Comparison of Melatonin and Ozone in the Prevention of Reperfusion Injury Following Unilateral Testicular Torsion in Rats

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OBJECTIVE
To compare the efficacy of ozone with melatonin, shown as the most powerful antioxidant in attenuation of testicular ischemia/reperfusion injury, in an experimental rat model of testicular torsion/detorsion.

METHODS
Twenty-four male Wistar rats were divided into 4 groups: sham-operated, torsion/detorsion, torsion/detorsion plus melatonin, and torsion/detorsion plus ozone. Melatonin (10 mg/kg) and ozone (4 mg/kg) were intraperitoneally injected daily beginning 15 minutes before detorsion for the following 7 days. At the seventh day, blood and tissue samples were obtained. Johnsen score, malondialdehyde, inhibin B, glutathione plasma total sulfhydryl group (RSH) levels, and total nitric oxide were studied.

RESULTS
Torsion/detorsion caused increase in tissue malondialdehyde and total nitric oxide along with a decrease in Johnsen score, tissue and plasma inhibin B, RSH, and glutathione levels. Melatonin prevented the rise in malondialdehyde and total nitric oxide levels and improved Johnsen score, tissue and plasma inhibin B, and tissue glutathione levels, along with a decrease in plasma RSH level. Ozone showed similar results except for the total nitric oxide level. Concomitantly, in contralateral testis, melatonin and ozone induced similar changes for Johnsen score, malondialdehyde, and inhibin B (not significant) and in glutathione (significant). Melatonin decreased the total nitric oxide level in both testes and ozone increased the same parameter.

CONCLUSION
On different pathways, ozone was comparable with melatonin in the amelioration of ischemia/reperfusion injury. Protective effects of ozone were associated with nitrous oxide. The potential for ozone as a treatment for torsion/detorsion therefore deserves to be further elucidated.

Torsion of the spermatic cord is a urologic emergency characterized by tissue hypoxia and eventually by necrosis of germinal cells that give rise to subfertility or infertility. Testicular salvage requires early reestablishment of blood flow. Although reperfusion is essential for the survival of ischemic tissue, reperfusion injury occurs because of the generation of reactive oxygen species (ROS) on re-establishment of blood flow. Although the basic pathologic mechanism underlying ischemia-reperfusion injury (IRI) of the testis has not been completely understood, this oxidative stress is characterized by an imbalance between ROS and the cellular antioxidative system. These ROS react with any biochemical component of the cell, particularly lipids in the cell membranes. Lipid peroxidation leads cellular injury ranging from increased membrane permeability to cell lysis.

An otherwise normal contralateral testis may also be damaged by unilateral torsion. However, controversial results have been obtained from several studies carried out on experimental animals. The different experimental and animal models used might be the causes of this discrepancy. Also, the pathogenesis of the contralateral testicular damage still remains poorly understood.

The current surgical treatment alone is inadequate and adjunct pharmacotherapy in an attempt to ameliorate reperfusion injury and contralateral testicular damage is needed. Several studies revealed that melatonin was the most powerful. It is a potent antioxidant with the features of being a free radical scavenger promoting the formation of antioxidant enzymes and inhibiting the lipids membrane peroxidation.
Ozone has been investigated as a therapeutic agent for the treatment of different physiopathologic events mediated by ROS.\textsuperscript{10-12} Judicious controlled ozone administration was able to stimulate the endogenous antioxidant defense systems and could prepare the host to face IRI.\textsuperscript{11-13} This phenomenon was called ozone oxidative preconditioning. It was reported as a simple and harmless method that provides a new tool to protect organs from IRI.\textsuperscript{13} The aim of this study was to evaluate the effects of ozone in testicular function and morphology in an experimental model of unilateral testicular torsion. Furthermore, the biochemical parameters characterizing the oxidative stress were evaluated under ozone treatment and compared with those obtained with melatonin, which is recognized as a potent antioxidant agent.

**MATERIAL AND METHODS**

**Animals**

Twenty-four healthy 3-month-old male Wistar rats weighing between 150 and 200 g were used. The rats were housed in a temperature of 24 ± 3°C and 12-hour light-dark cycle. The animals were fed with standard pellet diet and water ad libitum. A 7-day period of acclimatization was used.

