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Does Ozone Administration Have a Protective Effect Against Cisplatin-induced Histological Changes in Rat Testis?

Cisplatine Bağlı Rat Testisinde Meydana Gelen Histolojik Değişikliklere Karşı Ozon Tedavisinin Koruyucu Bir Etkisi Var mıdır?

İbrahim Aydoğdu[1], Rahmi Gökhan Ekin[2], Pelin Yıldız[3], Semih Lütfi Mirapoğlu[1], Ali Çay[1], Yaren Ece Aydoğdu[4], Hüseyin Kılınçaslan[5], Mehmet Bülent Semerci[6], Yusuf Özlem İlbey[2]

[1]Bezmialem Vakıf University Faculty of Medicine, Department of Pediatric Surgery, İstanbul, Turkiye
[2]University of Health Sciences, İzmir Tepecik Training and Research Hospital, Clinic of Urology, İzmir, Turkiye
[3]Bezmialem Vakıf University Faculty of Medicine, Department of Pathology, İstanbul, Turkiye
[4]Bezmialem Vakıf University Faculty of Medicine, İstanbul, Turkiye
[5]Avrasya Hospital, Clinic of Pediatric Surgery, İstanbul, Turkiye
[6]Ege University Faculty of Medicine, Department of Urology, İzmir, Turkiye

What's known on the subject? and What does the study add?

There is currently no study of the protective effect of ozone treatment against cisplatin-induced testicular damages.

Abstract

Objective: We investigated the protective and therapeutic effects of ozone therapy (OT) on cisplatin (CP)-induced testicular damage.
Materials and Methods: Thirty healthy adult male Wistar rats were divided into five groups consisting of 6 animals each: 1) control, 2) CP, 3) OT, 4) OT + CP and 5) CP + OT groups. Histopathological findings, Johnsen scores, thiobarbituric acid-reactive substances (TBARS), glutathione (GSH), superoxide dismutase (SOD), catalase, and GSH peroxidase (GPx) levels were evaluated.
Results: CP caused a significant decrease in testicular weight and Johnsen score compared to the control group. In addition, TBARS level was significantly higher, whereas GSH, SOD, catalase and GPx levels were significantly lower in the CP group when compared to the control group. Pre- and post-CP OT significantly increased GSH, SOD, catalase and GPx levels and decreased TBARS level. Also, testicular weight and Johnsen score were increased with OT.
Conclusion: The present study showed that OT is protective against CP-induced testicular damage. OT may be beneficial to patients who underwent CP chemotherapy.
Keywords: Ozone therapy, Cisplatin, Chemotherapy, Testicular histopathology, Testis

Öz

Amaç: Bu çalışmada, ozon tedavisinin (OT) cisplatin tedavisine (CT) bağlı gelişen testis hasarını önleyici ya da tedavi edici etkisini araştırdık.
Gereç ve Yöntem: Wistar cinsi 30 sağlıklı yetişkin erkek rat, her biri 6 rattan oluşan 5 gruba ayrıldı: 1) Kontrol, 2) CT, 3) OT, 4) OT + CT ve 5) CT + OT grubu. Histopatolojik bulgular, Johnsen skorları, tiyobarbitürik asit reaktif maddeler (TBARM), glutatyon (G), süperoksit dismutaz (SOD), katalaz, G peroksidaz (GP) düzeyleri değerlendirildi.
Bulgular: CP grubu, kontrol grubuna kıyasla testis ağırlığında ve Johnsen skorunda belirgin bir düşüşe neden oldu. Ayrıca, TBARM düzeyi anlamlı derecede yüksek iken; G, SOD, katalaz ve GP düzeyleri CP grubunda kontrol grubuna göre anlamlı derecede düşük bulundu. CP + OT öncesi ve sonrası GSH, SOD, katalaz ve GP düzeylerini önemli ölçüde artırdı ve TBARM seviyesini düşürdü. Testikül ağırlığı ve Johnsen skoru OT ile arttı.

Correspondence: İbrahim Aydoğdu MD, Bezmialem Vakıf University Faculty of Medicine, Department of Pediatric Surgery, İstanbul, Turkiye
E-mail: draydogdu@yahoo.com **ORCID-ID:** orcid.org/0000-0001-7900-8598
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Sonuç: Bu çalışma, OT'un cisplatine bağlı gelişen testis hasarını önleyici etkisi olduğunu göstermiştir. OT, cisplatin uygulanan hastalarda testis hasarını önlemede yararlı olabilir.

