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ORIGINAL ARTICLE

Antioxidant treatment ameliorates germ cell apoptosis induced by a high-dose ionizing irradiation in rats

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Abstract

Background Exposure to ionizing radiation results in cytotoxic and genotoxic effects caused mainly by the oxidative damage. In the present study, we investigated the radioprotective effect of novel antioxidant cocktail on germ cell apoptosis and spermatogenesis in rats subjected to whole body radiation (WBIR).

Methods Adult male rats weighing 250–270 g were divided into four groups, eight rats each. Group 1 served as untreated control, group 2 received an IP single dose of antioxidant cocktail (1 ml). Group 3 was exposed to a WBIR (6 Gy). Group 4 received antioxidant cocktail before WBIR. Rats from each group were killed after 48 h. MDA levels were measured in serum (TBARS assay). Johnsen's criteria and the number of germinal cell layers were used to categorize spermatogenesis. TUNEL assay was used to determine germ cell apoptosis. Statistical analysis was performed using one-way ANOVA test.

Results WBIR resulted in histological testicular damage (decrease in Johnsen's criteria, $p < 0.05$) that was accompanied by a significant increase in germ cell apoptosis, expressed as the number of apoptotic cells per 100 tubules (AI-1 apoptotic index) and the number of positive tubules per 100 tubules (AI-2 apoptotic index). Treatment with antioxidant cocktail resulted in a significant decrease in germ cell apoptosis (33% decrease in AI-1, $p < 0.05$ and 34% decrease in AI-2, $p < 0.05$) that was accompanied by an improved spermatogenesis (increase in Johnsen's criteria, $p < 0.05$).

Conclusions In a rat model of WBIR, antioxidant treatment ameliorates oxidative stress-induced testicular damage, decreases germ cell apoptosis and improves spermatogenesis.

Keywords Radiation · Antioxidant therapy · Germ cell · Apoptosis · Rat

Introduction

Radiation is an important modality in cancer treatment and estimates are that between one-third and one-half of all patients will require ionizing irradiation therapy. Radiation may be used as a single therapeutic option or as an adjuvant along with surgery and/or chemotherapy during some point in their clinical management. However, effective use of ionizing radiation is compromised by the side effects that result from radiation-induced damage to normal tissue [1, 2].

Radiation therapy to the testes and high cumulative dose of alkylating agents are the major factors decreasing the probability of fertility in cancer patients [3]. The gonadotoxic effect of radiotherapy depends on the gonadal dosage and the delivery method. The molecular pathways involved in the control of spermatogenesis in response to irradiation and the mechanisms of DNA damage response and homeostasis in spermatogonial stem cells are poorly understood. Spermatogonial stem cells are long-lived cells that

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support normal germ cell differentiation and must be preserved throughout life. However, after irradiation spermatogenesis recovery can be impaired as a consequence of the radiation-induced decline in spermatogonial stem cell [4]. Radiation doses begin to adversely affect spermatogenesis at 0.1–1.2 Gy, with irreversible damage at a 4 Gy dose [5]. Leydig cells are more resistant to radiation-induced injury, withstanding up to 30 Gy [6].

Recent evidence suggests that radiation stimulates oxidative stress and generates reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide and hydroxyl radicals, which may cause a direct damage [7]. Indirectly, radiation splits water molecules since the radiolytic products are highly reactive and more damaging to biomolecules [8]. Oxidative stress emerges when the production of ROS exceeds the capacity of cellular antioxidant defenses.

The use of chemical compounds (radioprotectors) represents an obvious strategy to improve the therapeutic index in radiotherapy. However, most of the synthetic radio protective compounds studied have shown inadequate clinical application owing to their inherent toxicity and high cost. These observations necessitated a search for alternative agents that are less toxic and highly effective. Studies in the recent past have shown that some medicinal plants possess radioprotective effects.

In the current study, a new antioxidant cocktail including ten antioxidants (including water-soluble and lipid-soluble molecules) was used to achieve a comprehensive and hopefully synergistic effect against radiation damage. The purpose of the present study was to observe radioprotective effect of this antioxidant cocktail on germ cell apoptosis and spermatogenesis in rats subjected to whole body radiation (WBIR).