**Treatment Schedule**

Melatonin ([N-acetyl-5-methoxytryptamine] M-250 Lot 25HO904, Sigma, St. Louis, MO, 10 mg/kg) was dissolved in ethanol and further diluted in isotonic saline to a final concentration of 1% ethanol and injected intraperitoneally (ip).

Ozone was generated by ozonator equipment (Medozon Compact-Hab Herrmann apparatabau, GmbH, Germany). Ozone was obtained from medical-grade oxygen by means of a silent electric discharge, representing about 3% of the ozone/oxygen gas mixture. The ozone concentration was measured by using a UV spectrophotometer at 254 nm. The ozone concentration in the ozone/oxygen mixture was 50 µg/mL. By knowing the body weight of the rat, the ozone dose was calculated as 4 mg/kg and administered ip.

**Experimental Protocol**

The experimental protocol was approved by the Ethical Committee on Animal Research at our institution. The rats were randomly allocated into 4 groups consisting of 6 rats each:

- **Group S** (sham): to determine the effect of the sham operation;
- **Group TD** (torsion and detorsion): to determine the effect of testicular torsion and detorsion;
- **Group M** (torsion/melatonin treatment/detorsion): to determine the effect of melatonin; and
- **Group Oz** (torsion/ozone treatment/detorsion): to determine the effect of ozone.

All surgical procedures were performed using a sterile technique under anesthesia with 50 mg/kg ketamine and 45 mg/kg xylazine (Ketalar and Citanest, 2%, Eczacıbaşı, Turkey) intramuscularly. A left inguinoscrotal incision was performed. Unilateral testicular torsion was created by rotating the left testis 720° in a clockwise direction and fixed within hemiscrotum with a 4/0 atraumatic silk suture. Torsion was maintained for 6 hours. After 6 hours, the spermatic cord was detorsed. After each surgical intervention the incision was closed and rats were returned to their cages under autoregulating thermal light to maintain body temperature at 37°C. Vehicle (1% ethanol in saline), 1 mL melatonin preparation and ozone/oxygen mixture were daily injected ip 15 minutes before detorsion and after daily injection for 7 days in the S and TD groups, and groups M and Oz, respectively. After 7 days, bilateral orchiectomies were performed, and blood from the vena cava inferior and tissue samples were obtained.

**Tissue Homogenization**

Fresh tissues were washed with ice-cold phosphate-buffered saline (PBS) (10 mM Na2HPO4, 10 mM KH2PO4, 0.9 g NaCl/100 mL, pH 7.4) and they were kept at −70°C until assayed.

**Tissue Malondialdehyde**

Malondialdehyde (MDA) levels were measured in the testicular tissue homogenates. MDA levels were measured by high-performance liquid chromatography using the solutions from Reciple (Reciple Chemicals + Instruments, GmbH-Germany). Intra- and interassay coefficients of variation were 3.1% and 2.4%, respectively.

**Inhibin B**

Inhibin B (InhB) assay was performed with an enzyme-linked immunosorbsent assay (ELISA, Diagnostic Systems Laboratories 10-84100, Germany). Standards, controls, and unknown tissue samples were incubated in microtitration wells, which were coated with anti-InhB subunit antibody. The absorbance measured was directly proportional to the concentration of InhB present. Intra- and interassay coefficients of variation were 6.2% and 3.5%, respectively.

**Glutathione**

Glutathione (GSH) levels were determined by a modified Ellman method.\textsuperscript{14} After centrifugation, 0.5 mL of supernatant was added to 2 mL of 0.3 M Na2HPO4, 2H2O solution and 0.2 mL solution of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). After 10-minute incubation at 25°C, the absorbance at 412 nm was measured spectrophotometrically.

