# The Effects of Medical Ozone in Rat Heart Exposed to Ischemia-Reperfusion Injury: Experimental Study

## İskemi-Reperfüzyon Hasarına Maruz Kalan Sıçan Kalbinde Medikal Ozonun Etkileri: Deneysel Çalışma

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**ABSTRACT Objective:** In this study, it was intended to investigate the effects of preconditioning with medical ozone on possible reperfusion injury, in which patients undergoing the operation due to coronary artery disease. **Material and Methods:** 37 female mature rats were categorized into 4 groups either as sham-control (Group I), ischemia (Group II), ischemic preconditioning by oxygen (Group III) or by medical ozone (Group IV). Through left thoracotomy, rats were subjected to 25 min of ischemia on left anterior descending (LAD) coronary artery and after that, reperfusion was provided for 75 min. At the end of this period, serum sampling for creatinine-kinase (CK)-MB, Troponin-I, superoxide dismutase (SOD) and malondialdehyde (MDA) and tissue sampling for histopathological examinations were performed. The results of pathological examination were divided into 3 groups according to cellular damage. **Results:** Statistically, there were no significant differences between the groups in case of CK-MB levels and SOD enzymatic activity ($p>0.05$). Nevertheless, Troponin-I was higher in Group III and MDA was meaningfully lower in Group IV in respect to control group ($p=0.005$). Moreover, MDA levels were significantly lower in Group IV, when compared to Group III. With these results, no significant differences were detected between ischemia and ozone group. Although it is important to detect lower MDA levels in ozone group when compared to oxygen group, it is hard to talk about protective effects of ozone just depending on this data. In histopathological examination, medical ozone had beneficial effects on cellular protection when compared to ischemic group; but when the groups were examined, no significant differences could be found between oxygen and ozone groups. **Conclusion:** Although the role of medical ozone on the prevention of reperfusion injury could not be demonstrated clearly, it might have some beneficial effects after biochemical and histopathological evaluation. To be able to show these effects precisely, more studies are needed.

**Key Words:** Ozone; reperfusion injury; ischemic preconditioning

**ÖZET Amaç:** Bu çalışmada, koroner arter hastalığı nedeniyle uygulanan operasyon sonrasında oluşabilecek reperfüzyon hasarının medikal ozonla önkoşullama yapılarak etkisinin araştırılması amaçlanmıştır. **Gereç ve Yöntemler:** 37 adet dişi erişkin sıçan sham-kontrol (Grup I), iskemi (Grup II), oksijenle iskemik önkoşullama yapılan (Grup III) ve medikal ozonla iskemik önkoşullama yapılan gruplar (Grup IV) olarak dörde ayrıldı. Deneklerde sol torakotomi ile sol ön inen arter (LAD)'de 25 dakikalık iskemi uygulandı ve ardından 75 dakika süre ile reperfüzyon sağlandı. Bu süre sonunda kreatinin-kinaz (CK-MB), Troponin-I, süperoksit dismutaz (SOD) ve malondialdehit (MDA) için serum ve histopatolojik inceleme amacıyla doku örneklemesi yapıldı. Patolojik inceleme ile sonuçlar hücresel hasara göre 3 ayrı sınıfa ayrıldı. **Bulgular:** İstatistiksel olarak CK-MB düzeyleri ve SOD enzim aktivitesi açısından anlamlı bir fark yoktu ($p>0.05$). Buna karşın Troponin I Grup III'te anlamlı olarak yüksek, MDA ise Grup IV'te kontrol grubuna göre anlamlı olarak düşük bulundu ($p=0.005$). Ayrıca MDA düzeyi Grup IV'te, Grup III'e göre de anlamlı olarak düşüktü. Bu sonuçlarla iskemi grubu ile ozon grubu arasında ciddi farklılıklar tespit edilememiştir. Oksijen grubu ile kıyaslandığında MDA seviyelerinin anlamlı olarak ozon grubunda daha düşük bulunması önemli olsa da, sadece bu veriyle ozonun koruyucu özelliklerinin olduğunu söylemek güçtür. Histopatolojik incelemede ise medikal ozonun iskemik gruba kıyasla hücre korunması açısından anlamlı olarak olumlu etkileri gözükmektedir. Yalnız burada gruplar kendi içinde incelendiklerinde oksijen ve ozon grubu arasında anlamlı bir fark bulunamamıştır. **Sonuç:** Bu çalışmada medikal ozonun reperfüzyon hasarını engellemedeki rolü açıkça ortaya konamasa da, biyokimyasal ve histopatolojik incelemeler sonucunda bazı yararlı etkileri olduğu gözükmektedir. Bu yararların kanıtlanabilmesi için daha farklı çalışmalara ihtiyaç vardır.

