Original Article

Does ozone administration have a protective or therapeutic effect against radiotherapy-induced testicular injury?

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ABSTRACT

Objective: We investigate the protective and therapeutic effects of ozone therapy (OT) in radiotherapy (RT)-induced testicular damage.

Methods: Thirty healthy adult male Wistar rats divided into five groups consisting of six animals each as follows: (1) Control (C), (2) RT, (3) OT, (4) OT + RT, and (5) RT + OT group. Histopathological findings, Johnsen scores, thiobarbituric acid-reactive substances (TBARS), glutathione (GSH), superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) levels were evaluated.

Results: RT 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 RT group when compared to the control group. Pre and postRT OT significantly increased GSH, SOD, catalase, and GPx levels and decreased TBARS level. Furthermore, testicular weight and Johnsen score were increased with OT.

Conclusions: The present study showed that OT is protective and therapeutic in radiation-induced testicular damage. OT may be beneficial to the patients who underwent RT.

KEY WORDS: Ozone treatment, radiation, radiotherapy, testicular histopathology, testis

INTRODUCTION

In children and young adults, testicular cancer, acute leukemia, and lymphoma are the common malignancies. These patients can be treated effectively with surgery, chemotherapy, or radiotherapy (RT). Therefore, reducing the long-term toxicity of treatment is important to maintain the quality of life.[1]

The radiation exposure damages the healthy tissues while killing the tumor cells and decreases the quality of life. Ionizing radiation-induced damage is mediated through free radicals, such as superoxide radical, hydroxyl radical, and hydrogen peroxide.[2] These reactive oxygen species (ROS) interact with proteins, lipids, and nucleotides.[3] These interactions disturb cell metabolism, proliferation, and differentiation. Eventually, they induce tumor and healthy cell apoptosis.[2,3] The adverse effect of RT on the testicle is abnormalities in spermatogenesis, decreased sperm production and low sperm count, abnormal spermatozoa, defective sperm function, apoptosis of germ cells, loss of testis weight, and dose-dependent temporary or permanent sterility.[4-7] This RT-induced testicular damage occurs through the overproduction of ROS and a reduction in the antioxidant level.[8]

The antioxidants are the natural defense mechanism against ROS in most of the organs including testicle. Oxidative damage arises when shift the equilibrium between the production of ROS and the antioxidant defense mechanism in favor of ROS.[9,10] Ozone is an inorganic molecule composed of three oxygen atoms. Ozone is a colorless gas with a unique pungent smell at room temperature.[10-12] After ozone therapy (OT) administration, it dissolves in biological water and instantly reacts with antioxidant mechanisms.[12] During these fast reactions, ozone is neutralized by the activation of antioxidant mechanisms which are superoxide dismutase (SOD), catalase, and glutathione

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peroxidase (GPx).[12] The stimulation of endogenous antioxidant prepares the host defense against ROS.[10-12] The other important effect of ozone is immune modulation, neoangiogenesis, and increased tissue oxygenation.[12]

To the best of our knowledge, there is no previous investigation concerning the radioprotective effect of OT on testicular histopathology. The aim of this investigation is to evaluate the protective and therapeutic effects of ozone administration on RT-induced oxidative stress in an experimental testicular rat model.

METHODS

All experimental protocols conducted on the animals were consistent with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Local Ethical Committee on animal research.

Animals

Thirty healthy adult male Wistar rats weighing between 250 and 300 g were used. The rats were kept in well-ventilated plastic cages at a room temperature of 25°C ± 3°C and 12-h light/dark cycle environment. The rats were nourished with standard laboratory chow and clean water *ad libitum*. A 1-week period of acclimatization was utilized.

Experimental design

Thirty rats were randomly separated into five groups comprising six animals each as follows:

- Group C (control): The rats received intraperitoneal oxygen as the vehicle
- Group OT: The rats received intraperitoneal OT to define the effect of OT on testicular histopathology
- Group RT: The rats received scrotal irradiation to determine the effect of RT on testicular histopathology
- Group RT + OT (RT after OT): The rats received scrotal irradiation and intraperitoneal OT after RT to determine the effect of post-RT OT on testicular histopathology
- Group OT + RT (OT after RT): The rats received intraperitoneal OT and scrotal irradiation after OT to determine the effect of preRT OT on testicular histopathology.