**Total Sulfhydryl Group**

In the total sulfhydryl group (RSH), 0.5 mL of each plasma sample was mixed with 1 mL of a solution containing 100 mM Tris-HCl (pH 8.2), 1% sodium dodecyl sulfate and 2 mM EDTA. The mixture was incubated for 5 minutes at 25°C and centrifuged to remove any precipitant. 0.3 mM DTNB was then added to each reaction volume and incubated for 15 minutes at 37°C. The absorbance of each sample was determined at 412 nm.\textsuperscript{14}

**Total Nitric Oxide**

Total nitric oxide (NOx) levels were obtained using an ELISA reader by vanadium chloride/Griess assay. Before NOx determination tissues were homogenized in 5 volumes of PBS and centrifuged at 2000xg for 5 minutes. Then 0.25 mL of 0.3 M NaOH was added to 0.5 mL supernatant. After incubation for 5 minutes at 25°C, 0.25 mL 5% (w/v), ZnSO4 was added for deproteinization. This mixture was then centrifuged for 20 minutes and supernatants were used for the assays.\textsuperscript{15}
Histopathologic Evaluation
The testicular tissues were fixed in Bouin's solution and dehydrated in 96% alcohol at room temperature for 24 hours. After routine tissue processing (Shandon, Excelsior tissue processor, Thermo-Fisher Scientific, Waltham, MA), the tissues were embedded in paraffin. Three 3-μm-thick slides prepared from the upper, lower, and mid portions of the testes, were stained with periodic acid-Schiff (Hotchkiss McManus, Milan, Italy) and hematoxylin-eosin staining. The testicular tissues were evaluated in random order under standard light microscopy by a pathologist in a blinded fashion. Testicular injury and spermatogenesis were graded on histologic sections with Johnsen score (JS), and a minimum 50 tubules were evaluated and each tubule was given a score from 1-10.

Analysis
All statistical analyses were performed using SPSS 16.0 software (SPSS, Inc., IBM, Armonk, NY). Differences among the groups were analyzed by the nonparametric Kruskal-Wallis one-way analysis of variance. Mann-Whitney U test was also used as a post hoc test for multiple comparisons. Significant differences were accepted at P < .05.

RESULTS
Our results demonstrated significant testicular damage in the TD group. For bilateral testes, there were statistically significant differences between the groups regarding all studied parameters (Tables 1 and 2).

MDA Levels
In ipsilateral testis, tissue MDA levels were significantly increased in the TD group compared with the S group. Both melatonin and ozone treatment significantly suppressed MDA level (Table 1). Their therapeutic effects were similar.

In contralateral testis, tissue MDA levels were significantly increased in the TD group compared with the S group, but this increase was less than that in ipsilateral testis (Table 2). This suggested that although the process of torsion may be unilateral, it induced bilateral effects. Both melatonin and ozone improved IRI, but not statistically significantly.

InhB Levels
In ipsilateral testis, tissue InhB levels as a marker of spermatogenesis were significantly decreased in the TD group compared with the S group. Both melatonin and ozone treatment significantly preserved spermatogenesis from IRI with similar effectiveness (Table 1).

In contralateral testis, spermatogenesis was also affected and tissue InhB level was significantly decreased after testicular torsion/detorsion. Both melatonin and ozone treatment significantly improved spermatogenesis to the same extent (Table 2).

After torsion/detorsion, plasma InhB level was significantly decreased in the TD group. Both melatonin and ozone treatment led to a similar significant improvement in plasma InhB level (Table 2).
GSH Levels
Torsion/detorsion caused significant depletion of GSH levels in bilateral testes. Both melatonin and ozone treatment led to significant improvement in tissue GSH levels (Tables 1 and 2).

RSH Levels
Plasma RSH levels were significantly decreased in the TD group than they were in the S group. Melatonin and ozone caused further decrease in the plasma RSH levels, but not statistically significantly.

NOx Levels
In bilateral testes, tissue NOx levels were increased, but not significantly, in the TD group compared with the S group. Significantly, tissue NOx level was decreased in the M group but was increased in the Oz group.

Histopathologic Results
The S group showed evidence of regular seminiferous tubular morphology with normal spermatogenesis. In the TD group, significant decrease in JS was higher in ipsilateral testis than contralateral testis. Melatonin and ozone significantly improved JS with similar success (Tables 1 and 2). In ipsilateral testis, after torsion/detorsion, there was evidence of marked congestion with the presence of interstitial edema, polymorphonuclear leukocytes (PMNLs) infiltration, and areas of focal hemorrhage. Almost all of the tubules showed either only Sertoli cells without germinal cells or germ cell necrosis. Both melatonin- and ozone-treated groups had tubules showing germ cell necrosis besides the most tubules and incomplete maturation arrest up to the level of primary and secondary spermatocyte, with significant rescue of testicular function. Mild to moderate interstitial edema was also detected (Fig. 1). The contralateral testes also showed minimally distorted seminiferous tubular morphology, but most with preserved spermatogenesis (Fig. 2).

