing red bone marrow (star) in the sixth week of the treatment group (H&E, X100)**

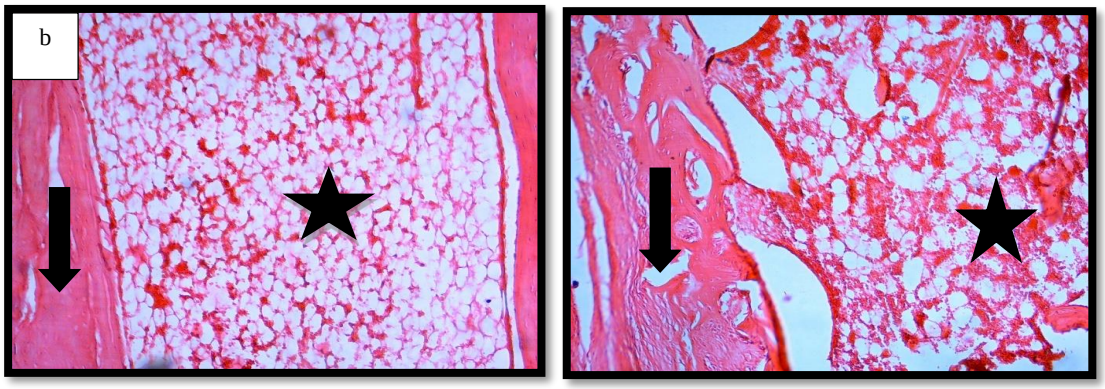


**Fig5: Histopathological comparison of control and treatment groups in the eighth week**
**a: union is relatively rearranged by cortical bone (arrow) and complete formation of the medullary canal containing red bone marrow (star) in the eighth week of the control group (H&E, X100)**
**b: Complete union of broken bone by cortical bone completely rearranged (arrow) and complete removal of cancellous bone from fracture space and complete formation of medullary canal with red bone marrow (star) in the eighth week in the treatment group (H&E, X100)**

**Discussion and conclusion**

Today, to speed up bone healing, various methods such as pulsed ultrasound with low density and electrical stimulation are used. Ozone therapy is one of the methods used to speed up bone healing. Based on the review of the literature, the effect of ozone on bone fracture repair has been conducted on animals such as rats, but no such study has been conducted on rabbits, so we conducted this research on rabbits.

In 2015, Irban et al conducted a study on the effects of ozone therapy on femur fractures in rats. Immunohistochemical evaluation on days 7 and 13 showed a significant increase in VEGF (Vascular endothelial growth factor) and β-catenin levels and on days 4, 7, and 13 in TGF-β (Transforming growth factor) levels in the treatment group. The expression of the VEGF gene, which plays an important role in angiogenesis, begins on the 14th day after a fracture. Bone repair is related to the formation of new blood vessels in the fracture area. During the fracture repair stage, blood circulation inside and outside the bone increases. In mice, it takes 2-4 weeks for the blood supply to return to its normal state before the fracture. VEGF gene expression stimulates the proliferation of endothelial cells, and this gene expression increases significantly during intrachondral ossification. Callus formation with VEGF gene expression mainly occurs in mesenchymal cells and osteoblasts during the first week of repair but decreases after 11 days. In their study, VEGF gene expression was higher in the ozone treatment group on days 7 and 13 compared to the control group. Thicker periosteum and better construction of cancellous bone along with more expression of VEGF, TGF-β, and β-catenin genes are all signs of better bone fracture repair. So, the final result indicated that rectal enema of ozone, with the effect of increasing the production of VEGF and TGF-β, causes better angiogenesis and more cell proliferation, and also causes the formation of a thicker bone matrix layer and, as a result, improves the bone healing process. In the present study, the effect of ozone therapy on bone healing was evident in the third and sixth weeks. Another study comparing growth factors in ozone therapy was done by Kim et al. in 2009, who investigated the local effects of ozone olive oil on pig wound healing and increased gene expression of VEGF factors and TGF observed.

In 2014, Zhang et al. investigated the growth factors effective on wound healing using oxygen-ozone therapy in the treatment of wounds of diabetic patients, they observed that the gene expression of VEGF, TGF-β, and PDGF factors in the oxygen group - Ozone was different compared to the control group; That is, in their study, oxygen-ozone therapy increased the wound healing process by potentially inducing VEGF, TGF-β and PDGF factors in the early stages of healing.

In 2018, Laçin et al investigated the comparative effects of ozone therapy in bone defects regenerated with allograft transplantation. They aimed to investigate the amount of ossification after the use of graft materials and also the histopathological effects of ozone therapy. Their study included 4 control groups, treatment with ozone, treatment with transplantation, and treatment with ozone and transplantation, and a defect was created in the skull in all groups. Their histopathological findings in the fourth and eighth weeks showed the best result regarding bone formation in the fourth group. The results of their study included preventing bleeding in the transplant area, inducing angiogenesis, speeding up the process of cell division, inducing the formation of cell matrix through the effect on the activity of osteoblasts, and in general improving the process of bone formation. In the fourth week, a significant difference was observed in cell infiltration, osteoblasts and osteoclasts activity, matrix and cancellous bone formation, vascular

expansion, and collagen fiber distribution. In the eighth week, a significant difference was observed in the formation of matrix and cancellous bone, vascular expansion, and collagen fiber distribution. Vascular dilation increased cell penetration between 4 and 8 weeks. The activity of osteoblasts also increased the formation of cancellous bone in these 8 weeks. In the upcoming study, the effects of ozone therapy on bone healing were fully evident in the third and sixth weeks, and a significant difference was observed in the control group and the group treated with ozone, which is consistent with the results of Laçin et al.