Each experimental procedure were performed under anesthesia with intraperitoneal 5 mg/kg xylazine hydrochloride and 50 mg/kg ketamine hydrochloride, then the rats were restrained from their four extremities on a table.

Ozone therapy

An ozone-oxygen mixture was generated by an ozone generator (Medozon Compact-Hab Herrmann apparatabau, GmbH, Germany). The ozone concentration was evaluated by an ultraviolet spectrophotometer at 254 nm. The ozone density in the blend was 50 μg/ml. The ozone mixture was applied 0.2 mg/kg/day (10 μg/ml ozone) for 3 days.

Irradiation procedures

Irradiation was delivered by a cobalt-60 (^{60}Co) treatment unit (CIS Bio International, Gif-sur-Yvette, France) at a source-surface distance of 65 cm. The rats were secured onto a 20 cm × 20 cm Styrofoam treatment couch (Med-Tec, Orange City, IA). Both testes were irradiated and each testis received the equal dose. The position of the field was planned for all animals using a treatment simulator (Mecaserto-Simics, Paris, France). Rats were treated with total 20 Gy dose (4 Gy/1 fraction/day was administered in 5 days).

Histopathological examination

Ten days after the end of experimental procedures, the rats were sacrificed by cervical dislocation and bilateral orchiectomy was performed. Each testis was weighed with the tunica albuginea. The right testis was used for histopathological examination and the left testis was used for biochemical analysis. Fresh testis samples were stored at −80°C until biochemical analysis. The right testis samples were processed for histopathological examination as described previously.[11] In brief, samples were fixed in Bouin's solution and dehydrated in alcohol for 24 h. After routine tissue processing, the samples were embedded in paraffin. Three 3-μm tissue slides were prepared from the upper, mid, and lower portions of the testes. The cross-sections were stained standard protocols for periodic acid–Schiff and h and e. The tissue slides were examined under the standard light microscope by a pathologist who is blinded to study experimental procedures. Johnsen score was determined for each tissue slide. A minimum level of 50 tubules were examined per tissue slide, and each tissue slide was scored from 1 to 10 based on the level of spermatogenesis [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 homogenizer. The homogenized tissues were centrifuged at 18.000 g for 30 min at 4°C. As an index of lipid peroxidation, the thiobarbituric acid-reactive substance (TBARS) levels were identified by the method of Yagi.[14] The absorbance was assessed spectrophotometrically at 532 nm. The results were expressed as nmol/g tissue. The glutathione (GSH) levels were identified using the method of Sedlak and Lindsay.[15] The absorbance was assessed spectrophotometrically at 412 nm.

Table 1: 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	<5 spermatozoa per tubule
9	Slightly impaired spermatogenesis
10	Full spermatogenesis

The results were expressed as nmol/ml. The SOD activity was determined using the method of Sun *et al*.[16] This method based on the inhibition of nitroblue tetrazolium reduction due to O_2^- generated by the xanthine/xanthine oxidase system. One unit of SOD activity was defined as the quantity of enzyme required to cause 50% inhibition of the rate of nitroblue tetrazolium reduction at 560 nm. The results were expressed as IU/mg protein. The catalase activity was determined using the method of Aebi.[17] This method based on that catalase present in the sample to convert H_2O_2 to H_2O and O_2. The decrease of H_2O_2 was evaluated spectrophotometrically at 240 nm. The results were expressed as k/mg protein. The GPx activity was determined using the method of Paglia and Valentine.[18] In this method, GPx activity was coupled with the oxidation of NADPH by glutathione reductase. The oxidation of NADPH was evaluated spectrophotometrically at 340 nm. The results were expressed as IU/mg protein.

Statistical analysis

Results were presented as the mean ± standard deviation. Normality distribution was tested using Kolmogorov–Smirnov test, and differences among the groups were analyzed using the nonparametric Kruskal–Wallis and Mann–Whitney U-tests. A value of $P < 0.05$ was considered statistical significance. Statistical analyses were performed using SPSS version 19.0 for Windows (IBM, New York, USA).

