Radioprotective effects of dexmedetomidine on X-ray-induced testicular damage

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Abstract. – OBJECTIVE: Approximately 70% of cancer patients require radiotherapy. However, despite its effectiveness in the treatment of cancer, radiotherapy can also affect and damage surrounding healthy tissues in addition to tumorous tissues. Since testicular tissues are highly radiosensitive, radiotherapy can cause impairments in spermatogenesis leading to infertility. The purpose of this study was to examine the potential radio-protective effect of dexmedetomidine (Dex), an α2-adrenoceptor agonist, on oxidative stress and apoptosis in testicular tissues caused by x-irradiation in rats.

MATERIALS AND METHODS: Thirty male Sprague-Dawley rats were allocated into three groups of ten (n=10): control, irradiation (IR), and IR + Dex groups. The IR group was exposed to a single dose of 2 Gy IR. The IR+Dex group was given a single intraperitoneal (i.p.) dose of 100 µg/kg Dex before IR. The control group received a single dose of saline solution i.p. Testicular tissues removed 24 hours after IR were subjected to histochemical, biochemical, and immunohistochemical analysis.

RESULTS: IR resulted in increased malondialdehyde (MDA) activity and significant changes in testis tissues. However, the application of Dex elevated glutathione levels by preventing MDA formation. In addition, Dex decreased tubular epithelial apoptosis *via* elevated Cleaved Caspase-3 expressions.

CONCLUSIONS: Dex exhibited a radio-protective effect against lipid peroxidation and apoptosis caused by IR.

Key Words:
  Apoptosis, Dexmedetomidine, Oxidative stress, Radioprotection, Testis, X-irradiation.

Introduction

Cancer has been one of the most important human health problems. It is responsible for a large proportion of deaths worldwide[1]. The International Agency for Research on Cancer (IARC) estimates that the number of cancer-related deaths will exceed 13 million by 2030[2]. Radiotherapy (RT) is used to kill or control tumor cells using high-energy photons such as x-irradiation[3]. Clinical studies[4] have shown that approximately 70% of cancer patients require RT. However, despite its effectiveness in the treatment of cancer, RT can also affect and damage surrounding healthy tissues in addition to tumorous tissues. Radiation applied to the pelvic region in particular can cause impairment of reproductive organ structures and functions[5].

Since the seminiferous tubule cells in testes are constantly multiplying and differentiating, they are highly radiosensitive even at low doses of radiation[6]. RT-induced damage can occur by direct testicular or neighboring tissue IR[7]. Reactive oxygen species (ROS) mediate the deleterious effects of radiation in biological systems. An increase in ROS triggers oxidative stress by causing lipid peroxidation in cell membranes. Increasing lipid peroxidation and oxidative stress produce changes in antioxidant activities with the activation of antioxidant systems. DNA injury thus occurs in immature germ cells[8-10]. This then triggers apoptosis in the cells of the spermatogenic series[11]. Therefore, increased oxidative stress can result in a decrease in spermatogenesis, and even infertility[7].

Testicular tissue contains a variety of cells with varying degrees of radiosensitivity. The spermatogonia are highly radiosensitive and die at doses lower than 3 Gy in the differentiation period[9]. Infertility following IR is caused by apoptosis of spermatogonia[9]. Renal and testis tissue damage induced by RT mostly occurs *via* oxidative stress resulting from overproduction of reactive oxygen/nitrogen species (ROS/RNS) and a decrease in antioxidant levels.

Dexmedetomidine (Dex), a powerful selective α2-adrenoceptor agonist, is used as a sedative and analgesic in intensive and post-anesthesia care units[12]. Studies[13] investigating the antioxi-

dant characteristics of sedatives have shown that Dex causes a decrease in lipid peroxidation and improves the antioxidant enzyme levels. Various studies[14-17] have also reported that Dex suppresses systemic inflammation and oxidative stress while exhibiting a reduction in apoptosis.

This study employed histochemical, biochemical, and immunohistochemical methods to investigate the effects on oxidative stress and apoptosis of Dex in testicular damage induced by 2 Gy IR.