Anahtar Kelimeler: Ozon tedavisi, Cisplatin, Kemoterapi, Testiküler histopatoloji, Testis

Introduction

One of the most effective chemotherapeutic drugs in the treatment of reproductive organs (ovary, testis), bladder, lung, head and neck cancers is cisplatin (CP) (1,2). The curative effect is well known when CP is used alone or in combination with other drugs (3). On the other hand, CP has quite a lot of adverse effects, especially testicular toxicity that affects spermatogenesis, chromosomal abnormalities in spermatozoa and fertility. Due to these unwanted effects, clinical use of CP is limited most of the time (4). In many previous studies, it has been confirmed that CP that induces testicular oxidative stress had both short-term and long-term effects (5). Oxidative stress is usually held responsible for the pathogenesis of testicular damage after CP exposure (6). Therefore, anti-oxidants are widely recognized to guard rapidly dividing testicular cells against damage by CP (3,7).

Antioxidants are the natural defense mechanism against reactive O_2 species (ROS) in most of the organs including the testicle. Oxidative damage occurs when there is a shift in the balance between production of ROS and the antioxidant defense mechanism in favor of ROS (8,9). Ozone is an inorganic molecular colorless gas composed of three O_2 atoms with a significant pungent odor at room temperature (9,10,11). After ozone therapy (OT) administration, it dissolves in biological water and instantly reacts with antioxidant mechanisms. During these fast reactions, ozone is neutralized by activation of antioxidant mechanisms, which are superoxide dismutase (SOD), catalase, and glutathione (GSH) peroxidase (GPx) (11). The stimulation of endogenous antioxidants prepares the host defense against ROS (9,10,11). The other important effects of ozone are immune modulation, neoangiogenesis and increased tissue oxygenation (11).

In the English literature, there is no previous study on the protective effect of ozone against CP-induced testicular injury. In this study, it was aimed to evaluate the effect of OT on testis histopathology before and after CP treatment in an experimental rat model.

Materials and Methods

The compatibility with ethical standards was provided using the experimental protocols for which the approval was officially taken from the local ethics committee on animal trials under the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The approval was taken from Bezmialem Vakıf University Ethics Committee (number: 24.11.2015/237).

Animals

Thirty healthy adult male Wistar rats weighing between 245 and 310 g were used. The rats were kept in well-ventilated plastic cages at a room temperature of 25±3 °C and 12-hour light/dark cycle environment. Ad libitum feeding was applied with appropriate chow to laboratory conditions and potable water. The setting was acclimatized at one-week intervals.

Experimental Design

Thirty rats were randomly divided into 5 groups consisting of 6 animals each:

Group control: the rats received intraperitoneal O_2,

Group OT: the rats were administered intraperitoneal OT to determine the effect of OT on testicular histopathology,

Group CP: the rats received a single dose of 7 mg/kg of CP intraperitoneally to determine the effect of CP on testicular histopathology as described by Beytur et al. (12),

Group CP + OT (CP after OT): the administration of a single dose of 7 mg/kg of CP was followed by intraperitoneal OT in order to determine the effect of OT on the histopathology of testicles of the rats undergoing CP chemotherapy,

Group OT + CP (OT after CP): the rats received intraperitoneal OT and then were administrated a single dose of 7 mg/kg of CP intraperitoneally to determine the effect of pre-CP chemotherapy OT on testicular histopathology.

All experimental procedures were performed under anesthesia by administering intraperitoneal 5 mg/kg xylazine hydrochloride and 50 mg/kg ketamine hydrochloride to the rats, then, the rats were immobilized by fixing them on the 4 corners of a tray.

Ozone Therapy

The ozone-oxygen mixture was produced by an ozone generator (Medozon Compact-Hab Herrmann Apparatebau, GmbH, Germany). The measured level for ozone concentration was found to be 254 nm on an ultraviolet spectrophotometer. The mixture included the ozone concentration measured at 50 µg/mL. Three-day administration of 0.2 mg/kg/day was made for the subjects to receive 10 µg/mL ozone.

Histopathological Examination

Four days after the end of the experimental procedures, the rats were sacrificed by cervical dislocation, and bilateral orchiectomy was performed. The tunica albuginea was used to individually weigh testes. As described in a previous study, the left testis samples were examined by biochemical analysis and the right testis samples were examined histopathologically (10). Briefly, the samples were fixed in Bouin's solution and dehydrated in alcohol for 24 hours. After routine tissue processing, the samples were embedded in paraffin. Tissue sections (3-µm thick each) from the upper, lower and middle testicular regions were mounted for microscope slides. Staining cross-sections were provided using periodic acid-Schiff and hematoxylin-eosin under the standard protocols. The observation of the tissue slides was made through the standard light microscope by a pathologist, who was uninformed of the results, to apply experimental procedures. Calculation of the Johnsen scores was performed for each one. At least 50 tubules per tissue slide were examined and scored from 1 to 10 for each tissue slide according to spermatogenesis level (Table 1) (13).