Materials and methods

Animals

Animal facilities and protocols were approved by Rappaport Faculty of Medicine (Technion, Haifa, Israel) Institutional Animal Care and Use Committee. Male Sprague–Dawley rats weighing 250–270 g were acclimatized at 21 °C on 12-h day and night cycles for 3–5 days before the experiment. The rats had free access to water and were pair fed with standard chow.

Materials

Antioxidant cocktails (capsule) include selected vitamins, minerals and herbs with research proven radio protective effect : vit A—5000 IU, Biotin—90 µg, vit C—500 mg, vit D3—200 IU, vit E—200 IU, vit B1—2.5 mg, vit B2—3 mg, vit B3—10 mg, vit B6—3 mg, folic acid—400 µg, vit B12—6 µg, Selenium—120 µg, N-acethyl cysteine (NAC)—60 µg, Coenzym Q-10 (Ubiquinone)—60 µg, herbal blend—465 µg that include Ginco—ginco-balboa, Ilex paraguriensis, Lycopene, Quercetin, Spirulina. One capsule was diluted in 10 ml of normal saline under sterile condition; 1 ml of the sterile condition was injected intraperitoneally.

Experimental design

Thirty-two rats were divided randomly into four experimental groups of eight rats each: Group A (CONTR)—control rats underwent IP injection of normal saline (1 ml); Group B (CONTR-AOX)—control-antioxidant rats received an IP single dose of antioxidant cocktail (1 ml). Group C (IR)—irradiation group was exposed to a whole body radiation (WBIR, 6 Gy). Group D (IR-AOX) IR-antioxidant rats received antioxidant cocktail before WBIR. 48 h following WBIR, rats from each group were anesthetized with an overdose of intraperitoneal pentobarbital (75 mg/kg) and were killed. Final body weight was measured. Blood samples (1 ml) were collected from rat heart at sacrifice. After centrifugation at 5000×*g* for 5 min, at 4 °C, the top plasma layer was transferred to a new tube and stored at − 80 °C. Both testes were quickly removed and weighed. Relative testis/body weight ratio was calculated.

Radiation exposure

Whole body gamma irradiation of rats was performed using Tc-99m Pertechnetate. Animals were placed in the specially designed tray and received a definite dose of 6 Gy delivered in four fractions at one day of interval at a dose rate of 0.5 Gy min^{-1}.

Malondialdehyde (MDA) levels

MDA levels were measured in serum by TBARS assay. Oxidative stress in the cellular environment results in the formation of highly reactive and unstable lipid hydroperoxides. Decomposition of the unstable peroxides derived from PUFAs results in the formation of malondialdehyde (MDA), which can be quantified colorimetrically following its controlled reaction with thiobarbituric acid. The measurement of these 'Thiobarbituric Acid Reactive Substances' (TBARS) is a well-established method for screening and monitoring lipid peroxidation. In the current experiment, MDA levels were measured in serum of experimental animals using Cayman's TBARS Assay Kit.

Histopathologic evaluation of spermatogenesis

The samples of testicular tissues were fixed in a buffered 4% formaldehyde solution and then were embedded in paraffin wax using standard techniques. Sections (5 µm each) were cut and stained with hematoxylin and eosin. Histological alterations were studied using a graded eye piece at ten times magnification.

The number of germinal cell layers and Johnsen's score were used to categorize the spermatogenesis. The number of germinal epithelial layers was counted in ten seminiferous tubules as described by Miller et al. [9] and the mean number was calculated. Each tubular section was given a score from 10 to 1 according to the presence or absence of the main cell types arranged in the order of maturity as described by Johnsen [10]: 10—complete spermatogenesis and normally organized tubules; 9—many spermatozoa present but germinal epithelium disorganized; 8—only a few spermatozoa present in the section; 7—no spermatozoa but many spermatids present; 6—only a few spermatids present; 5—no spermatozoa or spermatids but many spermatocytes present; 4—only a few spermatocytes present; 3—only spermatogonia present; 2—no germ cells but only Sertoli cells present; 1—no germ cells and no Sertoli cells present.