Materials and Methods

Animal Study

Forty male Sprague Dawley rats with an average weight of 290±8.5 g were used in this study. 10 of these were kept in reserve for anticipated loss due to IR. Throughout the experiment, the rats were treated in compliance with the Principles for the Use and Care of Laboratory Animals formulated in the Guideline for the Care and Use of Laboratory Animals published by the National Health Research Council and approved by the local Ethical Committee. Throughout the adaptation and experimental procedures, the rats were housed in rooms providing optimal temperature (22±2°C), humidity (55-60%), and a controlled light/dark cycle (12/12). *Ad libitum* access to standard rat chow and tap water was allowed throughout the experiment.

Animal experiments and procedures were performed by the national guidelines for the use and care of laboratory animals. The study protocol was approved by Institutional Animal Care Committee of Recep Tayyip Erdogan University (Approval No.: 2018/7, Approval date: 14/02/2018).

Experimental Protocol

The sample size of our study was calculated in accordance with the studies of Charan and Kantharia[18]. The rats were allocated randomly into 3 groups of 10.

The control group was given a single dose of 1 ml of saline solution only. The rats in the IR group were given a single dose of 2 Gy ionizing radiation to the abdominopelvic region. The rats in the IR+Dex group were given 100 µg/kg Dex (Mediteria Group, USA), 30 min before the application of a single dose of 2 Gy ionizing radiation to the abdominopelvic region. Animals from all groups were euthanized with lethal dose of anesthetics 24 hours after IR along with the controls. Testes were removed from the rats in all groups for use in histopathological and biochemical analyses.

IR Procedure

Before X-ray IR, a single dose of 50 mg/kg ketamine and 5 mg/kg xylazine was administered by intramuscular injection. The dose and exposure time of IR in rats were calculated using the treatment planning program of CMS XIO, version 5.0 (Maryland Heights, MO, USA). The calculation was performed with the convolution algorithm. Rats from the control group were placed in the prone position under anesthesia, but no IR was applied. Rats from the IR and IR+Dex groups were also placed in the prone position. The IR areas, the pelvic region together with the testes, were determined using the isometric method from the posterior and anterior with a 0- and 180-degree gantry. A tissue-equivalent bolus was then placed on the anterior and posterior aspects of the rats, and the SSD was adjusted to half depth, at 98.5 cm. 2 Gy single fraction radiation was applied to the selected regions schedules using a linear accelerator (Elekta, Synergy, Stockholm, Sweden model).

Biochemical Procedures

Blood was removed from the testes by rinsing with cold phosphate-buffered saline (PBS, pH: 7.4). PBS, twice the tissue weight, was subsequently added. The tissues were then homogenized for 5 min using a 30 Hertz homogenizer. Finally, the homogenates were centrifuged for 15 min at 3,000 g, and the resulting supernatants were used for biochemical study.

Malondialdehyde (MDA) Level Assay

This was carried out as described elsewhere[19]. Briefly, to 1,000 µL homogenate supernatant an equal amount of thiocarboxylic acid (10%) was added. The mixture was then placed in a water bath at 94°C. Then, to 400 µL supernatant, 200 µL thiobarbituric acid (67%) was added, and the mixture was again placed in a 94°C water bath. A pink color complex formed as a result of the reaction, and this was read at 532 nm on a spectrophotometer.

Reduced Glutathione (GSH) Assay

The Ellman method was employed to measure glutathione levels[20]. Briefly, to 100 µL supernatant 400 µL 3M disodium hydrogen phosphate and 100 µL Ellman reagent were added. The yellow color-forming with the Ellman reagent was read on a spectrophotometer at 412 nm. The results were expressed as nmol/g tissue.

Table I. Tubular damage score modified by Johnsen's Score.

