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Evaluation of the Healing Effects of Different Methods of Using Ozone in Third-Degree Skin Burns in Rat Experimental Model

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ABSTRACT

This study was conducted to evaluate the healing effects of different methods of using ozone in third-degree skin burns in the rat experimental model. To carry out this study, 45 adult male rats with an average weight of 250-300 g were divided into 5 groups of 9, including the first group of treatment with ozonated water, the second group of ozonated oil, the third group of ozonated gas, and the fourth group of systemic ozone gas. The fifth group was used as a control group. To create a burn, the innovative device, whose accuracy has been confirmed in previous studies, was used with a 2 × 3 cm probe with a temperature of 95 °C and 10 seconds of contact with the skin. Wound management was performed every other day for up to 14 days, and daily photographs were taken of the surface of the wounds. On the 4th, 8th, and 14th day, samples were taken for histopathologic examination, the amount of tissue hydroxyproline was also examined on the 14th day of the study. The statistical data obtained were analyzed with SPSS software and one-way analysis of variance statistical tests, values less than 5% were considered as significant levels. Morphologic examination of the wound healing process and histopathologic observations showed that the use of ozonated water and ozonated oil significantly accelerates ($p < 0.05$) the wound healing process compared to other groups and reduces the infiltration of inflammatory cells, improves angiogenesis and fibroplasia in the wound site. It was also found that the amount of tissue hydroxyproline in the ozonated oil group was lower than in the other studied groups ($p < 0.05$). According to the findings of the current study, it seems that the use of ozone in liquid phases, i.e. ozonated water and ozonated oil, has a better effect on the wound healing process than ozone in gaseous form.

Introduction

Active oxygen or ozone is a colorless gas with a rather pungent smell, which is produced in the atmosphere as a result of electrical discharge between the clouds and the earth, and there are three oxygen atoms in its chemical structure. High concentrations of this gas are poisonous and in combination with other pollutants create deadly acid compounds, for this reason, researchers are mainly focused on the harms of this chemical substance and have neglected its therapeutic and beneficial properties, many chemical elements in nature can Such a spectrum can have a double effect, but the expansion of studies and the progress of science always make it possible to use the useful aspect of these materials.[1]

Ozone was used for the first time in 1974 as a therapeutic agent to treat the gangrenous wounds of soldiers in World War I. Currently, the antibacterial properties of this chemical are not hidden from anyone, but depending on the treatment method and what dose

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of ozone is used, its therapeutic effects are different. The strong oxidation property and the ability to pass through cell membranes are the two main characteristics that form the bactericidal ability of ozone. Research has shown that the ozone molecule and the free radicals resulting from its decomposition are responsible for the death of microorganisms.[2,3]

The resistance of microbes against this molecule is different according to the environment, the method of application (in the form of gas, ozonated water, or ozonated oil), and the type of organism. In environments without starch, the effectiveness of this gas on disinfection is greater, but in environments containing fat, there is a high resistance protein against it. On the other hand, bacterial organisms are more sensitive to this chemical substance than fungi and molds, and Gram-positive bacteria are more sensitive to ozone than Gram-negative ones.[4]

The antioxidant property of ozone is one of its most important therapeutic effects, this molecule plays a role in the treatment of many diseases that are associated with increased oxidative stress by increasing the activity of some antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and catalase. Accelerating the healing of diabetic foot ulcers by using antioxidant properties and improving vascular function and blood supply is one of these cases. It has been found that the increase in the amount of 2,3-bisphosphoglycerate due to ozone therapy in red blood cells causes more oxygen release and accelerates wound healing. On the other hand, ozone, by affecting the metabolites caused by the oxidation of lipids, increases the release of nitric oxide from endothelial cells, which ultimately causes vasodilation, which increases not only oxygen but also other substances needed by the cell.[5,6]

Studies have also shown that ozone is effective in destroying some protozoa such as Giardia and its cysts, and it has also been effective on the human immunodeficiency virus.[7] Some studies also show that this gas has several anti-inflammatory properties in specific concentrations. have mentioned, it is said that this substance exhibits its anti-inflammatory effects by inhibiting nuclear factor kappa B (NF-κB) and inducing erythroid factor.[8] Due to the many therapeutic properties expressed by this molecule, it was seen that the healing effects of different methods of using ozone in third-degree skin burns were evaluated in the rat experimental model and the unknown aspects of using this chemical substance in therapeutic processes were determined.