COMMENT
The severity of testicular damage is related to the time and degree of torsion and reperfusion time. Testicular torsion/detorsion leads to testicular injury with 2 components: ischemic injury and reperfusion injury. During ischemia, the limited oxygen availability leads to the decrease in adenosine triphosphate (ATP) production, with degradation of ATP to adenosine and then to hypoxanthine. Loss of calcium-pumping activity allows increased intracellular Ca2+ concentration, which in turn led to the activation of the calcium-dependent metabolic activities with membrane damage and proteolytic conversion of xanthine dehydrogenase to xanthine oxidase.

Although reperfusion is essential for the survival of ischemic tissue, reperfusion itself causes additional cellular injury. Reperfusion injury occurs in 2 phases.
first phase occurs immediately after reperfusion, extends for a few hours, and is reversible. This phase is characterized by mitochondrial dysfunction, failure of oxidative phosphorylation, and toxic burst of ROS generation. Superoxide anions are generated by xanthine oxidase in parenchymal cells and PMNLs. Under physiologic conditions, ROS generated during cell metabolism are rapidly scavenged by endogenous antioxidant systems. However, oxidative stress is characterized by an imbalance between overproduced ROS and the antioxidant systems. Overexpressed ROS react with lipids in the cellular and mitochondrial membranes, proteins, and DNA, leading to cellular dysfunction and disruption of membrane integrity. The second phase extends for hours or days and is characterized by irreversible tissue damage and inflammation.

The effect of unilateral torsion on the contralateral testis has been controversial because of the different experimental and animal models used, which failed to produce a reliable testicular IRI. Experimental studies showed that ischemic periods longer than 6-8 hours and 720° rotation of one testis and minimum 24 hours of reperfusion result in meaningful biochemical and histologic changes in both testes. An optimal therapeutic agent should restore blood flow and prevent the formation of ROS as much as possible and neutralize the ROS as early as possible to prevent the reperfusion injury. It must be administered before detorsion to be effective in this critical phase. Among various tested agents, melatonin appeared to be the most powerful in preventing testicular damage after experimental unilateral torsion. Moreover, treatment of melatonin once a day for 7 days was more effective than one-dose melatonin against IRI, both biochemically and histopathologically. Therefore, in the present study, we selected a 6-hour duration of 720° torsion and 7 days of reperfusion to induce significant testicular damage in both testes to evaluate and compare the benefits of melatonin and ozone administered before detorsion.

Melatonin has antiapoptotic and antioxidative effects because it directly scavenges and inhibits generation of ROS, enhancing the production and the activities of the antioxidative enzymes, including GSH, by increasing the levels of their cellular mRNA. Ozone therapy acts as an efficient oxidative stress regulator and can trigger several useful biochemical mechanisms and reactivate the antioxidant system on the basis of the phenomenon of hormesis and together induce an adaptation to oxidative stress.

The mechanisms underlying ozone oxidative preconditioning remain unknown. When human blood is exposed to therapeutic ozone, ozone dissolves in the plasma and reacts immediately with several substrates, mainly polyunsaturated fatty acids and -SH groups, such as GSH, present in several compounds. The reaction generates H$_2$O$_2$ and lipid hydroperoxides at low
concentrations, which may act as cellular signals and activate the cellular cytoprotective signaling pathways, increasing the antioxidant capacity and some enzymes related to the glutathione synthesis against forthcoming oxidative stress, reducing the overproduction of ROS. Ozone exerted potent antiinflammatory and antiapoptotic properties and was able to reduce the ATP depletion and block the formation of xanthine oxidase. As a result, preserved adenosine regulates smooth muscle tone to induce vasodilation and other mechanisms of activation of antioxidant enzymes. Moreover, ozone regulates calcium levels, maintaining its homeostasis by protecting Ca⁺⁺⁺-adenosine triphosphatase activities. In addition, ozonated erythrocytes show an improved glycolysis with an increase of ATP and 2,3-DPG levels, which are able to shift the dissociation curve of HbO₂ to the right, leading to an improved delivery of oxygen in peripheral tissues. In total, ozone affects the balance in favor of vasodilators against vasoconstrictors, which in turn leads to regression of “no-reflow” phenomenon, characterized by failure of reperfusion of ischemic tissue after detorsion.