Grade	Findings
1	Absence of spermatogonia and spermatogenetics cells.
2	Absence of spermatogonia and spermatogenetics cells, whilst Sertoli cells are present.
3	Presence of spermatogonia only.
4	Only a few (<5) spermatozoa are available. Spermatids and spermatozoa are absent.
5	Spermatids and spermatozoa are absent. However, numerous spermatocytes are present.
6	Only a few spermatids (<5-10) are available, but spermatids and spermatozoa are lacking.
7	Numerous spermatids are available. However, spermatozoa do not exist.
8	Only a few spermatozoa (<5-10) are available.
9	Numerous spermatozoa are present. The germinal epithelium is decomposed with marked accumulation in the lumen.
10	Normal spermatogenesis with many spermatozoa. Germinal epithelium is well organized and has a regular thickness.

Histopathological Analysis

The testicular tissues were trimmed to a volume of 1.5 cm^3 and fixed for 24 h in Bouin solution. Specimens were then subjected to routine histological tissue processing using a tissue processor (Citadel 2000, Shandon, Thermo Scientific, Germany) and were embedded into paraffin blocks. 4-5 μm thick sections were then taken from the blocks using a rotary microtome (RM 2525, Leica Biosystems, Germany), and these were stained with Harris hematoxylin (Merck GmbH, Darmstadt, Germany) and Eosin G (Merck GmbH, Darmstadt, Germany) (H and E). Tissue sections were evaluated under a light microscope (Olympus Inc., Japan) attached to a digital camera (Olympus Inc., Japan) using the Johnsen's score system by two histopathologists blinded to the experimental groups[21].

Immunohistochemical Analysis

Tissue sections for immunohistochemical analysis were fixed in 10% neutral formalin for 24 h. The sections were then subjected to routine histological procedures in a processor (Citadel 2000, Shandon, Thermo Scientific, Germany) before embedding in paraffin blocks. 2-3 μm sections were cut using a rotary microtome (RM 2525, Leica Biosystems, Germany) and placed onto positively charged glass slides. Apoptotic cells in the testicular tissues were identified using the terminal deoxynucleotidyl transferase dUTP nick end labeling method (TUNEL, ab206386, Abcam, Boston, MA, USA).

Semi-Quantitative Analysis

Tissue sections stained with H+E were scored using the Johnsen's tubular damage scoring method in testicular tissues (Table I). The semi-quantitative analysis was done by two different histopathologists who were blinded to study groups.

Cells of the spermatogenetic series in the testicular tissue stained by the tunnel method were scored in thirty randomly selected areas for each rat by blinded histopathologists (Table II).

Statistical Analysis

Statistical data were analyzed using the SPSS 18.00 software (SPSS Inc., Chicago, IL, USA). Data distribution was analyzed using the Shapiro-Wilk Q-Q Plot, Skewness-Kurtosis and Levene's test analyses. Biochemical parametric data were calculated as mean ± standard deviation. One-Way ANOVA followed by the Tukey HSD test was used to evaluate intergroup differences. Data obtained from semi-quantitative analyses were calculated as median 25%-75% interquartile range. The differences between the experimental groups were calculated using the Kruskal Wallis followed by the Tamhane T2 test ($p < 0.05$ was considered statistically significant).

Table II. Apoptosis score.

Grade	
0	Lower than 5%
1	6-25%
2	26-50%
3	51-75%
4	Higher than 76%

Table III. Biochemical analysis.

Group	MDA (nmol/mg prt)	GSH (nmol/mg prt)
Control	0.17±0.64	399±4.51
Irradiation (IR)	0.34±0.89[a]	300±6.8[a]
IR+Dex	0.24±0.66[b,c]	885±9.87[a,c]

[a]p=0.000; *vs.* control; [b]p=0.012; *vs.* control; [c]p=0.002; *vs.* IR. One-Way ANOVA/Tukey HSD.

Results

Biochemical Analysis

While the MDA levels increased in the irradiated group (Table III; p=0.000), they decreased in the IR+Dex group compared to the control (Table III; p=0.012).

Similarly, the GSH levels increased in the IR group compared to the control (Table III; p=0.000) as they did in the Dex group compared to the IR group (Table III; p=0.000).