Materials and Methods

The present study was conducted in the Faculty of Veterinary Medicine, University of Tehran under the supervision of the professors of the Departments of Surgery and Pathology. This research was carried out in compliance with all the international rules of working with laboratory animals, the code of ethics (IR.UT.VETMED.REC.1402.032), and the code of the research project. All the laboratory stages of this research were carried out in the laboratories of the Department of Pathology, Faculty of Veterinary Medicine, University of Tehran. In order to prepare ozone in this study, a calibrated Gardina model MC80 generator was used. To prepare ozonated water, the outlet of the device was placed in three liters of twice-distilled water, and after 30 minutes of soaking, ozonated water with a concentration of 5 mg of ozone per liter was obtained. In order to prepare ozonated oil, olive oil was used as a vegetable oil base. The ozonation of the oil was done in such a way that 40 ml of olive oil was placed in the outlet of the ozone generator for 60 minutes with an output of 1.5 liters per minute. With this method, ozonated oil with a concentration of 45 mg/l was prepared. Systemic use of ozone was done through the rectum and with a lead connected to a cannula with a slow syringe. In this method, 0.5 mg/kg of ozone was administered rectally to the studied rats. In the method of using topical ozone gas, plastic containers and a concentration of 5 mg of ozone per liter were used for 15 minutes.[9]

Animals

Forty five adult male Wistar rats with an average weight of 250 to 300 grams were used for this study. Rats were kept for one week in an environment with standard conditions including 12-hour light exposure and a temperature of 23 degrees with free access to food and water. This study was conducted by obtaining the code of ethics and complying with all international laws regarding working with laboratory animals.

Method of Induction of Anesthesia and Wound Formation

In this study, anesthesia was induced by using xylazine 2% at a dose of 5 mg/kg and ketamine hydrochloride 10% at a dose of 40 mg/kg Intraperitoneally.[10] After anesthetizing, the rats were placed on sternal recumbency and the hair on dorsal of the body was clipped. In the next step, the surgical site was prepared with 70% ethanol and povidone-iodine 7.5%. To create a third-degree burn wound, an innovative device was used, which has been checked for burn accuracy in previous research. Based on this, first, the temperature of the device was set to 95 °C, and after preparation, by placing the probe of the device on the skin of the rat for 10 seconds, a third-degree burn of two by three centimeters was created. A wound in rectangle shape in 2 by 3 cm size was made on dorsum of each rat.

Ozone Use Method

In this study, rats were randomly divided into 5 groups of 9: the first group of ozonated water, the second group of ozonated oil, the third group of topical ozone gas, the fourth group of systemic ozone gas, and the fifth group as a control. It was considered that in this group, no treatment intervention was done in the created wounds. Wound management in this study was done every other day on days one, three, five, seven, nine, eleven, and thirteen.

Pathologic Investigation

To investigate the healing process of the wound, taking a picture of the wounds and measuring the area of the wound was done on days one, three, five, seven, nine, eleven, and thirteen, in addition to this, tissue sampling of the lesion was done on days fourth, eighth and fourteenth were performed, in this way, in addition to the wound, 2 mm from the healthy skin margin was also separated from the studied rats and placed in a container containing 10% formalin fixative. In the next step, the fixed tissue sample was processed in the histotechnique machine and paraffin molds were placed, and finally, 5-µm sections prepared from the wound site were stained with hematoxylin and eosin staining method, and the inflammatory processes were examined.