Evaluation of germ cell apoptosis

Germ cell apoptosis was evaluated by in situ TUNNEL assay for apoptotic cell detection using the I.S. Cell Death Detection kit (Boehringer Mannheim GmbH, Mannheim, Germany). Briefly, serial 5µ m thick paraffin-embedded sections were deparaffinized, rehydrated in graded alcohol and microwave-pretreated in 10 mM citrate buffer (pH 6.0) for 10 min. After washing in phosphate-buffered saline (PBS), the specimens were incubated with fluorescein-labeled deoxy-UTP and TdT at 37 °C for 60 min. After washing, the slides were incubated with blocking solution (3% H_2O_2 in methanol) for 10 min and were stained with anti-fluorescein antibody, Fab fragment from sheep and conjugated with horse-radish peroxidase (converter-POD) at 37 °C for 30 min. AES substrate (Zymed Laboratories) was applied for color development. For each group, the number of stained cells was counted in ten tubules in the areas without necrosis. For each group, the number of stained germ cells was counted. The apoptotic index AI-1 was defined as the number of apoptotic TUNEL-positive cells per 100 tubules and AI-2 as the number of tubules containing apoptotic cells per 100 tubules. Pathologists blinded to the source of testicular tissue performed all measurements.

Statistical analysis

The data are expressed as the mean ± SEM. A non-parametric Kruskal–Wallis ANOVA test was used for statistical analysis with p less than 0.05 considered statistically significant.

Results

Body weight and testicular weight

Table 1 compares the final body weight and testicular weight among the four experimental groups. As expected, exposure of whole body of rats to 6 Gy (Group C) resulted in a small but significant decrease in body weight (260 ± 3 vs 269 ± 2 g, $p < 0.05$) compared to control animals (Group A). Although IR-AOX (Group D) rats demonstrated a trend toward increase in final body weight compared to IR animals (Group C), this trend was not statistically significant.

γ-radiation (Group C) resulted in a significant decrease in testicular weight (13%, $p < 0.05$) as well as in the relative testis/body weight ratio (10%, $p < 0.05$) compared to control animals (Group A). Treatment of irradiated rats with antioxidant cocktail (Group D) resulted in a significant increase in testicular weight (10%, $p < 0.05$) as well as in the relative testis/body weight ratio (8%, $p < 0.05$) compared to IR-nontreated animals (Group C).

Malondialdehyde (MDA) levels

To determine whether excessive ROS production (oxidative stress) could be detected in these treated groups, we assessed levels of MDA, oxidative stress marker, in blood plasma (Fig. 1). As expected, CONTR-AOX rats demonstrated a trend toward decrease in MDA plasma levels compared to control animals (Group A); however, this trend was not statistically significant. Whole body radiation (WBIR, 6 Gy) (Group C) resulted in a significant increase in MDA plasma levels compared control rats suggesting elevated oxidative

Table 1 Body weight and testicular weight changes

Group	Body weight (g)	Testicular weight (g)	Relative testis/body weight ratio
CONTR	269 ± 2	1828 ± 36	6.8 ± 0.1
CONTR + AOX	271 ± 4	1772 ± 30	6.5 ± 0.2
IR	260 ± 3*	1593 ± 53*	6.1 ± 0.2*
IR + AOX	264 ± 3	1744 ± 42†	6.6 ± 0.6†

Value are average ± St error

CONTR control, *IR* irradiation, *AOX* antioxidant cocktail

*$p < 0.05$ vs CONTR, †$p < 0.05$ IR + AOX vs IR

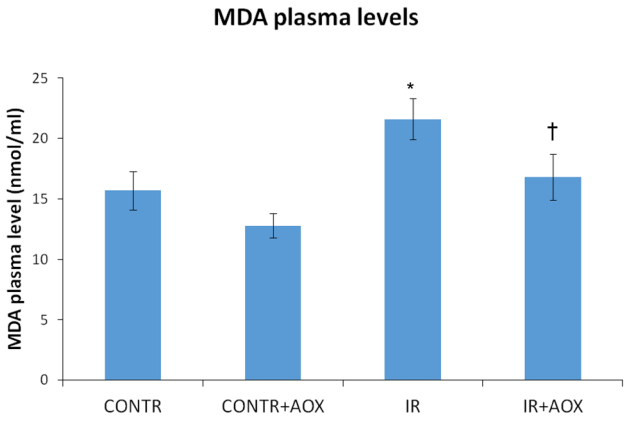

Fig. 1 The effect of whole body gamma irradiation and administration of antioxidant cocktail on plasma MDA levels. MDA levels were measured in serum by TBARS assay. Values are mean ± SEM. *CONTR* control, *IR* irradiation, *AOX* antioxidant cocktail. *$p < 0.05$ vs control, †$p < 0.05$ IR-AOX vs IR

stress. IR-AOX rats (Group D) demonstrated a significant decrease in MDA plasma levels compared to IR-nontreated animals (Group C) (16.8 ± 1.9 vs 21.6 ± 1.7 nmol/ml, $p < 0.05$).