RESULTS

None of the rats died during the experimental period. Histopathological examination and biochemical analysis were made on 30 rats.

We observed significant testicular damage in the RT group [Table 2]. Testis weights both right and left were significantly decreased in the RT group. However, pre and postRT OT was significantly prevented this decreases caused by radiation. In the C group, rat testes showed normal morphology and spermatogenesis, healthy seminiferous tubules containing plenty of spermatids and sperm in the lumen [Figure 1]. High Johnsen scores (9.66 ± 0.2) were detected in the C group. In the OT group, rat testes showed mostly same morphological characteristics as the C group, but a few seminiferous tubules were showed slightly impaired spermatogenesis. There was no significant difference between OT and C groups for Johnsen scores (9.56 ± 0.1 vs. 9.66 ± 0.2, $P = 0.461$). In the RT group, rat testes showed testicular interstitial fluid and interstitial edema, desquamation of germinal cells 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 RT group compared to the C (8.03 ± 0.6 vs. 9.66 ± 0.2, $P = 0.002$) and OT (8.03 ± 0.6 vs. 9.56 ± 0.1, $P = 0.004$) groups. In the pre and postRT OT groups, rat testes showed mild interstitial edema and tubules with incomplete maturation arrest. Most of the seminiferous tubules were preserved spermatogenesis. The Johnsen scores were significantly increased in RT + OT and OT + RT groups compared to the RT group (9.46 ± 0.2 vs. 8.03 ± 0.6, $P = 0.004$ and 9.51 ± 0.2 vs. 8.03 ± 0.6, $P = 0.004$, respectively). Meanwhile, no statistically significant alteration was found in Johnsen scores when compared to RT + OT vs C groups ($P = 0.256$), OT + RT vs C groups ($P = 0.291$), and RT + OT vs OT + RT groups ($P = 0.677$).

Levels of TBARS, GSH, SOD, catalase, and GPx in rat testis tissue are presented in Table 3. In rat testis tissue, TBARS level was significantly higher, whereas GSH, SOD, catalase, and GPx levels were significantly lower in RT group compared to C group. On the other hand, pre and postRT OT was caused significantly decrease in TBARS level and increase in GSH, SOD, catalase, and GPx levels when compared to RT group.

DISCUSSION

The present study showed that 20 Gy RT resulted a significant damage in the rat testis, increase in oxidative stress, and

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

	Control	OT	RT	RT + OT	OT + RT
Right testis weight (g)	1.306±0.04	1.301±0.01	0.748±0.02*	1.282±0.03#	1.291±0.02#
Left testis weight (g)	1.293±0.05	1.288±0.02	0.758±0.01*	1.289±0.03#	1.287±0.03#
Johnsen score	9.66±0.2	9.56±0.1	8.03±0.6*	9.46±0.2#	9.51±0.2#

Data expressed as the mean±SD. *Significantly different from the control group, #Significantly different from the radiotherapy group. OT=Ozone therapy, RT=Radiotherapy, SD=Standard deviation

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

	Control	OT	RT	RT + OT	OT + RT
TBARS (nmol/g tissue)	19.84±2.57	20.76±4.38	45.69±3.35*	22.64±4.08#	21.64±3.03#
GSH (nmol/ml)	187.57±2.66	191.03±4.19	113.43±9.44*	184.41±3.55#	185.37±5.14#
SOD (U/mg protein)	0.80±0.03	0.79±0.03	0.57±0.02*	0.78±0.02#	0.77±0.03#
Catalase (k/mg protein)	2.45±0.31	2.40±0.34	1.20±0.25*	2.33±0.26#	2.32±0.21#
GPx (U/mg protein)	214.41±24.52	205.33±29.09	122.58±37.64*	203.00±19.72#	198.17±21.82#

Data were expressed as the mean±SD. *Significantly different from the control group, #Significantly different from the radiotherapy group. OT=Ozone therapy, RT=Radiotherapy, TBARS=Thiobarbituric acid-reactive substances, GSH=Glutathione, SOD=Superoxide dismutase, GPx=Glutathione peroxidase, SD=Standard deviation

