*Research Article/Araştırma Makalesi*

# THE EFFECT OF OZONE MEDIATED PRECONDITIONING ON FLAP VIABILITY IN RATS: AN EXPERIMENTAL STUDY

# OZON ARACILI ÖN KOŞULLANDIRMANIN SIÇANLARDA FLEP YAŞAYABILIRLIĞI ÜZERINE ETKISI: DENEYSEL BIR ÇALIŞMA

**Abdulkerim OLĞUN[1]✉, Tahsin GÖRGÜLÜ[2], Ramazan GÜLER[3], Ahmet Eksal KARGI[4]**

1. Department of Plastic Reconstructive and Aesthetic Surgery, Faculty of Medicine, Atatürk University, Erzurum, Turkey
2. Department of Plastic Reconstructive and Aesthetic Surgery, Phi Clinic, Istanbul, Turkey
3. Department of Plastic Reconstructive and Aesthetic Surgery, The Private Clinic, Istanbul, Turkey
4. Department of Plastic Reconstructive and Aesthetic Surgery, Faculty of Medicine, Bülent Ecevit University, Zonguldak, Turkey

**ORCID**: 0000-0002-1493-5152, 0000-0002-4183-3020, 0000-0001-8897-1618, 0000-0003-4539-8107

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**Abstract**

**Objectives**: There are many type of preconditioning techniques in flap surgery. But ozone mediated preconditioning method was not studied sufficiently. The main purpose of our study is to show the effect of preoperative ozone gas on flap viability and to reveal that ozone therapy can be used as a preconditioning method in skin flaps. **Methods:** 40 rats were randomly divided into 4 groups and a caudal-based 11x3 cm modified Mac Farlane flap was elevated for all subjects. At the end of one week, back flaps were removed and evaluated macroscopically and histopathologically. **Results:** In terms of flap viability, all groups were statistically better than the control group not only macroscopically but also hystopathologically. Chronic inflammation scores, amount of granulation tissue and neovascularization were higher in all groups and especially in the preoperative ozone group compared to the control group ($p < 0.001$). **Conclusion:** In our study, it has been shown that ozone mediated pretreatment method has both macroscopically increased flap survival and beneficial effects at histopathological level.

**Keywords:** Skin flap, ozone gas, ozone mediated preconditioning, rectal ozone therapy.

**Özet**

**Giriş:** Flep cerrahisinde birçok ön koşullandırma tekniği vardır. Ancak ozon aracılı ön koşullandırma yöntemi yeterince çalışılmamıştır. Çalışmamızın temel amacı, ameliyat öncesi ozon gazının flep canlılığı üzerindeki etkisini göstermek ve cilt fleplerinde ozon tedavisinin bir ön koşullandırma yöntemi olarak kullanılabileceğini ortaya koymaktır. **Metot:** 40 adet sıçan rastgele 4 gruba ayrıldı ve tüm deneklere kaudal tabanlı 11x3 cm modifiye Mac Farlane flebi kaldırıldı. Bir hafta sonunda sırt flepleri çıkarılarak makroskopik ve histopatolojik olarak değerlendirildi. **Bulgular:** Flep canlılığı açısından, tüm gruplar sadece makroskopik olarak değil aynı zamanda histopatolojik olarak da kontrol grubuna göre istatistiksel olarak daha iyiydi. Kronik inflamasyon skorları, granülasyon dokusu miktarı ve neovaskülarizasyon tüm gruplarda ve özellikle preoperatif ozon grubunda kontrol grubuna göre daha yüksekti ($p<0,001$). **Sonuç:** Çalışmamızda ozon aracılı ön tedavi yönteminin hem makroskopik olarak flep sağkalımını arttırdığı hem de histopatolojik düzeyde faydalı etkileri olduğu gösterilmiştir.

**Anahtar kelimeler:** Cilt flep, ozon gazı, ozon aracılı ön koşullandırma, rektal ozon tedavisi.

## 1. INTRODUCTION

Flap losses are one of the most common complications of this type of surgery because their blood supply are disrupted when they are transferred from the donor site to the recipient site for reconstruction.