Testicular parameters of spermatogenesis

Histological examination using light microscopy showed that the control rats demonstrated normal testicular tissue with no changes in the ultrastructure configuration. Treatment of control rats with AOX cocktail (Group B) did not change significantly testicular architecture and showed unchanged parameters of spermatogenesis (Fig. 2). The irradiated rats (Group C) have demonstrated a degeneration of sertoli cells that contain swelling mitochondria, degeneration of spermatids and cluster of spermatids with a characterized chromosomal "cap". IR rats (Group C) demonstrated a significant decrease in mean testicular score (Johnsen criteria) (8.5 ± 0.2 vs 9.3 ± 0.1, $p < 0.05$) and a trend toward a decrease in the number of germ cell layers compared to control rats; however, this trend was not statistically significant. Treatment of irradiated rats with antioxidant cocktail (Group D) resulted in less severe testicular damage that was accompanied by a significant increase in germ cell layer count (threefold, $p < 0.05$) and in the mean testicular score (Johnsen's criteria) (8.8 ± 0.08 vs 8.5 ± 0.2, $p < 0.05$) compared to IR animals (Group C).

Evaluation of germ cell apoptosis

Figure 3 represents data concerning germ cell apoptosis in all experimental groups. As expected, control rats (Group A) exhibited a low apoptotic index in both testes. Treatment with AXO cocktail of control rats (Group B) did not change significantly germ cell apoptosis compared to control untreated animals. γ-radiation (Group C) resulted in a significant increase in programmed germ cell death, expressed as the number of apoptotic cells per 100 tubules (AI-1, fivefold increase, $p < 0.001$) and the number of positive tubules per 100 tubules (AI-2, threefold increase, $p < 0.001$). Treatment with AOX (Group D) resulted in a significant decrease in germ cell apoptosis, expressed as the number of apoptotic cells per 100 tubules (AI-1, 33% increase, $p < 0.05$)

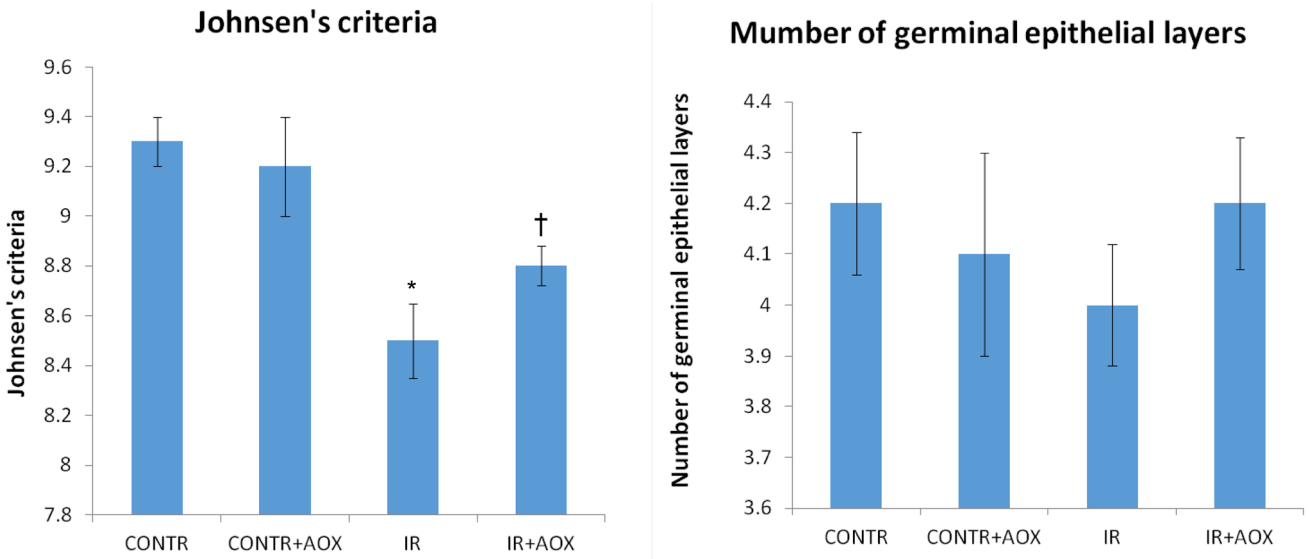

Fig. 2 The effect of whole body gamma irradiation and administration of antioxidant cocktail on spermatogenesis. The number of germinal cell layers and Johnsen's score were used to categorize the spermatogenesis. Values are mean ± SEM. *CONTR* control, *IR* irradiation, *AOX* antioxidant cocktail. *$p < 0.05$ vs control, †$p < 0.05$ IR-AOX vs IR