**Anahtar Kelimeler:** Ozon; reperfüzyon hasari; iskemik önkoşullama



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Ozone ($O_3$) is a molecule that is made up of three oxygen atoms. In medical practice, ozone is used as oxygen and ozone ($O_2/O_3$) mixture. With reinfusion of ozonated blood, it has been shown to increase the nitric oxide (NO) levels, decrease hypoxia as a result of vasodilatation in ischemic fields and decrease oxidative stress by superoxide dismutase (SOD) activation and decrease glutathione levels.[1,2] If we could ever compress medical ozone in forms of tablets, pack them in suitable dosage and save them as stable infusion solutions and even sell them as over the counter forms, it might be possible that most of our problems about some of complex diseases be resolved in the future. But, is medical ozone really science, or fiction?

Ischemia-reperfusion (I/R) injury is one of the biggest problems in cardiac surgery. Although there are numerous experimental and clinical studies held by hundreds of chemical materials, this problem has not completely been resolved yet. In recent years, it has been shown that medical ozone had beneficial effects in diabetic foot injuries, decrease claudication and reperfusion injury in peripheral artery diseases.[3,4] Numerous ozonetherapy clinics are in use but their effectiveness is not known and can not be shown by objective scientific data and studies. In this study, we tried to investigate the effects of medical ozone in an experimental I/R model.

## MATERIAL AND METHODS

This study was performed in collaboration with biochemistry and pathology departments after taking permission from local ethics committee using 37 Sprague-Dawley adult female rats (190-236 grams). All animals were subjected to humanitarian behavior as described in Guide for the Care and Use of Laboratory Animals. The subjects were divided into four groups either as sham-control (Group I), ischemia (Group II), ischemic preconditioning by oxygen (Group III) or by medical ozone (Group IV). The average weight of the rats were similar (Group I: 218,28±15,42 g; Group II: 219,80±9,99 g; Group III: 217,10±12,13 g and Group IV: 233,00±14,24 g). Rats were anesthetized using ketamine hydrochloride (60 mg/kg, intraperitoneal). After tracheotomy, the rats were connected to ventilator (SAR-830 Ventilator, CWE, Incorporated, Ardmore, PA) by a tracheotomy cannula and respiration was supported by a frequency of 60/min with 400 ml/min room air. Thereafter 50 IU heparine sodium and 1 ml of 0.9% NaCl was injected through tail vein. Through left 4$^{th}$ intercostal space, we reached the heart.

Before the standard procedures, as a sham-control, we injected 5 ml of free room air intraperitoneally in Group I for 3 days. At the end of 3 days, via left thoracotomy pericardium was opened and the rats were followed-up for 75 min without performing ischemia. At the end of this period, blood samples were taken from the left ventricle, and the heart was excised for tissue sampling. The blood samples were centrifuged and the serum was conserved at -80 $^0$C. In Group II, 5 ml of free room air was injected intraperitoneally for 3 days but after pericardiectomy, left anterior descending (LAD) artery of the heart was occluded using a mini-bulldog clamp and a 25 min of ischemia was performed. After this period, clamp was removed and transient ischemia was terminated. LAD field was reperfused for 75 min and blood and tissue samples were taken as explained before. In Group III, 5 ml of 100% oxygen was injected intraperitoneally for 3 days. Using the same procedures, 25 min of ischemia was performed after clamping of LAD and the rats were subjected to reperfusion for 75 min. At the end, blood and tissue sampling was performed as explained before. In Group IV, ischemic preconditioning was performed using intraperitoneal medical ozone injection for 3 days. Medical ozone was generated using portable ozone generator (Dr. Hánsler Ozonosan, photonic). Ozone concentration was adjusted as 50 μg/ml. Generated ozone and oxygen mixture was immediately injected (1 mg/kg of ozone dose, in 5 ml of mixture) in order to prevent destruction of ozone. Thereafter, 25 min of ischemia was performed after clamping of LAD and the rats were subjected to reperfusion for 75 min. Then, the rats were sacrificed after blood and tissue sampling in a routine manner.