✉Abdulkerim OLĞUN, Atatürk University, Faculty of Medicine, Department of Plastic and Reconstructive Surgery, Erzurum,TÜRKİYE.
E-mail: abdulkerimolgun@gmail.com

Therefore, various methods have been studied in order to increase flap viability especially in postoperative period. One of these methods is the delay method.

The delay method, which is also considered as a preconditioning method, is the only realistic method that has been shown to clinically improve flap viability (1). Alternatively non-surgical preconditioning techniques are also available in the literature. Some of these are hyperthermic preconditioning, hypothermic preconditioning, pharmacological preconditioning (2). The common features of the aforementioned preconditioning techniques are the activation of antioxidant systems in order to prevent the negative effects of oxidative stress and ischemia-reperfusion injury on the harvested flap.

Oxidative stress stimulation is the most commonly used method in the activation of antioxidant mechanisms. One of the methods that produce controlled oxidative stress is the application of ozone (3). Oxidative stress resulting from ozone application, increasing the levels of antioxidant enzymes and by vasodilation via receptors on endothelial, increases blood supply to the skin flap removed and thereby increase the flap (4). Ozone, which activates the antioxidant mechanisms and thus stimulates the blood-raising effect desired in the skin flap, is 1.6 times more dense than oxygen and 10 times more soluble in.

Ozone acting in liquid environments, like all gases, follows Henry's law, its solubility depends on the pressure, concentration and temperature of the gas (5).

The ozonization process almost always produces oxidative stress, whether in the blood, intervertebral disc or within the muscle. This oxidative stress creates beneficial effects by stimulating antioxidant mechanisms (5). For this purpose, we applied our ozone-mediated preconditioning study in the Mac Farlane flap operation in Wistar Rats, which we think is not sufficiently studied between the preconditioning techniques applied in flap surgery.

## 2. METHODS

Our study was initiated with the approval of BEU Animal Experiments Local Ethics Committee with the meeting dated 07/11/2012 and numbered 2012/09 from Bülent Ecevit University (BEÜ) Animal Experiments Ethics Committee. The study was carried out in BEU Faculty of Medicine Animal Experiments Research Laboratory.

### 2.1. The subjects

40 female Wistar rats weighing 250-300 g were randomly divided into four groups. Groups; Group 1 (control group), Group 2 (preoperative rectal ozone group), Group 3 (preoperative + postoperative rectal ozone group) and Group 4 (postoperative rectal ozone group) was named.

**Rectal ozone therapy:** This method can be applied to rectal gas delivery in patients with vascular problems or in children (5). 10-25 µg ozone / ml oxygen-ozone mixture 150-300 ml volume in adults, 10-20 µg / ml ozone / ml oxygen-ozone mixture with a volume of 10-30 ml in children is given slowly by means of a rectal tube (6). Especially in rectal ozone therapy, ozone gas rapidly dissolves in the aqueous environment in the rectum lumen. Soluble ozone gas reacts with the biomolecules in the rectum wall to form lipid oxidation products (LOPs). These products are introduced into the circulation through the lymphatic and venous capillaries, passing through the muscularis mucosa. In this case, systemic efficacy of rectal ozone therapy is equivalent to major autohemotherapy. However, in order to achieve this level of activity, it is necessary to prevent the ozone gas from contacting with the faeces. The most effective way to achieve this is absolute bowel cleansing (7). Based on this theory, the dose of rectal ozone was determined and given at a dose of 0.5 mg / kg.

### 2.2. Groups

**Group 1 (n = 10): Control group**

No preoperative and postoperative procedures were performed and flap viability was monitored without exposure to any agent.

**Group 2 (n = 10): Preoperative rectal ozone group**

The rats were treated with rectal route at a dose of 0.5 mg / kg (O3 / O2) in the ozone reactor at a rate of 10 mg / L (O3 / O2) for 4 days preoperatively. Before the ozone application, all rats were stimulated with metal cannula rectally and defecation was provided. After defecation, ozone gas was applied in the appropriate dose (0.5 mg / kg O3 / O2) rectally via metal cannula and ozone gas was passed to the systemic circulation.