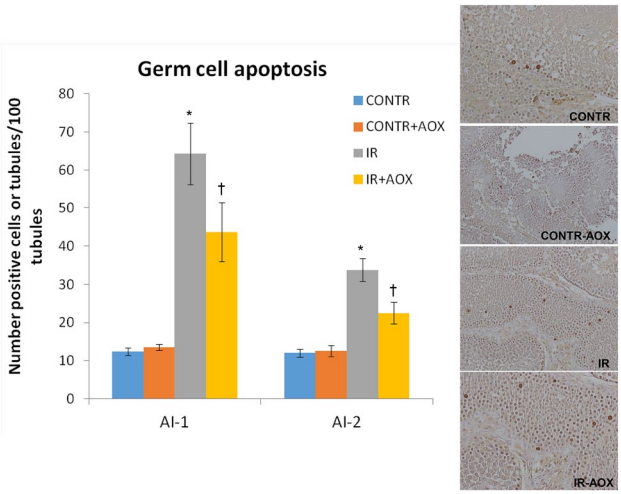

Fig. 3 The effect of testicular irradiation and antioxidant therapy on germ cell apoptosis [apoptotic cells per 100 tubules (AI-1) and positive tubules per 100 tubules (AI-2)]. Immunohistochemistry for apoptosis in the four experimental groups was assessed using TUNEL assay. Single apoptotic cells are present in sections from control and control-AOX rats. Labeled cells are frequently found in IR rats. The number of apoptotic cells decreases following administration of AOX. Values are mean ± SEM. *CONTR* control, *IR* irradiation, *AOX* antioxidant cocktail. *$p < 0.05$ vs control, †$p < 0.05$ IR-AOX vs IR

and the number of positive tubules per 100 tubules (AI-2, 33% increase increase, $p < 0.001$) compared to IR nontreated animals (Group C).

Discussion

Radiotherapy remains a treatment mainstay for many malignancies in men of reproductive age. However, exposure to high-dose ionizing irradiation, such as in radiation therapy or incidental radiation exposure, can cause injuries in susceptible tissues. There is extensive and growing literature on the temporal and spatial characterization of ionizing irradiation–induced tissue injuries. Radiation induces genetic and epigenetic changes that result in altered immune system, abnormal brain development with resultant cognitive impairment, cataractogenesis, abnormal embryonic development, circulatory diseases, weight gain, premature menopause in female animals, tumorigenesis and shortened lifespan [11]. On a cellular level, the primary cause of cellular injury by ionizing irradiation includes DNA damage, in which severely damaged cells commit to apoptosis [12, 13].

Apoptosis, or programmed cell death, is an evolutionarily conserved and highly regulated process of nonfunctional cells' death. It is a physiologic process whereby the body disposes of unwanted cells by self-destruction and is our utmost defense against damaged cells. Germ cell apoptosis has been reported by several investigators to play an important role in the normal testicular physiology. Programmed germ cell death is crucial during embryonic development of the human gonads [14]. Apoptosis control is important for regulation of the population of germ cells in the adult testis [15]. Recent evidence suggests that enhanced germ cell apoptosis is related to a decrease of germ cell mass in the aged testis with impaired spermatogenesis [16]. Besides its role in normal testicular physiology, apoptosis has been identified as important in the development of a variety of testicular disorders including undescended testes [17], varicocele [17] and testicular ischemia–reperfusion [18].