All blood samples were centrifuged and the serum was conserved at -80$^0$C and pathological specimens were kept in 10% formaldehyde solution after labeling. At the end of the study, serum CK-MB, Troponin-I (Tn-I), SOD and MDA enzyme activities were studied. Ischemia-reperfusion injury was assessed using histopathological evaluation under light microscopy.

CK-MB and Tn-I levels were measured using chemiluminescent method by Immulite 2000 (Immulite, Siemens, Germany) commercial kit in hormone analyzer. CK-MB concentrations were given as ng/ml. To measure SOD activity, a SOD activity kit (Cayman Superoxide Dismutase Assay Kit, Cayman Chemical Company, USA) was used and enzyme activity was given as U/mL. MDA levels were measured by establishing the color of tiobarbituric acid reacting with MDA at 532 nm and given as nmol/ml.

Histopathological evaluation was performed by a pathologist blinded to groups. After preparation of the cardiac tissues, they were stained by hematoxylene-eosine (H&E) and examined under light microscopy. Histomorphological grading scale was shown in Table 1.

Data from the study were analyzed in computer using a programme (SPSS for Windows v.16.0, SPSS Inc., Chicago, Illinois, USA). First, the data was analyzed for normal distribution and multi-groups representing normal distribution were compared using one-way variant analyses (One-way ANOVA) and then Tukey's post-hoc parametric tests and coupled groups were using Student's t-test. Data of not representing normal distribution, histopathological damage of the heart and coupled comparison of each group by control group were performed by non-parametric Mann-Whitney U test. Any p value of <0.05 was regarded as statistically significant.

**TABLE 1:** Histomorphological grading scale of specimens.

| Grade | Histomorphological features |
|---|---|
| 0 | Normal histomorphology without any cardiac damage |
| 1 | Cardiac muscles with striation loss and cytoplasmic vacuolization, intercellular lymphocytic infiltration and edema |
| 2 | Partial necrotic muscle fibers |

**TABLE 2:** The levels of creatinine-kinase MB (CK-MB), troponin-I and superoxide dismutase (SOD) enzyme activity in research groups.

| | Group I (n=7) | Group II (n=10) | Group III (n=10) | Group IV (n=10) |
|---|---|---|---|---|
| CK-MB (ng/ml) | 0.66±0.25 | 0.59±0.25 | 0.55±0.18 | 0.89±1.01 |
| Troponin I (ng/ml) | 0.19±0.01 | 0.24±0.06 | 0.25±0.05* | 0.26±0.01 |
| SOD activity (U/ml) | 4.01±0.76 | 3.50±0.30 | 3.50±0.18 | 4.35±0.24 |
| MDA (nmol/l) | 3.96±0.30 | 3.71±0.34 | 3.57±0.14 | 3.14±0.14*# |

Data were given as mean ± standard error. n= number of subjects in each group, *p< 0.05, when compared to Group I, #p< 0.05, when compared to Group III.

## RESULTS

All subjects completed the study protocol without any problem and sacrificed by removal of the cardiac tissue for tissue sampling. CK-MB, Tn-I and MDA levels and SOD enzyme activities were shown in Table 2. Between the groups, there were no statistically significant differences in CK-MB levels and SOD enzyme activity (p>0.05). However, Tn-I was significantly higher in Group-III and MDA was lower when compared to control group (p=0.005). Also, MDA levels were statistically lower in Group IV, when compared to Group III.

In MDA binary group comparisons, the results for Groups I and II was p=0.632; for Groups I and III was p=0.20; for Groups I and IV was p=0.015 (p<0.05); for Groups II and III was p=0.699; for Groups II and IV was p=0.140 and for Groups III and IV was p=0.043 (p<0.05). In explanation, we were able to find statistically significant differences between the control and ozone groups and between the oxygen and ozone groups.

In our study, we could not detect any differences between the ischemia and medical ozone groups biochemically. Although it may be considered to be important to find lower MDA levels in ozone group when compared to oxygen group, it is hard to mention ozone's protective effect just relying on one parameter.

### HISTOPATHOLOGIC EVALUATIONS

After being stained with H&E, tissue samples were examined under light microscopy with x100 and x200 magnification. Grade 0 represents heart muscles with normal histomorphological features (Figure 1). Grade 1 injury represents muscle fibers with basically striation loss, cytoplasmic vacuolization, intercellular lymphocytic infiltration and intercellular edema (Figure 2). Finally, Grade 2 injury represents characteristic partial necrotic muscle fibers (Figure 3).