In our study, increased tissue level of MDA and histologic evidence of hemorrhage between tubules and degeneration of germinative cells were seen, as indicators of oxidative stress, in both ipsilateral and contralateral testes. We showed that melatonin and ozone decreased MDA level with the finding of limited tissue injury.

InhB is a unique testicular product that is not detectable in the sera of orchidectomized men. InhB level is a clinically important marker of Sertoli cell function and the state of spermatogenesis. In the TD group, we found decreased serum and tissue levels of InhB, JS, and increased MDA levels in both testes. This result indicated that torsion/detorsion had an adverse effect on spermatogenesis. Both melatonin and ozone restore spermatogenesis effectively in contralateral testis and in acceptable level in ipsilateral testis.

The measurement of changes in protein sulphydryl and GSH is often used as an index of oxidative stress to biological systems. Some studies have reported that ozone therapy increased GSH levels and decreased plasma sulphydryl. In our study, in agreement with the previous, GSH and RSH levels decreased in the TD group. GSH levels were reversed, but RSH levels continued to drop after ozone therapy. It has been reported that exposure to ozone leads to oxidation of plasma sulphydryls. This may cause a decrease in RSH levels after ozone therapy.

Moreover, in the present study, nitric oxide (NO) levels in both testes were moderately elevated in the TD group, but were significantly decreased in the M group and increased in the Oz group. NO acts locally to regulate the distribution of oxygen, nutrients, and hormones by the testicular vessels. The properties of...
NO depend on which isoform of NOS is activated. Endothelial nitric oxide (eNOS) and neuronal nitric oxide (nNOS) are constitutively expressed in the testis and contribute to the physiologic regulation of vascular tone.\textsuperscript{29} eNOS is expressed in Leydig cells, Sertoli cells, spermatogonia, and spermatocytes, whereas nNOS is distributed to Leydig cells.\textsuperscript{30} No inducible nitric oxide (iNOS) expression is observed in Sertoli, Leydig, and peritubular cells.

The role of NO in IRI is still controversial. NO may have a protective effect in vasodilatation, antiapoptotic action, inhibition of platelet plug formation, and reduction of the inflammatory response.\textsuperscript{20} By contrast, NO rapidly reacts with superoxide, yielding very reactive peroxynitrite, which can induce tissue injury.\textsuperscript{5} In IRI, nNOS expression is unmodified and endogenous NO is synthesized by eNOS and iNOS. IRI may reduce the transcription of eNOS and activate iNOS with the time of reperfusion.\textsuperscript{5,29} After reperfusion, the infiltration of PMNL in the interstitial tissue further expressed iNOS, promoting germ cell death with the predominance of necrosis over apoptosis.\textsuperscript{29} We also realized apparent necrosis in the TD group. However, the role of iNOS in IRI is still controversial. Some recent studies confirmed that eNOS-generated NO played a pivotal protective role in IRI and iNOS-generated NO inhibited IRI.\textsuperscript{13} iNOS deficiency produces unanticipated genetic alterations that render mice more sensitive to IRI. Melatonin exerts its protective effect by decreasing iNOS expression.\textsuperscript{22} However, ozone activates the genes associated to both eNOS and iNOS, which promotes NO formation in the required concentrations for protecting against IRI.

The limitation of this study is that types of NOS and adenosine levels were not analyzed. Further studies with well-designed experimental models are needed to clarify the mechanisms by which ozone exerts its effects.

CONCLUSIONS

This study showed that ozone therapy had benefits in the treatment of testicular torsion with respect to the biochemical and histopathologic parameters. Its effectiveness was comparable with melatonin. Some differences between these approaches, such as the effect on NO, may provide a new insight to treatment of IRI. Therefore, it deserves to be considered as a therapeutic agent in clinical trials aimed to improve the outcome of patients with ischemia-reperfusion injury of the testicle.

References