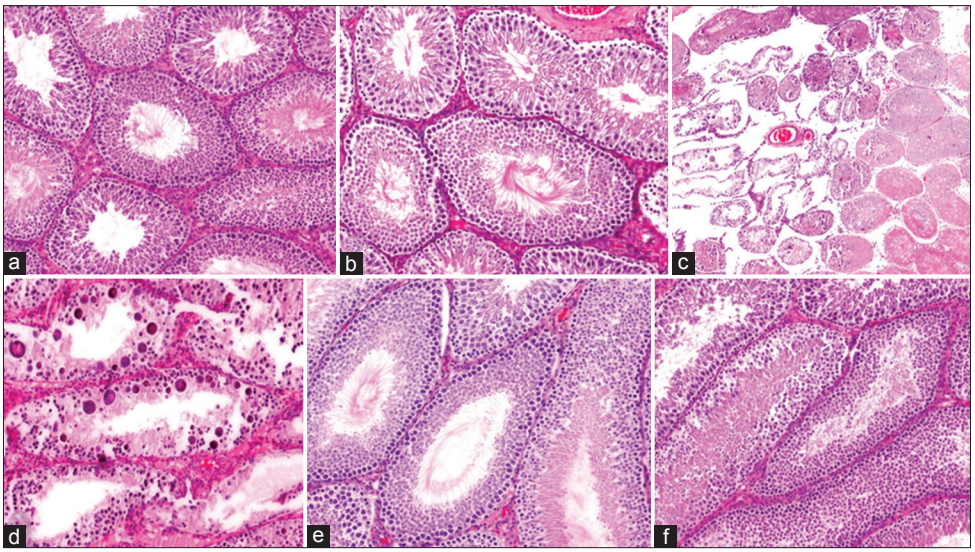

Figure 1: (a) Normal testis histology (control group) (H and E, ×100). (b) Full spermatogenesis and normal testis histology (ozone therapy group) (H and E, ×100). (c) Necrosis of testicular tubules, impaired spermatogenesis (radiotherapy group) (H and E, ×40). (d) Multinucleated giant cells in distributed testicular tubules (radiotherapy group) (H and E, ×40). (e) Full spermatogenesis and normal testis histology (ozone therapy + radiotherapy group) (H and E × 100). (f) Many early spermatids and disorganized epithelium (radiotherapy + ozone therapy group) (H and E, ×100)

decrease in antioxidants. On the other hand, we showed that OT had protective and therapeutic effects of radiation-induced testicular injury. Pre and postRT OT administration lead to increased antioxidant enzyme levels and decreased oxidative stress.

The human testis is sensitive to radiation. Adjuvant or palliative RT is administered to patients with various types of cancers. However, the testicles take a considerable amount of scatter (secondary) radiation when patients are irradiated.[19] Radiation-induced testicular damage is dose dependent. Low-dose external beam RT (such as scattered radiation) to the testis can affect fertility.[19] High-dose RT can cause permanent azoospermia by depletion of spermatogonial stem cells instead of differentiation.[9] The predictors of radiation-induced testicular damage degree are plasma levels of inhibition B, follicle-stimulating hormone and luteinizing hormone, epithelial height, diameter of the seminiferous tubule, and weight of testis.[1,19-21]

RT triggers oxidative stress. Oxidative stress plays an important role in male reproductive dysfunction.[7] The detrimental effect of RT is commonly mediated through the water radiolysis and increased production of ROS in cells.[6,9,21,22] Antioxidants are inactivating ROS and maintain normal cell function.[9,10] In irradiated rats, testicular enzymatic antioxidants, such as SOD and catalase are decreased.[6,9,20,21] SOD converts superoxide anion into water and oxygen. Catalase converts peroxide radicals into water and oxygen. The reason for decrease in enzymatic antioxidants is increased ROS production.[9] When shift the balance between the production of ROS and the antioxidant defense mechanism in favor of ROS, ROS interact with proteins, lipids, and nucleotides in cell structure. These pathways induce apoptotic process.[9,10] Sensitivity to radiation-induced apoptosis is highest in the spermatogonia. Apoptotic germ cells are reached a maximum after 12–48 h posttesticular-radiation.[21] Thus, the use of radioprotector can prevent radiation-induced male infertility.