Measuring the Amount of Tissue Hydroxyproline

On the 14th day of study, in addition to preparing the histopathologic slide, the amount of tissue hydroxyproline was also measured. The method used to obtain the amount of tissue hydroxyproline was modified by spectrophotometry, which determines the amount of this tissue protein with high accuracy. In this method, tissue samples were first hydrolyzed in 6 M hydrochloric acid at 105 °C for 16 hours. Then, by adding chloramine T solution to the hydrolyzed samples, the hydroxyproline in it was oxidized. By adding Ehrlich's reagent to the hydroxyproline oxidation product, a colored mixture was created. Then, the color composition created by using toluene was extracted during two steps, and using a spectrophotometer, its optical absorption was read at a wavelength of 550 nm and compared with existing standards.[11]

Statistical Analysis

The obtained data were analyzed by SPSS version 21 software and using a one-way analysis of variance test. Differences in values less than ($p < 0.05$) were considered significant. The findings were shown as mean ± standard deviation.

Results

Evaluation of Wound Size

The results of the evaluation of different methods of ozone therapy on the healing process of wounds caused by third-degree skin burn in rats showed that the use of ozonated oil and ozonated water caused a significant reduction ($p < 0.05$) of the wound size compared to other groups in The final day has been studied, but ozone therapy by systemic method did not create a significant difference in the process of reducing the wound size in the fourth group compared to the control group ($p > 0.05$). Also, there was no significant difference in the wound healing process comparing the two methods of systemic ozone gas group and the use of topical ozone gas ($p > 0.05$). In this study, the lowest wound size on the 13th day of the study was related to the second experimental group, ozonated oil, and the largest was related to the control group. Also, the examination of the percentage of wound healing in different groups showed the highest healing rate in the groups of ozonated oil, ozonated water, topical ozone gas, systemic ozone gas, and the control group, respectively, at the rate of 44.61, 41.66, 32.77, 31.55, and 44. 24% was observed (Table 1, Figure 1).

Examining the Amount of Tissue Hydroxyproline

The results of the statistical analysis showed that on the fourteenth day after the wound was created, the amount of tissue hydroxyproline in different groups had a significant difference ($p < 0.05$), so the amount of hydroxyproline in the second group (ozonated oil) was significantly ($p < 0.05$) lower compared to the other groups, and the investigations showed that there was no significant difference in the amount of hydroxyproline tissue in third group (topical ozone gas) and fourth group (systemic ozone gas) with the control group ($p > 0.05$). Finally, the results showed that the highest amount of

Table 1. The trend of wound size (cm) change in different treatment days in the treatments.

Group	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13
Ozonated water	6 ± 0	6 ± 0	5/07 ± 0/15^{a}	4/62 ± 0/19^{a}	4/14 ± 0/24^{a}	3/75 ± 0/08^{a}	3/50 ± 0/08^{a}
Ozonated oil	6 ± 0	6 ± 0	5/10 ± 0/18^{a}	4/62 ± 0/19^{a}	4/27 ± 0/13^{ac}	3/73 ± 0/12^{a}	3/32 ± 1/10^{a}
Topical ozone gas	6 ± 0	6 ± 0	5/83 ± 0/25^{b}	5/15 ± 0/19^{b}	4/71 ± 0/25^{bc}	4/30 ± 0/09^{c}	4/03 ± 0/33^{c}
Systemic ozone gas	6 ± 0	6 ± 0	5/62 ± 0/12^{b}	5/21 ± 0/20^{b}	4/86 ± 0^{b}	4/53 ± 0/09^{bc}	4/10 ± 0/09^{bc}
Control	6 ± 0	6 ± 0	5/91 ± 0/13^{b}	5/25 ± 0/18^{b}	5/13 ± 0/16^{b}	4/83 ± 0/22^{b}	4/53 ± 0/09^{b}

^{abc} Different superscript letters in the same column indicate significant differences ($p < 0.05$).