Biochemical Analysis

A 10% homogenate of testicular tissue was prepared in 0.1 mol KCl buffer (pH 7.4) with Teflon-glass homogeniser. Following homogenization of the tissues, centrifugation was conducted at 18.000 g at 4 °C for 30 min. To establish a lipid peroxidation index, Yagi's (14) method was effective to identify the levels of thiobarbituric acid-reactive substances (TBARS). The spectrophotometrical measurement was 532 nm for the absorbance level. The results for the tissues were in nmol g^{-1} tissue. The Sedlak and Lindsay (15) method was applied to obtain the GSH levels. Absorbance occurred at 412 nm as displayed by spectrophotometer, and the records were presented in nmol mL^{-1} tissue. The technique introduced by Sun et al. (16) provided with identifying the levels of SOD activity.

Table 1. The Johnsen score (13)

Score	Level of spermatogenesis
1	No seminiferous epithelial cells, tubular sclerosis
2	No germ cells, Sertoli cells only
3	Spermatogonia only
4	Few spermatocytes, arrest of spermatogenesis at the primary spermatocyte stage
5	Many spermatocytes
6	Few early spermatids, arrest of spermatogenesis at the spermatid stage
7	No late spermatids, many early spermatids
8	Less than five spermatozoa per tubule
9	Slightly impaired spermatogenesis
10	Full spermatogenesis

In this method, inhibition of O_2-induced nitroblue tetrazolium reduction, produced by the xanthine/xanthine oxidase system, is decisive. For one unit of SOD activity, the quantity of enzyme was used, which is necessary to result in the half-inhibition of nitroblue tetrazolium reduction rate at 560 nm. The results in the study were expressed as IU mg^{-1} protein. Catalase activity was determined using the Aebi method (17). This method is based on the concept that catalase present in the sample converts H_2O_2 to H_2O and O_2. The spectrophotometrical measurement of H_2O_2 has decreased from 240 nm. The results were displayed in k mg^{-1} protein. The observations of the GPx activity levels were attained, thanks to the Paglia and Valentine's (18) method. This technique was established to match GPx activity and nicotinamide adenine dinucleotide phosphate oxidation using GSH reductase. The latter was spectrophotometrically measured at 340 nm. The results were showed in IU mg^{-1} protein.

Statistical Analysis

The statistics were exhibited in the format of mean ± standard deviation. The Kolmogorov-Smirnov test was used for normal distribution compatibility, and non-parametric tests of the Kruskal-Wallis and Mann-Whitney U for between-groups analysis. Significance level was defined as $p<0.05$. Statistical analyses were conducted by SPSS package (version 19.0 for Windows; IBM, NY, USA).

Results

None of the rats in the study, performed on a total of 30 rats, died during the experiment. Histopathological examination and biochemical analysis were performed on the rats.

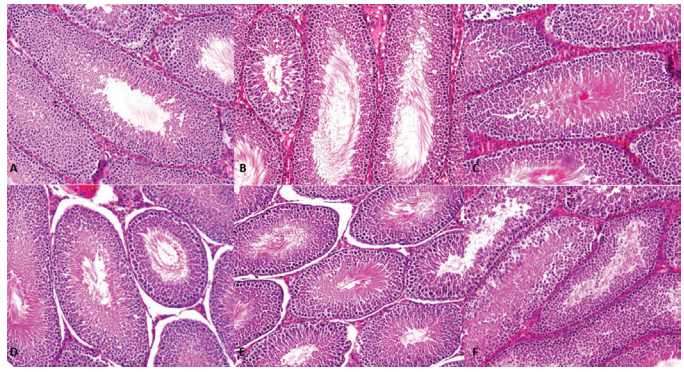

Figure 1. A) Normal testis histology (control group, hematoxylin and eozin, 100x), B) full spermatogenesis and normal testis histology (ozone therapy group, hematoxylin and eozin, 100x), C) necrosis of testicular tubules, impaired spermatogenesis (cisplatin group, hematoxylin and eozin, 40x), D) multinucleated giant cells in distributed testicular tubules (cisplatin group, hematoxylin and eozin, 40x), E) full spermatogenesis and normal testis histology (ozone theraphy + cisplatin group, hematoxylin and eozin, 100x, F) many early spermatids and disorganized epithelium (cisplatin + ozone theraphy group, hematoxylin and eozin, 100x)