**Group 3 (n = 10): Preoperative and postoperative rectal ozone group**

The rats were treated with rectal route at a dose of 0.5 mg / kg (O3 / O2) in the ozone reactor at a rate of 10 mg / L (O3 / O2) for 4 days preoperatively and 7 days postoperatively. Before the ozone application, all rats were stimulated with metal cannula rectally and defecation was provided. After defecation, ozone gas was applied in the appropriate dose (0.5 mg / kg O3 / O2) rectally via metal cannula and ozone gas was passed to the systemic circulation.

**Group 4 (n = 10): Postoperative rectal ozone group**

The rats were treated with rectal route at a dose of 0.5 mg / kg (O3 / O2) in the ozone reactor at a rate of 10 mg / L (O3 / O2) for 7 days postoperatively. Before the ozone application, all rats were stimulated with metal cannula rectally and defecation was provided. After defecation, ozone gas was applied in the appropriate dose (0.5 mg / kg O3 / O2) rectally via metal cannula and ozone gas was passed to the systemic circulation.
All rats were sacrificed at the end of postoperative 7th day. Following surface measurements, the flaps were excised and stored in appropriate containers for pathological examination.

### 2.3. The Experiment

**Preparation before surgery**
Animals were injected intraperitoneally 40 mg / kg pentothal sodium (Ie Ulugay, Turkey) and 8 mg / kg im. Ketamine (Pfiser, Turkey). After the depth of anesthesia was observed with jaw and skeletal muscle tone, back areas of all subjects were shaved with the help of electric razor.

**Elevation of flaps**
Preoperative anesthesia depth was checked again. The modified McFarlane flap was caudal-based, with the size of 3 × 11 cm in the rat's back as a random flap, including pannicus carnosus. After hemostasis, flap were sutured with 3/0 silk to it's former bed (Figure 1).

**Euthanasia process and taking samples**
On postoperative day 7, cervical dislocation was performed under anesthesia after pentothal injection at a dose of 40 mg / kg intraperitoneally to all animals. The necrosis areas were measured after sacrification and digital photographs were taken. The flaps were then excised and fixed in 10% neutral formalin solution for pathological examination, in separate containers.

**Surface Area Measurements**
Before the flaps were excised on the seventh day, necrosis flap areas were drawn and measured by the Visitrak digital measuring device (Smith and Nephew) following euthanasia.

**Histopathological Evaluation Method**
In this study, excisional biopsy was performed removing flaps totally (4 groups, 40 samples). Then, the flaps were divided into strips containing 2 mm wide including distal and proximal ends, and fixed with 10% neutral formalin solution. The paraffin blocks were subsequently buried. Microtome knife sections were taken from the samples in paraffin blocks. Each sample was routinely stained with hematoxylin & eosin dye. The prepared preparations were examined by the same pathologist under the light microscope with x40, x100, x200 magnifications.

In pathological examination, wound healing parameters such as acute inflammation, chronic inflammation, amount of granulation tissue, fibroblast maturation, collagen deposits, reepithelization, neovascularization and ulcer depth were evaluated. The scoring of the parameters was determined based on the article examining the histological characteristic of the healing of vaginal and abdominal surgery wounds (8). (Table 1).

### 2.4. Statistical analysis
Statistical analysis was performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA) program. Descriptive statistics for numerical variables were expressed as median (minimum-maximum) and categorical datas was expressed as number and percentage (%). The categorical variables (acute inflammation, chronic inflammation, granulation amount, fibroblast maturation in granulation tissue, collagen deposition, reepithelization, neovascularization and ulcer depth) were evaluated by chi-square test. The difference between the groups in terms of flap viability ratio was examined by Kruskal-Wallis variance analysis. When Kruskal Wallis variance analysis was found significant, group pairs were compared with Bonferroni Corrected Mann-Whitney U test. For results, $p < 0.05$ was considered statistically significant.