The ability of recovery of spermatogenesis based on the survival of stem cell and their capability to repopulate and differentiation. Progression of the cell cycle is essential for proliferation of spermatogenic cells. PreRT melatonin administration is enhanced the expression of S-phase protein which is called proliferating cell nuclear antigen.[23] Histopathological examination showed that testicular architecture of untreated irradiated rats was disrupted.[8,9] The blood-testis barrier is an important physical ultrastructure between the blood vessels and the seminiferous tubules of the mammalian testis. High-dose RT induces the blood-testis barrier damage by regulation of tight junction proteins. This damage can be reduced by antioxidant N-Acetyl-L-Cysteine.[24] Epigallocatechin-3-gallate in green tea exerts antiapoptotic activity and prevents radiation-induced testicular damage through the mitogen-activated protein kinase/BCL2 family/caspase-3 pathway.[25] While the preRT epigallocatechin-3-gallate administration is strengthen the germ cells against RT, postRT administration support recovery of the germ cells. Another protective effects of this molecule are ameliorated RT-induced blood-testicular barrier permeability and suppressed testicular steroidogenesis.[25]

The discovery of antioxidant defense mechanisms and condition of testicle during oxidative stress is important for identifying the novel treatments. L-carnitine has an antioxidant and free radical scavenging activity.

Administration of L-carnitine improved the spermatogenic recovery and decreased the germ cell apoptosis after RT.[21] Melatonin pre and postRT increased glutathione and total antioxidant capacity, thus it decreased DNA strand breaks, lipid peroxidation, and apoptotis.[23] In another study, ameliorative effects of kolaviron and vitamin C on testicular lipid peroxidation and sperm characteristics were evaluated after RT. Both of these antioxidants increased testes weights, antioxidant enzymes, and decreased lipid peroxidation.[9] A recent study was evaluated the protective and therapeutic effect of aminoguanidine on RT-induced testicular damage. Aminoguanidine is a selective inhibitor of inducible nitric oxide synthase. This study showed that aminoguanidine treatment decreased oxidative stress index, total oxidant status, and malondialdehyde levels, and increased SOD, catalase, GPx, and glutathione levels.[8] According to these studies, RT caused testicular dysfunction by depleting the antioxidant mechanism, while administration of antioxidants ameliorated the radiation-induced testicular damage.

The antioxidant mechanism of ozone remains unknown. When therapeutic ozone administrated, ozone dissolves in the plasma and reacts with polyunsaturated fatty acids and –SH groups. These reactions generate hydrogen peroxide and lipid hydroperoxides at low concentrations. Low-dose OT may work as cellular signal molecules and trigger the cytoprotective pathways which are increasing the glutathione synthesis and antioxidant capacity.[12] Another antioxidant pathway of OT is inhibition the formation of xanthine oxidase, thereby reduce the ATP depletion. Preserved adenosine induces the vasodilation in the smooth muscle. In addition, ozone inhibits nuclear transcriptional factor kappa B responsible for inflammatory responses and tissue injury.[26] Thus, ozone regulates the inflammation and limit the tissue injury. Furthermore, ozone increases the blood flow into tissues, augments oxygen delivery by activation of hypoxia-inducible factor-1a, vascular endothelial growth factor, erythropoietin, and glycolytic enzymes.[12,26]

Molecular mechanism of radioprotective substance is essential for drug approval. To the best of our knowledge, no study has published molecular mechanism of ozone in recovery or protection of radiation-induced testicular damage. Limitation of our study is an insufficient evaluation of tissue damage parameters.

CONCLUSIONS

The present study showed that OT is protective and therapeutic in radiation-induced testicular damage. OT may be beneficial to patients who underwent RT. Further studies are needed to confirm our results and transition from experimental animal studies to clinical trials.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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