In another study, Duman et al. 2017 investigated the effect of rectal administration of ozone in the gaseous form on the process of femur bone regeneration in a rat model; The second week of their histopathological evaluation showed that there was a significant difference between the control and treatment groups in terms of union and bone marrow formation so that in the treatment group, endochondral fusion was observed in all cases and bone marrow was also formed in all cases, while in The control group had no bone marrow. In both groups, the cancellous formation was observed, while the cortex was not formed in any of the groups. In the fourth week, no significant difference was observed between the two groups in terms of union, cancellous bone, bone marrow, and cortex. In the sixth week, only bone marrow formation was higher in the treatment group, but in terms of union, cancellous bone formation, and bone cortex, high scores were observed in each group.

In 2013, Ozdemir et al investigated the effects of ozone therapy in the use of bone grafts in the repair of the defect created in the skull bone, and in their histopathological findings, significant results were found regarding the strengthening of ossification as well as the increase in the number of osteoblasts between the two control and treatment groups.

According to the study of Irban, Kim, and Zhang, each of them observed a significant increase in the gene expression of growth factors such as VEGF, TGF-β, β-catenin, and PDGF in the early stages of bone repair in the treatment group, and considering that in our study, a significant difference was observed in the process of making bone cortex and cancellous bone in the first weeks, especially in the third week ($p=0.006$ and $p<0.001$). It can be assumed that this process is caused by increased angiogenesis during bone repair.

Also, according to the study by Laçin et al. in 2018, who observed the best result in the group of treatment with ozone combined with transplantation, and a significant difference in preventing bleeding in the transplant area, inducing angiogenesis, speeding up the process of cell proliferation, They observed the construction of cellular matrix through the effect on the activity of osteoblasts in their treatment group compared to the control group, so it can be assumed that the significant difference observed in our study before the eighth week can be caused by the effects of ozone on vascular expansion, distribution Collagen fiber is the activity of osteoblasts, which ultimately improves the process of bone formation.

In the study of Duman et al. in 2017, in the second week of their histopathological assessment of union and bone marrow formation, there was a significant difference between the two control and treatment groups, so in the treatment group, endochondral fusion was observed in all cases, and bone marrow was also It was formed in all cases. Compared to our initial results, we can say that there are similarities in the comparison between rats and rabbits, but considering that the results of our investigation were significant up to the sixth week, in the study of Duman et al. in 2017, the difference in the results up to the week The second was significant, it can be concluded that bone repair is done faster in rats than in rabbits, so ozone therapy is more

useful in rabbits, which have a slower repair.

According to the study by Ozdemir et al. in 2013, which observed significant results regarding the strengthening of bone formation as well as the increase in the number of osteoblasts between the two control and treatment groups, considering that osteoblasts are responsible for collagen production and actions such as production, regulation, sorting the extracellular matrix. Therefore, they have a direct role in fracture repair, so it can be said that one of the reasons for the increase in the speed of bone repair in our treatment group could be the increase in the number of osteoblasts due to the administration of ozone.

Considering the limitations caused by not using implants in this study, it is suggested to use implants in future studies to better immobilize the position.

Based on the similar studies conducted above and the aforementioned topics, as well as the results obtained from this research, which are mentioned in the results chapter, it can be concluded that the injection of saline solution is effective in accelerating the healing of bone fractures in the rabbit model animal.

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**Conflict of interest**

The authors of the article have no conflict of interest.

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# بررسی اثرات درمانی اُزُن‌تراپی بر ترمیم شکستگی تجربی استخوان زند زبرین در مدل خرگوش

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چکیده

ترمیم شکستگی یک فرایند زیستی پیچیده است که به همکاری انواع مختلفی از سلول‌ها و مواد نیازمند است. امروزه برای سرعت بخشیدن به التیام استخوان از روش‌های مختلفی مثل اولتراسونوگرافی ضربانی با تراکم پایین و تحریک الکتریکی استفاده می‌شود. اثرات درمانی اُزُن در ترمیم استخوان در چند سال اخیر مورد توجه قرار گرفته است. هدف ما بررسی اثر گاز اُزُن بر سرعت التیام نقیصه تمام ضخامت استخوانی بود. بدین منظور ۳۰ رأس خرگوش نیوزلندی ۱ ساله (± ۲ ماه) با میانگین وزنی ۰/۵ ± ۲ کیلوگرم انتخاب شد و در شرایط آسپتیک جراحی یک نقیصه استخوانی به عرض ۳ میلی‌متر در تمام ضخامت بخش دیافیز استخوان زند زبرین ایجاد شد. سپس آن‌ها به صورت تصادفی و به دو گروه مساوی کنترل و دریافت‌کننده‌ی اُزُن تقسیم شدند و در یک دوره ۵۶ روزه مورد بررسی رادیوگرافیک و هیستوپاتولوژیک قرار گرفتند. در هفته‌های اول، دوم، سوم و ششم از استخوان جراحی شده رادیوگراف تهیه شد، همچنین در هفته‌های سوم، ششم و هشتم از هر گروه در هر مرحله ۵ خرگوش آسان کشی شد و استخوان جراحی شده آن مورد ارزیابی هیستوپاتولوژی قرار گرفت. نتایج هفته‌های دوم، سوم و ششم حاصل از این تحقیق، بین گروه درمان با اُزُن و گروه کنترل تفاوت معنی‌داری را نشان داد. این تفاوت نشان‌دهنده‌ی افزایش سرعت ترمیم استخوان در گروه درمان بود. پس می‌توان اُزُن‌تراپی را در روند بهبود استخوان مؤثر دانست.

**کلمات کلیدی:** اُزُن تراپی، ترمیم استخوان، خرگوش، زند زبرین

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