Histopathological Analysis

Light microscopic examination of the sections from the control group revealed that the seminiferous tubules were filled with cells of the spermatogenic series consisting of normal spermatogonia, spermatocytes, spermatids, and spermatozoa [Figure 1A-B, Table IV, Johnsen's score: 10 (9-10)]. In contrast, sections of the irradiated group exhibited widespread edematous areas accompanying a significant decrease in spermatogenic cell numbers [Figure 1C-D, Table IV, Johnsen's score: 4 (3-4)]. In the Dex treatment group, cells of the spermatogenic series consisting of typical spermatogonia, spermatocytes, spermatids, and spermatozoa were observed in the seminiferous tubules [Figure 1E-F, Table IV, Johnsen's score: 9 (8-9)].
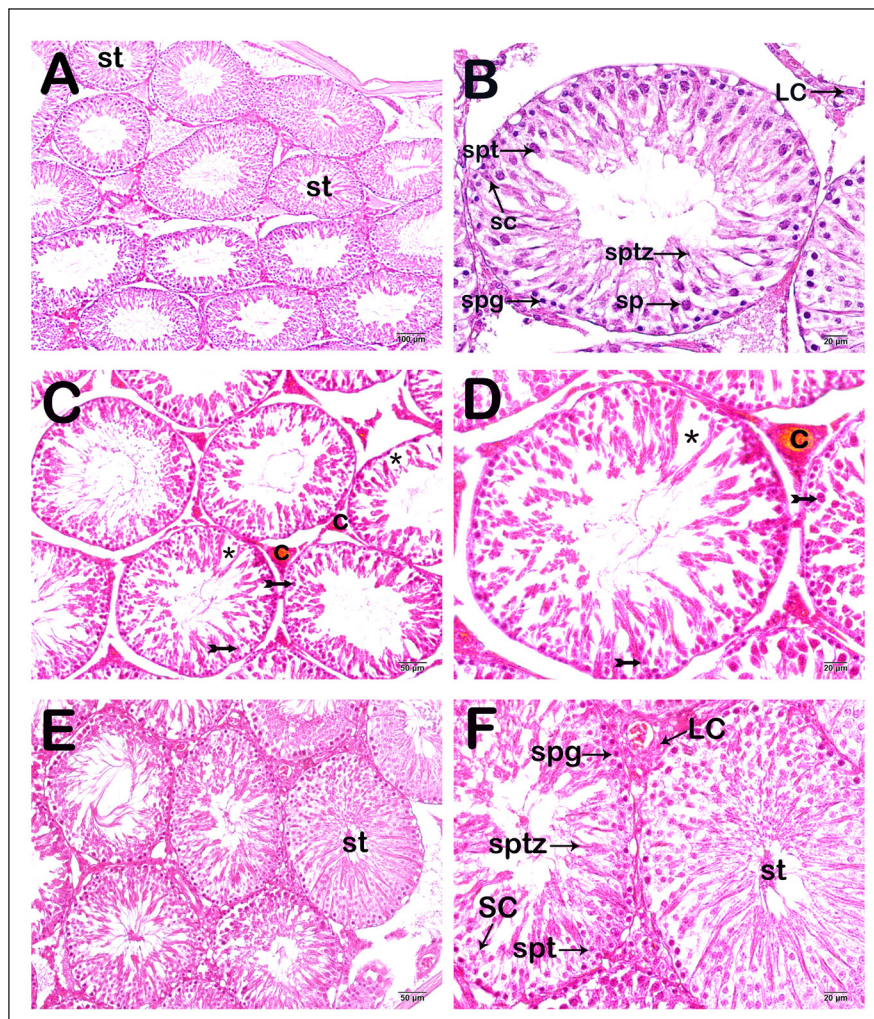
Figure 1. Light microscopic image of testis tissue sections stained with H+E. Seminiferous tubules (st), Spermatogonia (spg), Spermatid (spt), Spermatozoa (sptz), Sertoli cell (SC), Leydig cells (LC). **A-B**, Normal seminiferous tubules consisting of spermatogonia (spg), spermatocytes (spt), and spermatozoa (spt) in sections from the control group [Johnsen's damage score: 10 (9-10)]. Magnification: A, x20; B, x40. **C-D**, Widespread edematous areas (*) in seminiferous tubules associated with loss of spermatogonia, spermatocytes, and spermatozoa in the irradiation group. Diffuse vascular congestion (C) can also be seen in interstitial areas [Johnsen's damage score: 4 (3-4)]. Magnification: C, x20; D, x40. **E-F**, Seminiferous tubules consisting of normal spermatogonia (spg), spermatocytes (spt), and spermatozoa (spt) in sections from the Dex application group [Johnsen's damage score: 9 (8-9)]. Magnification: E, x20; F, x40.