## 3. RESULTS

### 3.1. Macroscopic Findings
At the end of the seventh day, the necrosis of the flaps of all rats in the groups was measured using the rat visitrak digital grup device. Then, all the necrosis part of the flaps of each groups were documanted. All the other three groups had better survival rate in a comparison with control (Table 2).

### 3.2. Histopathological Evaluation Findings
Mixt type inflammatory response was observed in all groups with varying degrees of neutrophil leukocytes. There was no statistically significant difference between the groups in terms of acute inflammation ($p > 0.05$).
The chronic inflammatory response, which is dominated by lymphocytes, plasma cells and histiocytes, was observed in all groups, and chronic inflammation scores were significantly higher in all groups than in the control group. The amount of granulation tissue was higher in all groups compared

to the control group and this difference was statistically significant ($p < 0.001$). It was noted that granulation tissue developed better in the preoperative ozone group than in the other groups.

When the groups were examined for fibroblast maturation, it was observed that fibroblasts were more dense and morphologically mature in the preoperative ozone group in parallel with the development of granulation tissue and this difference was statistically significant ($p = 0.036$) (Figure 2). The amount of collagen was significantly more intense in the preoperative + postoperative ozone group than in the other groups ($p = 0.007$) .

Although no statistically significant difference was found between the groups in terms of reepithelization ($p = 0.083$), 20% of the preoperative and preoperative + postoperative ozone-receiving groups had minimal-immature reepithelization and 80% partial-immature reepithelialization so it was observed that reepithelization was better in these groups (Figure 2). When examined in terms of neovascularization, it was found that all groups were significantly different from each other. In pairwise comparisons, the number of newly formed vessels was significantly higher in the preoperative ozone group than in the other groups ($p < 0.001$) (Figure 2). Although, there was no significant difference between the groups in terms of the presence and depth of ulcer ($p=0.313$), in the control group, the presence of deep ulcers with subcutaneous tissue and muscle layer was observed in 40% of the samples and this ratio ranged from 10-20% in the other groups. As a result, granulation tissue, fibroblast maturation, reepithelization, neovascularization, and collagen deposition were found to be significantly higher in groups preconditioned with ozone (preoperative ozone group and preoperative +postoperative ozone group) comparing non preconditioned groups (control group and postoperative ozone groups) and ulcer depth was found to be more superficial in preconditioned groups.

**Figure 1:** After hemostasis, flap were sutured with 3/0 silk to it's former bed

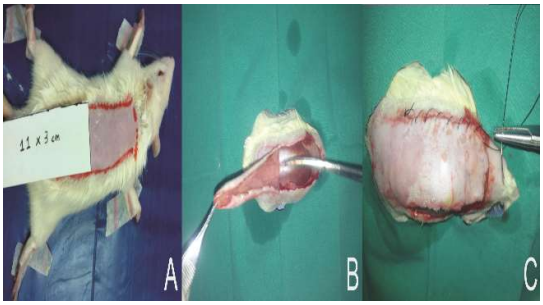
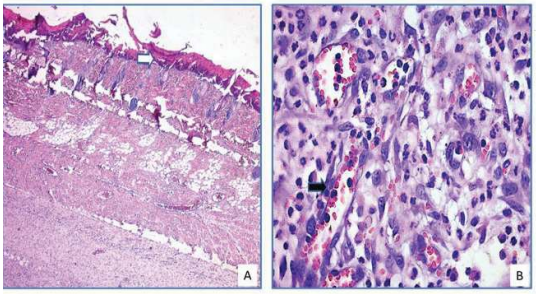



**Figure 2:** Light microscopic views of the subcutaneous tissue samples taken from the preoperative ozone group (A-B, H & E).




A) Epidermis limited necrosis (white arrow) and subcutaneous tissue granulation tissue is observed (A, x40). B) Immature fibroblasts in granulation tissue and angiogenic activity increase (black arrow) are noted (B, x1000).