We observed significant testicular damage in the CP group (Table 2). The weight of both the right and left testicles in the CP group was significantly lower than in controls. But pre- and post-CP OT was significantly prevented this decreases caused by CP. In the control group, the rat testes showed normal morphology and spermatogenesis, healthy seminiferous tubules containing plenty of spermatids and sperm in the lumen (Figure 1). High Johnsen scores (9.71±0.3) were detected in the control group. In the OT group, the rat testes showed mostly the same morphological characteristics as in the control group, but a few seminiferous tubules showed slightly impaired spermatogenesis. There was no significant difference in Johnsen score between OT and control groups (9.64±0.2 vs 9.71±0.3, p=0.833). In the CP group, the rat testes showed testicular interstitial fluid and interstitial edema, germ cell desquamation and irregular spaces in the epithelium, decreased spermatogenic cells and seminiferous tubules containing Sertoli cells and germ cell necrosis. The Johnsen scores were significantly decreased in the CP group compared to that in the control (8.16±0.8 vs 9.71±0.3, p<0.001) and OT (8.16±0.8 vs 9.64±0.2, p<0.001) groups. In the pre- and post-CP OT groups, the rat testes showed mild interstitial edema and tubules with incomplete maturation arrest. Spermatogenesis was maintained in the majority of the seminiferous tubules. The Johnsen scores were significantly increased in the CP + OT and OT + CP groups compared to that in the CP group (9.54±0.3 vs 8.16±0.8, p<0.001; 9.57±0.2 vs 8.16±0.8, p<0.001, respectively). Meanwhile, no significant difference was found in Johnsen score between CP + OT and control groups (p=0.381), OT + CP and control groups (p=0.377), and CP + OT and OT + CP groups (p=0.891).

Levels of TBARS, GSH, SOD, catalase and GPx in rat testis tissue are presented in Table 3. When the control group and CP group were compared in the testis tissues of the rat, the levels of TBARS were significantly higher in the CP group, whereas the GSH, SOD, catalase and GPx levels were significantly lower. On the other hand, pre- and post-CP OT caused a significant decrease in the TBARS level and an increase in GSH, SOD, catalase and GPx levels when compared to the CP group.

Discussion

It is well known that human testis is sensitive to CP. This study showed that CP chemotherapy induced significant damage in the testes of rats. Besides, pre- and post-CP OT was protective against spermatogenic cell damage.

CP is a highly effective anti-tumor drug used in the treatment of many different types of tumors, including testicular, ovarian, endometrium, cervical, bladder, lung and head and neck cancers. However, its usage is limited by its toxic effects on the reproductive system (4,19,20). The main unwanted effects of CP are ototoxicity, nephrotoxicity, peripheral neuropathy, azoospermia, sperm morphology, and motility modifications (19,21). As a result, prevention of side effects of CP is very important in terms of treatment protocol, benefits in quality of life and extending the limits of dose (4). CP targets testicular cell types such as Leydig cells, Sertoli cells and germ cells thus leading to male reproductive toxicity (22). In this research, testicular destruction induced simply by CP medication was seen as significant raises in lipid peroxidation, reduced antioxidant status and changed testicular histopathology in rats.

Oxidative stress plays a significant role in male reproductive dysfunction (23). Oxidative stress is an abnormal condition where the balance between the production of ROS and their adequate

Table 2. Comparison of testes weight and the Johnsen score among the five groups

	Control	OT	CP	CP + OT	OT + CP
Right testis weight (g)	1.321±0.05	1.317±0.04	0.868±0.03*	1.282±0.03$^{\gamma}$	1.291±0.02$^{\gamma}$
Left testis weight (g)	1.310±0.04	1.308±0.03	0.845±0.05*	1.289±0.03$^{\gamma}$	1.287±0.03$^{\gamma}$
Johnsen score	9.71±0.3	9.64±0.2	8.16±0.8*	9.54±0.3$^{\gamma}$	9.57±0.2$^{\gamma}$

OT: Ozone therapy, CP: Cisplatin, *significantly different from the control group, $^{\gamma}$significantly different from the cisplatin group, data expressed as mean ± standard deviation

Table 3. Comparison of thiobarbituric acid-reactive substances, glutathione, superoxide dismutase, catalase and glutathione peroxidase levels among the five groups