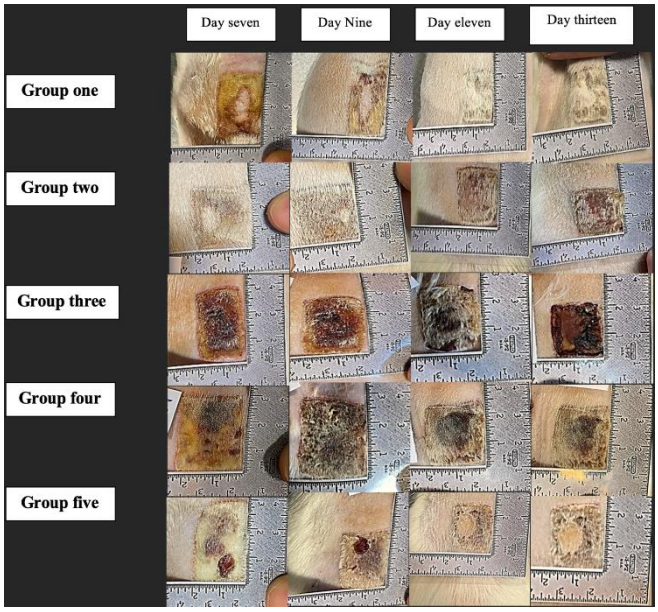

Figure 1. Measurement of wound size in different groups of ozone treatment in different forms.

Table 2. Comparison of average hydroxyproline amount in treatments on the last day of the study (mg / gram).

Group	Day 14
Ozonated water	34/57 ± 2/02[a]
Ozonated oil	25/04 ± 1/79[b]
Topical ozone gas	46/95 ± 3/93[c]
Systemic ozone gas	41/24 ± 2/97[ac]
Control	49/21 ± 1/52[c]

[abc] Different superscript letters in the same column indicate significant differences ($p < 0.05$).

hydroxyproline in the lesion site is related to the third group (topical ozone gas), and the lowest is related to the second group (ozonated oil) in the experimental groups (Table 2).

Pathologic Findings

The results of histopathologic investigations showed that on the fourth day after the wound was created, the presence of inflammatory cells in the fourth group (systemic ozone gas) was higher than in other experimental groups, the majority of these cells were neutrophils. On the eighth day of sampling, there was no significant difference ($p > 0.05$) in the amount of infiltration of inflammatory cells in different treatments, and pathologic examinations on the fourteenth day showed that the highest amount of infiltration of inflammatory cells into the wound was in the fourth group (systemic ozone gas).

On the fourth day of the study, the formation of new blood vessels in the second group (ozonated oil) occurred more intensively than the other experimental groups, but with time on the eighth day, due to the presence of fibroblasts and collagen deposition, the number of new blood vessels in the second group ozonated oil decreased sharply. In other experimental groups, on the eighth day, angiogenesis was studied more intensively than on the fourth day. In the sampling on the last day of the study, it was found that the highest number of blood vessels was related to the third group (topical ozone gas), and the lowest in the experimental group was related to ozonated oil.

Table3. Pathologic scores.

Slide No.	Epidermis necrosis	Reepithelialization	Epidermis thickness	Hyper keratosis	Dermal fibrosis	Infl. cell type	Inf. cell infiltration	Panniculitis	Granulation tissue	Neovascularization	Bacterial colony
G1D4	4	0	0	0	0	L/N	1	2	0	0	0
G2D4	4	0	0	0	0	N	1	3	2	2	0
G3D4	4	0	0	0	0	N	2	1	1	1	1
G4D4	4	0	0	0	0	N	2	2	3	3	1
G5D4	4	0	0	0	0	M	1	3	2	2	0
G1D8	4	0	0	0	0	N	1	2	0	0	1
G2D8	4	0	0	0	0	N	3	1	2	2	1
G3D8	4	0	0	0	2	N/L	3	1	1	1	2
G4D8	4	0	0	0	1	L/M	1	1	1	1	2
G5D8	4	0	0	0	0	L/M	1	2	2	1	2
G1D14	4	2	0	0	1	M/N	3	1	2	2	0
G2D14	4	1	0	0	3	N	2	1	1	1	1
G3D14	4	1	0	0	2	N	2	1	2	1	2
G4D14	4	1	0	0	2	N/L	3	2	3	2	2
G5D14	4	1	0	0	0	L/M	1	1	1	1	2

N: Neutrophil. L: Lymphocyte. M: Macrophage. G: Group. D: Day.