Table IV. Johnsen's modified tubular damage score results.

GGroup	Score [Median (25-75% interquartile range)]
Control	10 (9-10)
IR	4 (3-4)[a]
IR+Dex	9 (8-9)[b]

[a]*p*=0.000; *vs.* the Control Group, [b]*p*=0.002; *vs.* the IR Group. Kruskal-Wallis – Tamhane's T2 test**.**

Table V. Apoptosis Score [Median (25-75% interquartile range)].

Group	Apoptosis Score
Control	0.00 (0-0)
IR	3.00 (3-4)[a]
IR+Dex	1.00 (1-1.50)[b]

[a]*p*=0.000; *vs.* the Control Group, [b]*p*=0.002; *vs.* the IR Group. Kruskal-Wallis – Tamhane's T2 test.

Immunohistochemical (IHC) Analysis

Light microscopic examination of the control group revealed that the seminiferous tubules were filled with normal cells of the spermatogenic series by the TUNEL method [Figure 2A; Table V; Apoptosis score: 0 (0-0)]. In contrast, an increase in apoptotic spermatogenic cells was monitored in the IR group versus the control group [Figure 2B; Table V; apoptosis score: 3 (3-4)]. However, the numbers of apoptotic spermatogenic cells in the Dex group were reduced in comparison to the IR Group [Figure 2C; Table V; apoptosis score: 1 (1-1.5)].

Discussion

Despite being highly effective in the treatment of several types of cancers, RT can also damage healthy surrounding tissues along with the targeted tumorous tissue[22]. Testis tissues are highly radiosensitive and can be damaged following IR of the pelvis or the entire body[7]. Studies[23] have shown that the application of RT to male patients with malignancies leads to impairment of the normal spermatogenic cycle and that this can result in severe complications, such as infertility. This study is the first to investigate the effect of Dex on oxidative stress and apoptosis in X-ray-induced testis injury.

Although the mechanism by which RT causes damage to the testicular tissue is not fully understood, previous studies[11,24] showed that it is associated with an increase in ROS. Lipid peroxidation and oxidative stress, which cause an imbalance between the production of free oxygen radicals and antioxidant capacity, cause damage to biological macromolecules. This, in turn, leads to impairment of normal metabolism and physiology. ROS can attack polyunsaturated fatty acids in biological membranes and induce free radical chain reactions that lead to an increase in lipid peroxidation[24]. MDA, a product of lipid peroxidation, is a marker of oxidative stress[25]. In accordance with the present results, the application of RT caused an increase in MDA activity in the target tissues. Various
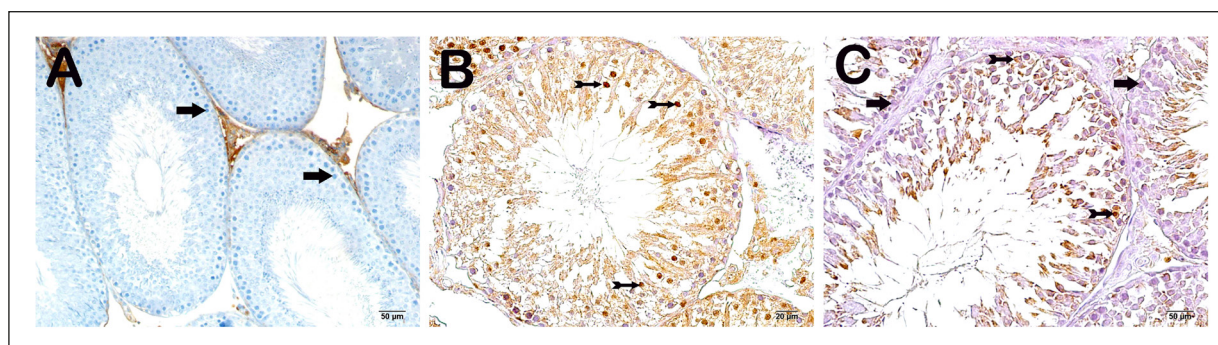
Figure 2. Representative light microscopic images of testicular tissue in which apoptotic cells are marked using the TUNEL method. **A**, Normal spermatogenetic cells in testis tissue from the control group *(arrow)* [apoptosis score: 0 (0-0)]. Magnification: x20. **B**, Apoptotic spermatogenetic cells in testis tissue from the IR group *(tailed arrow)* [apoptosis score: 3 (3-4)]. Magnification: x20. **C**, Decreased apoptotic spermatogenetic cells in testis tissue from the Dex group *(tailed arrow)* [apoptosis score: 1 (1-1.50)]. Magnification: x20.