**Table 1:** Wound-healing histologic scoring system. HPF, high-power field.

| **Variable** | **Score** | | | |
|---|---|---|---|---|
| | **0** | **1** | **2** | **3** |
| **Acute inflammation** | None | Scant | Moderate | Abundant |
| **Chronic inflammation** | None | Scant | Moderate | Abundant |
| **Granulation tissue amount** | None | Scant | Moderate | Abundant |
| **Fibroblast maturation** | Immature | Mild maturation | Moderate maturation | Fully matured |
| **Collagen deposition** | None | Scant | Moderate | Abundant |

| | | | | |
|---|---|---|---|---|
| **Reepithelialization** | None | Partial | Complete but immature or thin | Complete and mature |
| **Neovascularization** | None | Up to five vessels per HPF | 6–10 vessels per HPF | More than 10 vessels per HPF |
| **Ulcer depth** | None | Loss of surface epithelium | Dermal tissue limited ulcer | Ulcer of the muscular layer. |

**Table 2:** Statistical documentation of flap viability rates. It is noteworthy that the flap survival rates of the group that received the preoperative ozone group were high

| **Groups** | **Group 1 (Control) (n:10)** | **Group 2 (Postoperative ozone group) (n:10)** | **Group 3 (Preoperative + Postoperative ozone group) (n:10)** | **Group 4 (Preoperative ozone group) (n:10)** | **p** |
|---|---|---|---|---|---|
| **Mean± SD** | 49,09±25,98 | 86,04±7,03 | 90,56±6,90 | 96,09±3,12 | <0,05 |
| **Average living area of flaps(%)** | 49,09 | 86,04 | 90,56 | 96,09 | |
| **The lowest measured living area of flaps(%)** | 2.8 | 90,0 | 79,4 | 78,8 | |
| **The highest measured living area of flaps(%)** | 71,3 | 99,4 | 98,8 | 97,9 | |

Values are expressed mean ± standart deviation or number.

## 4. DISCUSSION

Studies have shown that the blood flow in the skin is mainly regulated at the arteriolar level and that the blood flow in the pre-capillary sphincter, arteriole and arteriovenous anastomosis is regulated with sympathetic effect and this sympathetic stimulation leads to the contraction of the precapillary sphincter and direct the blood to the arteriovenous anastomosis (9). This sympathetic vascular smooth muscle contraction is counterbalanced by beta adrenergic vasodilatation (10). Flap elevation leads to a lack of blood supply to the flap and, consequently, to a sympathetic activation triggered by this condition, resulting in a vicious cycle. Immediately after this event, the release of vasoconstriction with epinephrine, norepinephrine, serotonin, PGI2, PGF2α and thromboxan A2 is further increased and the blood flow is decreased. These mediators lose their activity in about 12-24 hours. However, if the ischemia in the flap continues for more than 6 hours and if the flap blood supply is provied again, reperfusion injury begins to be seen (11). While the energy needed in ischemic conditions is tried to be created under anaerobic conditions, a number of reactive oxygen radicals begin to emerge by anaerobic glycosylation. These oxygen radicals cause structural changes in the cell membrane with lipid peroxidizing effects and thus the activation of the inflammatory process and adhesion of the leukocytes, resulting in endothelial damage that would ultimately lead to microvascular occlusion (12). In the endothelial damage, not only microvascular occlusion occurs, but also edema and micro-bleedings occur in the tissue by increasing the permeability of the vessel (13). Therefore, the pathophysiology of ischemic necrosis induced by flap elevation is associated with reactive oxygen radicals induced by oxidative stress and the role of lipid peroxidation formed by these radicals. Therefore, the preconditioning principle is to cope with this oxidative stress through anti-oxidant mechanisms that will activate the oxidative stress environment created before the flap elevation. In our study based on these principles, we performed rectal ozone administration in group 2 and group 3 subjects with preconditioning models created with ozone-mediated oxidative stress for 4 days before flap elevation and last administration of rectal ozone preoperatively was performed 12 hours before surgery. We predicted that the oxidative effect caused by ozone therapy, which we applied 12 hours