Histopathological evaluation shows significant increase of injury in all ischemia-reperfusion groups when compared to control group ($p<0.05$), meaning that we could create successful I/R injury model. When we compared Group I and II in case of injury grades, we found $p<0.001$ ($p<0.05$); in Groups I and III, $p=0.014$ ($p<0.05$); in Groups I and IV, $p=0.019$ ($p<0.05$); in Groups II and III, $p=0.052$; in Groups II and IV, $p=0.019$ ($p<0.05$) and in Groups III and IV, $p=0.739$.

These results showed us that we could generate successful ischemia model during this study. Combining histopathological and biochemical parameters, since cellular damage could be seen in such a small period, pathological changes became an important determinant. After cellular evaluation, medical ozone seemed to have protective effects when compared to ischemia group. But in dual examination, we couldn't detect any differences between the oxygen and ozone groups. However detecting no difference between oxygen and ozone groups partly limits our hypothesis, since whether this protection was only owing to ozone or not.

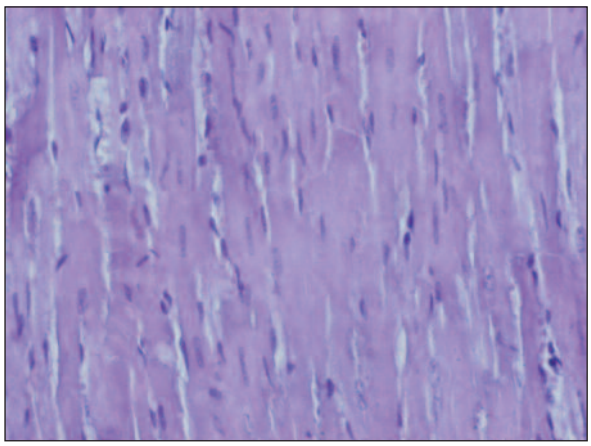

**FIGURE 1:** Myocytes with grade 0 properties; normal histomorphology (HE, x100).
HE: Hematoxylene and eosine stain.
(See for colored form http://cardivascular.turkiyeklinikleri.com/)

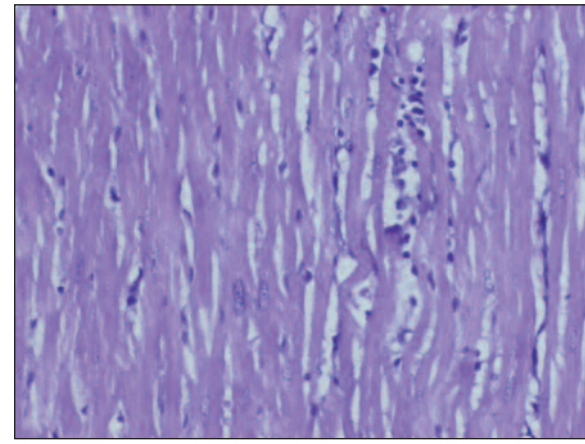

**FIGURE 2:** Myocytes with grade 1 injury; striation loss, cytoplasmic vacuolization, intercellular lymphocytic infiltration and edema of myocytes (HE, x100).
HE: Hematoxylene and eosine stain.
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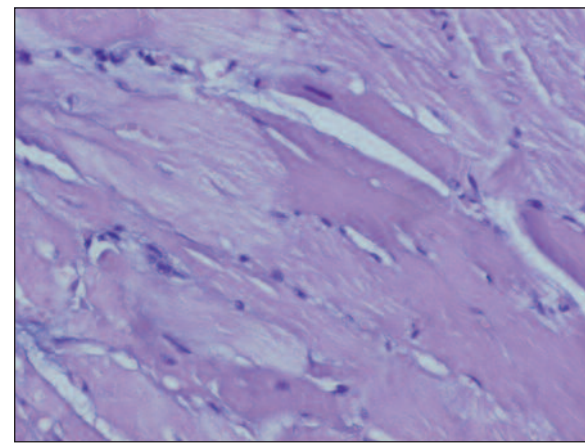

**FIGURE 3:** Myocytes with grade 2 injury; partial necrotic muscle fibers (HE, x200).
HE: Hematoxylene and eosine stain.
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## DISCUSSION

Today, one of the most important topics that have been discussed is myocardial protection. Despite numerous studies, there is no consensus for optimal myocardial protection technique and surgical

decision is made with the guidance of past medical researches and surgeon's personal practice.