researchers[9,24] have also reported that RT leads to lipid peroxidation in association with an enhancement in ROS.

The increase in ROS after RT leads to an increase in oxidant capacity and the activation of antioxidant enzyme systems[24]. Studies[9,11,26] have reported that the application of RT causes a decrease in levels of the antioxidant enzyme GSH. This study confirms that GSH activity decreased following IR.

Free radicals are known to cause cellular damage by decreasing membrane viscosity and permeability and by increasing membrane protein denaturation. ROS forming after RT trigger structural damage in tissues[24]. Naeimei et al[27] demonstrated that 2 Gy IR causes damage in cells of the spermatogenic series in the testes and thinning of the germinal epithelial wall. Ekici et al[9] also showed that IR led to impairment in the seminiferous tubular structures, vacuolization, and necrosis. Similarly in the present study, 2 Gy IR caused a decrease in spermatogenic cells and led to vascular congestion and widespread edematous areas in the seminiferous tubules.

IR induced cellular damage results in ROS formation that can trigger apoptosis by caspase activation[28,29]. Caspase-3 is a protein whose levels rise following cell damage and is pivotal in the apoptotic process[30]. Previous studies[11,31] have reported that IR causes an increase in Caspase-3 levels in testis tissue. An increase in Caspase-3 immunoreactivity was also observed following IR in the present study.

Dex, a selective and potent α2-adrenoreceptor agonist, is currently used for its excellent sedation and analgesia with minimal cardiovascular effects[32]. Previous studies[33-35] noted the importance of the anti-inflammatory and anti-apoptotic effects of Dex. Wang et al[36] reported that the application of Dex reduced oxidative stress and apoptosis in an acute kidney injury model in mice. In addition, Tuglu et al[37] reported that Dex application exhibited an antioxidant effect in testicular ischemia-reperfusion injury.

Dex has been shown[32] to have a reducing effect on lipid peroxidation in various tissues. Wang et al[36] reported that Dex caused a decrease in MDA activity in acute kidney injury. Hanci et al[38] also reported that Dex application led to a decrease in MDA levels in a testicular torsion/detorsion model in rats. This study confirms that Dex exhibits inhibitory effect on lipid peroxidation by decreasing the MDA levels. In addition, Dex application also increases GSH levels. Various authors[13,37] have reported that Dex causes an improvement in antioxidant levels.

Due to its antioxidant activity, Dex is also known to play an ameliorating role in structural damage in various tissues[39,40]. However, no previous studies have examined the effectiveness of Dex in preventing IR induced testicular damage, which this study demonstrates.

Dex also exhibits anti-apoptotic effects[33,36,41,42,43]. In this study, too, it suppressed apoptosis in cells of the spermatogenic series.

Some of the limitations of this study include measurement of oxidative stress by additional parameters in randomized controlled studies.

Conclusions

This study shows that the α2-adrenoreceptor agonist Dex exhibits a protective effect by suppressing lipid peroxidation and apoptosis in testes induced by 2 Gy abdomino-pelvic irradiation.

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