prior to surgery, would disappear within the time period mentioned above, and that the anti-oxidant and other mechanisms that will be activated after the flap elevation will continue to have beneficial effects. In our pre-conditioning model, we aimed to activate the mechanisms that can cope with oxidative stress due to ischemia caused by flap elevation. Several studies have been carried out to reduce the oxidative load of inflammatory mediators and cells created by flap elevation and positive results have been obtained from the studies. Steroids are one of these agents. It has been reported that dexamethasone inhibits the development of necrosis by reducing the production of free radicals, by attenuating calcium-free inducible nitric acid synthase activity in leukocyte, smooth muscle and endothelial cells, and by reducing neutrophil accumulation and edema formation (14) (15) (16). In these studies, dexamethasone has been shown to reduce myeloperoxidase activity, which is a marker of neutrophil accumulation (17).

One of the theses proposed to improve flap survival is to reveal the mediators that stimulate angiogenesis by using some preconditioning methods. Application of VEGF to the donor site 7 days before the flap removal and reported that the VEGF pretreated group had better results in flap survival compared to the VEGF injected group after flap elevation (2, 18).

Therefore, there are studies suggesting that the aforementioned growth factors can be achieved by the ozone mediated activation we use in our study (19). Considering the beneficial effects of growth factors on flap survival and studies demonstrating that ozone therapy increases growth factor synthesis, it is clear that the results of our study are in fact not a coincidence.

Ozone is a molecule that reacts with organic substances in the organism and oxidizes them.

It has also been reported that ozone increases the level of antioxidant enzymes such as superoxide dismutase (SOD), GSH-peroxidase (GSH-Px), GSH-reductase (GSH-Rd) and catalase (CAT) in combination with reactive oxygen molecules (20). With ozone therapy, vasodilatation and antioxidant activity have been achieved to increase blood flow to maintain flap viability (21).

In an experimental study , it is reported that the ozone pre-treatment given by colorectal route inhibited CCl4-mediated hepatotoxicity and activated antioxidant systems in rats (22). It has been reported that intrarectal ozone therapy protects tissue from ischemia-reperfusion injury by releasing adenosine, blocking the xanthine / xanthine oxidase pathway and suppressing ROS production after reperfusion (21, 22). It has also been shown that intrarectal ozone therapy activates endogenous antioxidant systems in renal and liver ischemic reperfusion models and suppresses ROS production (22).

In the data obtained in our study, it was seen that the histopathological data obtained in group 2 and group 3, which were preconditioned with rectal ozone, were better than group 1 and group 4 which were not pre-treated. Some of these histopathological data are chronic inflammation, amount of granulation, fibroblast maturation, and amounts of neovascularization.Although the numerical data of ulcer depth in the control group were not statistically significant with other treatment groups, we found that the depth of ulcers was higher in the control group without ozone therapy. However, when the outcome parameters were evaluated at the level of statistical significance, only the neovascularization ($p = 0.036$), granulation amount and fibroblast maturation values of the group receiving the pre-operative rectal ozone were significantly higher than the other treatment groups ($p < 0.001$).

In addition, flap survival rates of all groups with rectal ozone significantly higher flap survival rates compared to the control group clearly show the beneficial effects of ozone in flap surgery. The highest rate of flap survival of the preoperative rectal ozone group revealed the beneficial effect of ozone pre-conditioning on flap survival.

The high survival rate of flap survival in postoperative and preoperative + postoperative rectal ozone groups indicates that ozone therapy has improved flap survival in all conditions In our study, the statistically significant increase in neovascularization in the histopathological studies in group 2, which explained the positive effects of ozone mediated preconditioning on angiogenesis.

It is proved that ozone-mediated pre-conditioning, which is non-invasive, inexpensive, easily applicable and effective, compared with other pre-conditioning methods, was evaluated not only in terms of macroscopic results, but also in terms of histopathological parameters. We believe that the positive results of our study, which considers the concept of ozone mediated preconditioning in skin flap surgery and which will lead to the development of this understanding of application, will be among the principles of plastic surgery in a short time.

**Conflict of Interest:** The authors declare that no conflict of interest.

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