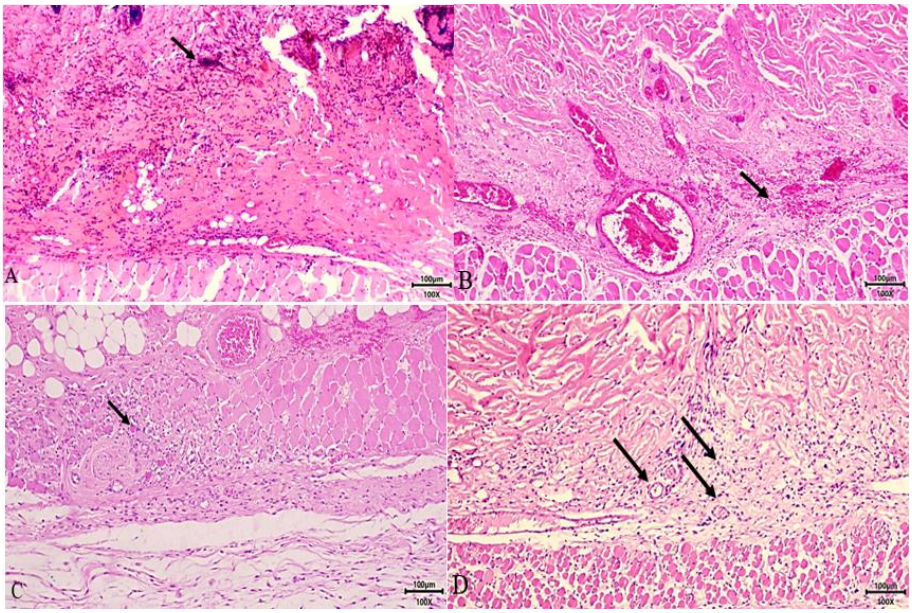

Figure 2. Wound site 4 days after treatment. A: Diffuse full-thickness necrosis of the skin with widespread leukocyte infiltration (arrow) to the wound area in the systemic ozone therapy group. B: Severe hyperemia, hemorrhage, and inflammatory infiltrates (arrow) in the histologic location of the dermis and hypodermis in the topical ozone gas therapy group. C: Neovascularization (arrow) and fibroplasia in the ozonated water treatment group. D: Neovascularization and pronounced fibroplasia (arrows) in the ozonated oil treatment group.