In addition to stimulating cell apoptosis, ionizing irradiation also results in the generation of reactive free-radical species, which attack vital cellular components and can cause cell death including germ cells. Antioxidants are proposed as a biological protection agents against radiation damage in humans [19]. Antioxidants can act at several different stages in an oxidative sequence; by removing oxygen or decreasing local O_2 concentrations, scavenging initiating radicals, quenching or scavenging singlet oxygen, breaking the chain of an initiated sequence and more [20]. The range of antioxidant defense mechanisms available within the cell and extracellularly should be adequate to protect against oxidative damage. However, the balance can be lost because of overproduction of free radicals by exposure to sources that overwhelm the antioxidant defense, such as X-ray and gamma ray radiation. Therefore, the extent of tissue damage is the result of the balance between the free radicals generated and the antioxidant protective defense system [21]. For the antioxidants to protect cells from primary free radical damage, they need to be present in the tissue at the time of radiation and in sufficient concentration. Many antioxidants were found to protect against the harmful effects of ionizing radiation when administered prior to exposure [22].

The purpose of the present study was to evaluate whether treatment with a new antioxidant cocktail including ten antioxidants (water-soluble and lipid-soluble molecules) could affect germ cell apoptosis following whole body gamma irradiation (Tc-99m Pertechnetate, 6 Gy) in a rat model.

To determine oxidative stress we assessed levels of MDA, oxidative stress marker, in blood plasma. Our data show that CONTR-AOX rats demonstrated a trend toward decrease in MDA plasma levels compared to control animals; however, this trend was not statistically significant. Whole body radiation (WBIR, 6 Gy) resulted in a significant increase in MDA plasma levels compared to control rats suggesting elevated oxidative stress. The elevation of MDA level may be due to the effect of O^{2-}, H_2O_2, OH and $ONOO^-$ radicals which interact with polyunsaturated fatty acids in the phospholipids of cell membrane inducing LPO in testicular tissue [23]. Treatment of irradiated animals with antioxidant cocktail resulted in a significant decrease

in MDA plasma levels compared to IR-nontreated animals suggesting antioxidative effect.

Our data demonstrate that administration of antioxidant cocktail in normal rats did not change significantly germ cell apoptosis and histologic criteria of compared to control untreated animals. Both control and control-AOX rats showed normal histologic architecture of the seminiferous tubules in testis. Consistent with literature data [4, 5], whole body gamma irradiation induced degeneration of germ cells and impaired spermatogenesis. A marked decrease in the average number of germinal epithelial cell layers and Johnsen's criteria in the irradiated rats support this conclusion. The irradiated rats showed degeneration of sertoli cells that contain swelling mitochondria, degeneration of spermatids and cluster of spermatids with a characterized chromosomal "cap". The measurement of germ cell apoptosis in irradiated testis demonstrated an increase cell programmed death following testicular IR. This is evident from the increase in the number of apoptotic cells per 100 tubules (AI-1) and the number of tubules containing apoptotic cells per 100 tubules (AI-2) compared to the control animals. These results agree with the previous studies which reported that irradiation induced DNAF activates p53, increases Bax (pro-apoptotic) and decreases Bcl2 protein expression (antiapoptotic), activates pro-caspases and stimulates apoptosis [24, 25].

Treatment with antioxidant cocktail prevents damage caused by testicular irradiation and improved recovery of testicular tissue. Antioxidant treatment resulted in a significant decrease in the germ cell apoptosis. This is evident from the decrease in the number of apoptotic cells per 100 tubules (AI-1) and the number of tubules containing apoptotic cells per 100 tubules (AI-2) compared to the IR-untreated animals. With the observed decrease in germ cell apoptosis, the majority of seminiferous tubules in this group maintain normal architecture. Further experiments are needed to clarify the mechanisms of the affect of antioxidant treatment on germ cell apoptosis following whole body irradiation. Decreased production of prostanoids that are involved in signal transduction pathways activated by distinct interleukins as well as a decrease in production of nitric oxide may be responsible of this anti-apoptotic effect of antioxidant therapy on germ cell apoptosis after irradiation.

In conclusion, the use of antioxidant cocktail produces a strong inhibitory effect on germ cell programmed death, prevents testicular damage and improves spermatogenesis in a rat model of whole body gamma irradiation. The use of antioxidant therapy may be beneficial in preserving germ cell mass and in preventing fertility loss after ionizing irradiation therapy in cancer patients.