	Control	OT	CP	CP + OT	OT + CP
TBARS (nmol g^{-1} tissue)	23.51±1.23	24.86±3.12	49.82±3.95*	25.63±3.88$^{\gamma}$	24.90±4.13$^{\gamma}$
GSH (nmol mL^{-1})	192.74±3.81	194.41±4.22	103.13±8.74*	187.04±4.15$^{\gamma}$	190.07±5.99$^{\gamma}$
SOD (U mg^{-1} protein)	0.82±0.04	0.80±0.04	0.60±0.03*	0.79±0.03$^{\gamma}$	0.78±0.04$^{\gamma}$
Catalase (k mg^{-1} protein)	2.43±0.81	2.41±0.94	1.23±0.45*	2.36±0.66$^{\gamma}$	2.34±0.91$^{\gamma}$
GPx (U mg^{-1} protein)	220.11±26.52	215.63±28.68	151.18±28.40*	209.39±12.63$^{\gamma}$	211.55±16.27$^{\gamma}$

OT: Ozone therapy, CP: Cisplatin, TBARS: Thiobarbituric acid-reactive substances, GSH: Glutathione, SOD: Superoxide dismutase, GPx: Glutathione peroxidase, *significantly different from the control group, $^{\gamma}$significantly different from the cisplatin group, data were expressed as mean ± standard deviation

elimination by antioxidant systems available in the body is severely disturbed (4,19). Within pathological circumstances, ROS could be produced not merely by macrophages and neutrophils but likewise spermatozoa and various other cell types (20,24,25).

O_2 free radical formation is associated with impaired GSH metabolism, changes in the antioxidant enzymes, and lipid peroxidation (4,20). Antioxidants are inactivating ROS and they maintain normal cell function (8,9). The enzymes such as GPx, SOD and catalase are endogenous defense mechanisms that remove the activation of superoxide, hydrogen peroxide and hydroxyl radicals (3,4,19,20). SOD changes superoxide anion in to water and O_2, whilst catalase changes peroxide radicals in to water and O_2. The reason for the decrease in enzymatic antioxidants is increased ROS production (8). TBARS are also a by-product of the damage that results from oxidative stress. TBARS produced from peroxidation of fatty acids by ROS are considered to be an indicator of lipid peroxidation (19). When there is a shift in the balance between the production of ROS and the antioxidant defense mechanism in favor of ROS, ROS interacts with proteins, lipids and nucleotides in the cell structure. These pathways induce an apoptotic process (8,9). Testis is a main organ for oxidative stress as a consequence of its large content material of polyunsaturated membrane lipids (12,20). Thus, sensitivity of testis to oxidative agents such as CP is high. Therefore, use of antioxidants can prevent CP-induced male infertility. In this study, we found that CP treatment caused increased oxidative stress and decreased antioxidant enzymes.

The antioxidant mechanism of ozone remains unknown. Once therapeutic ozone is administrated, ozone dissolves in the plasma and responds with polyunsaturated fatty acids and sulfhydryl groups. These reactions lead to the production of lipid hydroperoxides and hydrogen peroxides at low concentrations. Low-dose OT may act as cellular signal molecules and activates the cytoprotective pathways which increase the GSH synthesis and antioxidant capacity (11). Another antioxidant pathway of OT is inhibitory formation of xanthine oxidase thereby reducing the adenosine triphosphate depletion. Preserved adenosine induces vasodilation in the smooth muscle. It also inhibits the nuclear transcription factor kappa B, which is responsible for ozone, tissue damage, and inflammatory responses (26). Thus, ozone regulates inflammation and limits tissue injury. Ozone also has effects such as increasing blood flow to the tissues, activation of hypoxia-inducible factor-1a, vascular endothelial growth factor, activation of erythropoietin and glycolytic enzymes and increased O_2 delivery (11,26).

Molecular mechanism of radio protective substances is essential for drug approval. To our knowledge, no study investigating the molecular mechanism of ozone in recovery or protection against CP-induced testicular damage has been published.

Study Limitations

In our study, inadequate evaluation of tissue damage parameters and ROS is a limitation.

Conclusion

The current investigation demonstrated the fact that OT is shielding in CP-induced testicular destruction. OT might be effective in patients who go through CP chemotherapy. Further studies are needed to confirm our results and transition from experimental animal studies to clinical trials.

Ethics

Ethics Committee Approval: The approval was obtained from Bezmialem Vakıf University Ethics Committee (number: 24.11.2015/237).

Informed Consent: Experimental study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: İ.A., P.Y., Concept: İ.A., Y.Ö.İ., R.G.E., Design: İ.A., Y.Ö.İ., Data Collection or Processing: İ.A., R.G.E., S.L.M., A.Ç., Y.E.A., H.K., Analysis or Interpretation: İ.A., R.G.E., Y.E.A., M.B.S., A.Ç., S.L.M., H.K., Literature Search: İ.A., Y.Ö.İ., Y.E.A., A.Ç., S.L.M., M.B.S., H.K., Writing: İ.A., Y.Ö.İ., R.G.E., M.B.S.

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