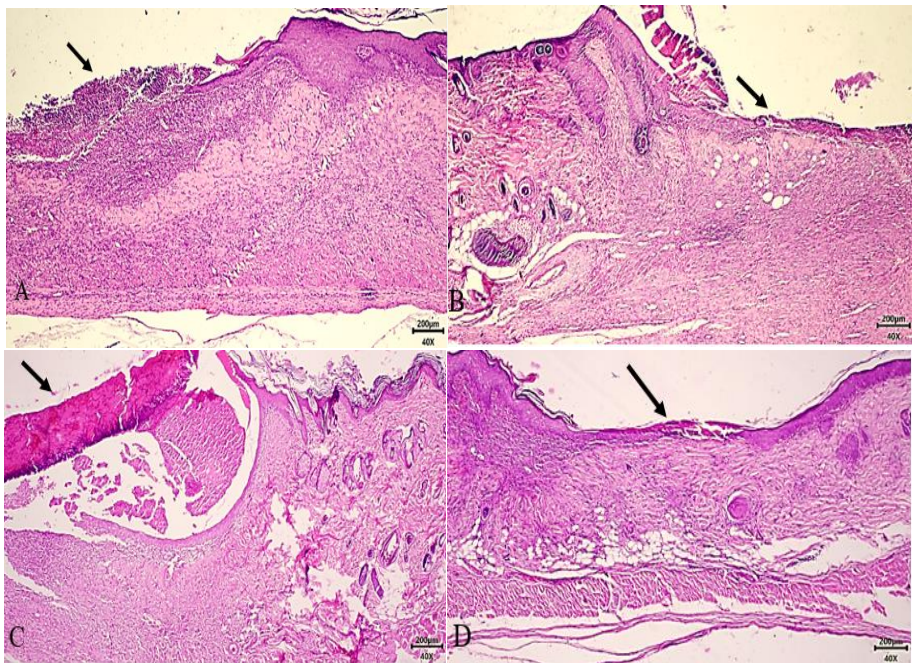

Figure 3. Wound site 14 days after treatment A: Complete lack of re-epithelialization is evident as an ulcerated area (arrow) in the systemic ozone treatment group. B: Re-epithelialization in the absence of keratinization of the epidermis (arrow) is depicted in the topical ozone gas treatment group. C: Complete re-epithelialization and mild keratinization (arrow) of the epidermis in the ozonated water treatment group. D: Complete re-epithelialization and well-formed keratin layers (arrow) of the ozonated oil treatment group.

The formation process of granulation tissue in different treatments on the fourth day of the study was significantly different from each other ($p < 0.05$), the highest amount of granulation tissue formation was observed in the second group (ozonated oil) on the fourth day. On the eighth day after wounding, this index was higher in the third group (topical ozone gas) than in other experimental groups. On the last day of sampling, the formation of granulation tissue was the highest in the fourth group (systemic ozone gas) (Table 3, Figures 2 and 3).

Discussion

The present study was conducted to evaluate the healing effects of different methods of using ozone in third-degree skin burns in the rat experimental model. The specific results of this research showed that the use of ozonated water and ozonated oil significantly speeds up the healing process of skin wounds caused by third-degree burns in rats.

The use of ozone to heal third-degree burn wounds in rats is different depending on the phase used, in this

research it was found that the use of ozone in liquid phases, i.e., dissolved in water and vegetable oils, significantly in the wound healing process compared to other experimental groups. This is while the use of ozone in the form of topical ozone gas or its rectal (systemic) injection does not make a significant difference compared to the healing process of the control group. In the initial stages of the study, after burning the skin, due to the infiltration of inflammatory cells, some purulent secretions were seen from the wounds. With the use of ozone therapy in all treatments in the following days of the study, the amount of this inflammation was reduced. In addition to the rate of inflammatory cell infiltration, with the passage of time and repetition of treatment, the rate of angiogenesis in ozonated water and ozonated oil groups started with high intensity, and the transition from the inflammatory phase of wound healing in these groups was faster than other treatments. Studies predict that the anti-inflammatory effects of ozone lie mainly in the ability of this substance to break double bonds in molecules such as arachidonic acid and its derivatives such as prostaglandins and leukotrienes. This group of mediators are actually the factors that initiate and survive inflammation in the body.[12] Ozone in different ways, by removing these biological mediators, reduces the inflammatory phase of the wound. In 2022, Özalp *et al*. showed in a study that the use of ozone in the healing of oral wounds in rats improves the score of inflammatory indicators compared to the control group and speeds up the healing process.[13] This conclusion is consistent with the findings of the current study, in both studies, the use of ozone reduced tissue inflammation and the speed of wound healing increased under its influence.

Another effect of ozone therapy on damaged tissues is to increase the release of tissue growth factors, including transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF). So, ozone increases vascular regeneration and increases new blood vessels in the wound site, not only providing oxygen but also providing other nutrients needed by the damaged tissue for repair.[14,15] In the present study, histopathologic investigations showed that angiogenesis occurred more intensively in the ozonated water and ozonated oil group than in the control group, and the healing process was significantly higher in these groups than in other groups. In a similar study by Acikan *et al.* in 2023, it was found that the use of ozone to heal palatal wounds increases the amount of some tissue factors such as VEGF, and accelerates the formation of new blood vessels. In this study, it was also found that the formation of fibrotic tissue in the treated groups was more with ozone compared to other groups.[16]