References

1. Mantel F, Flentje M, Guckenberger M (2013) Stereotactic body radiation therapy in the re-irradiation situation–a review. Radiat Oncol 5:8:7–10
2. Sun Z, Ng KH (2012) Use of radiation in medicine in the Asia–Pacific region. Singap Med J 53(12):784–788
3. Jahnukainen K, Ehmcke J, Hou M, Schlatt S (2011) Testicular function and fertility preservation in male cancer patients. Best Pract Res Clin Endocrinol Metab 25(2):287–302
4. Marjault HB, Allemand I (2016) Consequences of irradiation on adult spermatogenesis: between infertility and hereditary risk. Mutat Res 770(Pt B):340–348
5. Ståhl O, Eberhard J, Jepson K (2006) Sperm DNA integrity in testicular cancer patients. Hum Reprod 21:3199–3205
6. Shalet SM, Tsatsoulis A, Whitehead E, Read G (1989) Vulnerability of the human Leydig cell to radiation damage is dependent upon age. J Endocrinol 120:161–165
7. Limon-Pacheco J, Gonsebatt ME (2009) The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. Mutat Res 674:137–147
8. Cadet J, Mouret S, Ravanat JL, Douki T (2012) Photoinduced damage to cellular DNA: direct and photosensitized reactions. Photochem Photobiol 88:1048–1065
9. Miller DC, Peron SE, Keck RW, Kropp KA (1990) Effects of hypothermia on testicular ischemia. J Urol 143:1046–1048
10. Johnsen SG (1970) Testicular biopsy score count—a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. Hormones 1:2–25
11. Tang FR, Loke WK, Khoo BC (2017) Low-dose or low-dose-rate ionizing radiation-induced bioeffects in animal models. J Radiat Res 2017 58(2):165–182
12. Harms-Ringdahl M, Nicotera P, Radford IR (1996) Radiation induced apoptosis. Mutat Res 366:171–179
13. Lavin MF (1998) Radiation-induced cell death and its implications in human disease. Res Probl Cell Differ 24:213–232
14. Modi DN, Sane S, Bhartiya D (2003) Accelerated germ cell apoptosis in sex chromosome aneuploid fetal human gonads. Mol Hum Reprod 9:219–225
15. Bartke A (1995) Apoptosis of male germ cells, a generalized or cell type-specific phenomenon. Endocrinology 136:3–4
16. Kimura M, Itoh N, Takagi S, Sasao T, Takahashi A, Masumori N, Tsukamoto T (2003) Balance of apoptosis and proliferation of germ cells related to spermatogenesis in aged men. J Androl 24:185–191
17. Hikim AP, Lue Y, Yamamoto CM, Vera Y, Rodriguez S, Yen PH, Soeng K, Wang C, Swerdloff RS (2003) Key apoptotic pathways for heat-induced programmed germ cell death in the testis. Endocrinology 144:3167–3175
18. Sukhotnik I, Voskoboinik K, Lurie M, Bejar Y, Coran AG, Mogilner JG (2009) Involvement of the bax and bcl-2 system in the induction of germ cell apoptosis is correlated with the time of reperfusion after testicular ischemia in a rat model. Fertil Steril 92(4):1466–1469
19. Löbrich M, Rief N, Kühne M, Heckmann M, Fleckenstein J, Rübe C, Uder M (2005) In vivo formation and repair of DNA double-strand breaks after computed tomography examinations. Proc Natl Acad Sci USA 102:8984–8989
20. Gutteridge JMC (1994) Biological origin of free radicals, and mechanisms of antioxidant protection. Chem Biol Interact 91:133–140
21. Machlin LJ, Bendich A (1987) Free radical tissue damage: protective role of antioxidant nutrients. FASEB J 1(6):441–445
22. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, Durazo-Arvizu RA, Gallagher JC, Gallo RL, Jones G, Kovacs

CS, Mayne ST, Rosen CJ, Shapses SA (2011) The 2011 report on dietary reference intakes for calcium and vitamin D from the institute of medicine: what clinicians need to know. J Clin Endocrinol Metab 111(4):524–527

23. Shaban NZ, Helmy MH, El-Kersh MA, Mahmoud BF (2003) Effects of *Bacillus thuringiensis* toxin on hepatic lipid peroxidation and free-radical scavengers in rats given alpha-tocopherol or acetylsalicylate. Comp Biochem Physiol C Toxicol Pharmacol 135:405–414
24. Batista LF, Kaina B, Meneghini R, Menck CF (2009) How DNA lesions are turned into powerful killing structures: insights from UV-induced apoptosis. Mutat Res 681:197–208
25. Borrás C, Gómez-Cabrera MC, Viña J (2011) The dual role of p53: DNA protection and antioxidant. Free Radic Res 45:643–652