In medical practice, ozone is used as a mixture of $O_2/O_3$. Medical ozone is mainly used in neurology, orthopedics, internal medicine, sports medicine and endocrinology. Ozone can be accepted as a pro-drug. Antioxidant enzymes, NO pathway and other cellular activities could be regulated by low doses of ozone.[5] Controlled ozone use could decrease damage induced by reactive oxygen species by oxidative preconditioning or adaptation to oxidative stress.[2] Sanchez and colleagues showed that ozone facilitated glycemic control by decreasing hyperglycemia, increased insulin sensitivity and had beneficial effects on wound healing in neuroinfectious diabetic foot patients.[3] In another experiment with rats, controlled ozone helped glycemic control and prevented damage generated by reactive oxygen species.[6]

Oxidative preconditioning with ozone may stimulate endogenous antioxidant systems against liver damage by creating medium sized oxidative stress. Protective mechanisms are provided by protein synthesis and ozone treatment protects mitochondrial functions and cellular balance.[7] Calunga and colleagues showed that ozone therapy increased antioxidant enzyme activity and protected kidneys against reactive oxygen species in rats subjected to warm ischemia.[8] In another study, autohemotherapy with ozone increased walking distance and decreased subjective clinical findings of ischemia in dialysis patients with peripheral arteriel disease.[4] Medical ozone use also increased ischemic ulcer healing and their pain resolved in a great extent.[9] Medical ozone is also shown to be effective for acute and chronic painful intraarticular injections, acute and chronic joint illnesses including knee and shoulder disorders and for antiaging purposes in anesthesia and algology.[10] Altinel and colleagues showed that the therapeutic effect of medical ozone is more pronounced than that of hyperbaric oxygen therapy in acute distal colitis.[11] Its possible effect is by means of decreasing inflammation, edema, and oxidative stress. In limited studies performed by heart, Merin O. and colleagues showed that ozone use in isolated rat heart decreased reperfusion damage.[12]

In our study an experimental model was designed in order to demonstrate a patient taken into cardiac operation for revascularization and show whether ozone was protective in reperfused heart tissue. In this study unchanged levels of CK-MB and Tn-I could be explained by a time necessity of at least 4 hours; but histopathological examination of cardiac tissue samples showed us that we created successful ischemia. Time period for ischemia (25 min) might be considered enough both for arising tissue damage after reperfusion and also show similarities with cardioplegia intervals in cardiac surgery. The main difference was hypothermic surgical conditions. Perhaps we would get better results if we could have generated hypothermic conditions. Another important time factor in this model was to excise the heart after 75 min of reperfusion and made histopathological examination, which also simulated 3-4 coronary arterial bypass time and provide reperfusion. In this study we detected significant differences between control and the ozone group in MDA. Our expectations were to detect protective effects in ozone group without any change in control group but showed significant differences when compared to ischemia group. The only result supporting our hypothesis was protective effect of ozone against reperfusion when compared to oxygen group. This can be explained by not providing ideal myocardial protection in all ischemia performed groups (Groups II-IV) as a lack of afore mentioned myocardial protection techniques; but histopathological examination of cardiac tissue, which can be defined as early postoperative period, probably gave us more valuable data because of detecting instant cellular changes. As a result of the evaluations, we found beneficial effects in medical ozone group when compared to ischemic group in case of cellular protection. However detecting no difference between oxygen and ozone groups partly limits our hypothesis, since whether this protection was only owing to ozone or not.

## CONCLUSION

As a result, we can say that ozone is easily provided, safe, not expensive material with several treatment potentials. In most countries several ozone therapy clinics are in use but their effects are not served scientifically although subjective and objective effects are widely known in certain fields. So, is medical ozone science, or fiction?

Our study was one of the first studies made in cardiac field. Although we could not definitely show its role in preventing reperfusion injury, we found some beneficial effects histologically, and to some extent biochemically. Owing to literature, just like different systems, medical ozone might have beneficial effects in myocardial protection; but in order to demonstrate these, perhaps we should design different models with better myocardial protection, longer follow-up periods after reperfusion and provide longer myocardial vitality. If its effectiveness could clearly be shown in human studies, it might become an alternative treatment option in cardiac surgery or percutaneous coronary interventions in order to decrease myocardial damage after reperfusion and increase operative success rates and save lives.

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