In another study by Peker *et al.* in 2020, it was found ozone beneficial in second degree skin burns in rat with positive effects on development of inflammation, fibrosis and granulation by increasing tissue enzymes such as 6PGD, GR, and G6PD.[17] In another study by Sanguanini *et al.* in 2020, it was found positive effects of ozone therapy on wound healing. It was found that ozonated water was beneficial on wound retraction and assists in the maturation and remodeling phase and ozonated oil was beneficial on neovascularization with higher amount of VEGF factor and higher deposition of type I collagen.[18]

In fact, fibroblasts are one of the main cells of the connective tissue in the wound healing process, these cells help a lot in the final phases of wound healing both by building a framework for mounting the epithelial tissue and by creating tension on the edges of the wound. The improvement in the activity of fibroblasts due to ozone therapy can be due to the increased expression of proteins such as vimentin and fibronectin.[19] The results of Acikan *et al*.'s study are considered consistent with the findings of the current research.[16] These studies show the effect of ozone therapy in the wound healing process by increasing angiogenesis and improving fibrotic tissue and flesh bud.

Hydroxyproline is a non-protein amino acid that is produced by the post-translational hydroxylation process of proline and during collagen biosynthesis. Determining the tissue values of this factor provides useful information about the healing processes and provides the healing rate and the effects of therapeutic agents in the proliferative phase of the wound.[20] In this research, it was also found that the amount of tissue hydroxyproline in the second group (ozonated oil) is lower than the other groups studied. The tissue amounts of this protein in the third (ozonated gas) and fourth (systemic ozone gas) groups were not significantly different from the control group. Research has shown that the increase in the amount of hydroxyproline in the damaged tissue actually indicates the presence of more collagens. In the processes of wound healing, the filling of the wound surface is necessary for the next phase of healing and the presence of epithelial cells. Normally, this index increases rapidly in the early stages of the wound and after the inflammatory phase and reaches its peak tissue concentration in the proliferation stage of wound healing, finally, the amount of this factor decreases with the entry into the final stages and epithelization of the wound surface.[21] In this study, although the speed of wound healing in the first group (ozonated water) and the second (ozonated oil) was higher than in other experimental treatments, the amount of hydroxyproline in the groups that had faster wound healing was lower in the final day sample, this process can indicate the wound of the first and second groups should pass through the proliferative stage of wound healing faster. In ozonated water and ozonated oil treatments,

due to the effect of ozone on healing, the wound quickly went through the initial stages of healing, and for this reason, on the last day (fourteenth), the amount of tissue hydroxyproline was the lowest in the second group and the highest in the control group. The high amount of this amino acid in the control group also confirms that the wounds of the control groups are still in the proliferative stages.

In the study conducted by Karakaya *et al*. in 2021, which aimed to investigate the effect of subcutaneous ozone therapy on burn wound healing in the rat model, the results showed that the use of ozone subcutaneously improved the wound healing process compared to the group has been controlled, in this study the measurement of tissue values showed a significant difference in the tissue concentration of this amino acid in ozone treatments compared to the control group.[22] The results of this research are not consistent with the current study. In Karakaya's study, sampling on days 7 and 14, tissue hydroxyproline values were higher than the control group.[22] Also, in another study by Roshanmaram *et al*. in 2023, the findings showed that in the process of full-thickness wound healing in rats, the amount of Hydroxyproline increased in the first days of tissue repair, and with time, until the 21st day of the study, its amount decreased continuously.[23] The findings of this research are consistent with the current study. In both studies, the amount of tissue hydroxyproline decreased in the last days of wound healing in the treatment groups.

Finally, the results of this research stated that the use of ozone in the form of ozonated water and ozonated oil significantly accelerates the wound healing process caused by third-degree burns in the rat experimental model, while ozone in the form of gas and systemic has no significant effect on this process. Based on the results of the present study, it seems that the use of ozone in liquid phases has a significant effect on the wound healing process.

Conflict of Interest

No conflicts of interest have been reported between the authors.

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