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Circadian Clock on Ionizing Radiation-Induced Testicular Injury

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ABSTRACT

Objective: This research focuses on the interaction between the circadian clock and the damage caused to testicular tissue by x-ray ionizing radiation. By examining the links between circadian rhythms and radiation-induced testicular damage, a deeper understanding of the underlying mechanisms may emerge, potentially leading to new strategies to mitigate or prevent such damage and its subsequent consequences.

Methods: Twenty-four Sprague-Dawley rats were divided into 3 groups. Rats in group 1 (control group) did not undergo any procedures. Rats in group 2 (day) received 6 Gy total body external x-ray radiation in a single fraction between the hours 05:00 and 06:00. Rats in group 3 (night) received 6 Gy total body external x-ray radiation in a single fraction between the hours 19:00 and 20:00.

Results: The day group was compared with the control group, and a decrease in spermatogenetic cells and edematous areas was observed. In addition, there was vacuolar accumulation in the cytoplasm of spermatids in the germinal epithelium and necrotic Leydig cells in the intertubular spaces. In the night group, we observed that the changes observed in group 2 were significantly restored. In terminal deoxynucleotide transferase dUTP nick end labeling and 8-hydroxy-2′-deoxyguanosine immunohistochemical analyses, we observed significantly increased immunopositivity in group 2 compared to the control group and group 3.

Conclusion: In conclusion, it reveals that the circadian clock protects against testicular damage caused by x-ray ionizing radiation. By regulating DNA repair processes, antioxidant defense mechanisms, and other important pathways, the circadian clock appears to increase the resistance of testicular tissues to radiation stress.

Keywords: Chronoradiotherapy, circadian clock rat, testis, x-ray irradiation

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Introduction

The complex dance of life is regulated by a rhythmic synchronization of various biological processes managed by an internal timekeeping mechanism known as the circadian clock. This internal timekeeper regulates a multitude of physiological processes, such as the sleep–wake cycle, hormonal secretion, metabolism, and cell repair, to be consistent with the natural 24-hour day–night cycle.[1] Most recent scientific research has revealed that the circadian clock extensively affects various physiological systems, shedding light on its critical role in protecting overall health and well-being.[2]

The potential to modulate the effects of ionizing radiation on biological systems is an area of increasing interest. Ionizing radiations such as x-rays possess both beneficial and harmful effects on human health.[3] Although ionizing radiation offers very important advantages in medical diagnosis and cancer treatment, it also entails potential risks, especially when exposure occurs outside the boundaries of biological tolerance.[3] Ionizing radiations, such as x-rays, harm cells and tissues, including the testicles.[4] In the field of radiation biology, the complicated interaction between the circadian clock and the effects of ionizing radiation has received considerable attention.[5] One of the areas where the interaction between circadian rhythms and ionizing radiation becomes important is testicular health.[4] The testicles comprise an inseparable component of male reproductive function and are extremely susceptible to environmental effects such as ionizing radiation due to a high cell division rate.[6] While the harmful effects of ionizing radiation on testicular health have been documented, the role of the circadian clock in modulating these effects remains an interesting area of research. This relationship has great importance, as it not only broadens our understanding

of the basic mechanisms underlying radiation-induced testicular damage but also provides potential ways to ameliorate the effects of this damage.

This study investigates the relationship between the circadian clock and x-ray ionizing radiation-induced testicular damage. We aim to unravel the underlying mechanisms that determine the susceptibility of testicular tissues to ionizing radiation at different times of the day by exploring the complex connections between circadian rhythms, radiation exposure, and testicular physiology. We also want to contribute to the strategies for the optimization of radiation-based medical interventions that will minimize the potential effects and the risk of testicular damage with these findings in the context of both clinical applications and public health policies. Through this study, we hope to open the door to a more comprehensive understanding of circadian clock-mediated modulation of x-ray ionizing radiation-induced testicular damage and, ultimately, to contribute to advanced strategies concerning protecting male reproductive health.

Materials and Methods

Experimental Animals and Study Design

This study was performed in the Recep Tayyip Erdoğan University, Faculty of Medicine, Experimental Animal Research Laboratory in accordance with the Animal Research: Reporting of In Vivo Experiments guidelines regarding the care and use of experimental animals.[7] Approval for this study was granted by the Recep Tayyip Erdoğan University University animal experiments local ethical committee (Rize, Turkey) (approval date: August 29, 2023; approval number: 2023/38). This study used 24 3- to 4-month-old male Sprague-Dawley rats (n = 24) weighing 283 ± 20 g. All rats were kept in conditions of a 12-hour light/12-hour dark cycle, with a temperature and humidity of 55%-60% and free access to food and water. The radiation doses in this study were determined based on previous work on radiation-induced acute side effects.[8]

Twenty-four rats were randomly distributed into cages of 8. Randomization was performed using a computer-based number generator. Rats in group 1 (control group) did not undergo any procedures. Rats to undergo x-ray radiation were divided into 2 groups: a morning group and an evening group. At the time of the study, the time of sunrise was between 05:00 and 06:00, and the time of sunset was between 19:00 and 20:00 in our region. Group 2 (day group, n = 8) underwent 6 Gy total body irradiation in a single fraction between 05:00 and 06:00 (sunrise). Group 3 (night group, n = 8) underwent 6 Gy total body irradiation in a single fraction between 19:00 and 20:00 (sunset). The irradiation procedure was performed by administering 6 Gy total body external x-ray radiation in a single fraction through an anterior field using a 6 MV linear accelerator (Elekta, Crawley, UK) at 100 cm source to skin distance (SSD) under anesthesia. Prior to irradiation, conformal planning was performed using the CMS XiO planning system (version 13.2). The rats were sacrificed by decapitation under high-dose anesthesia (100 mg/kg ketamine HCl and 10 mg/kg xylazine HCl) 24 hours after irradiation. Right and left testicular tissues excised from the rats were placed in formalin solution for histopathological and immunohistochemical evaluations.

Histopathological Analysis

Testicular tissue samples excised from male Sprague-Dawley rats were placed in tissue-embedding cassettes and fixed in 10% neutral formalin solution (Sigma Aldrich, St. Louis, Mo, USA) for 24-36 hours. After fixation, testicular tissue samples were subjected to a routine histological tissue processing procedure that involved dehydration by passing through an ethanol series of ascending concentrations (Merck, Darmstadt, Germany) using a tissue processor (Shandon Citadel 2000, Thermo Fisher Scientific168, Third Avenue,Waltham, MA, USA 02451), clearing in 2 series of xylol solution (Merck), and embedding in soft and hard paraffin (Merck). Following the histological tissue processing procedure, the testicular tissue samples removed from the tissue processing cassettes were embedded in tissue embedding cassettes with hard paraffin using a tissue embedding center (Leica 115EG, Leica Biosystems, Ernst-Leitz-Strasse, 17-3735578, Wetzlar, Germany). From the resulting paraffin blocks of testicular tissue, sections of 4-5 µm were obtained using a rotary microtome (Leica RM2525, Leica Biosystems). The obtained testicular tissue sections were stained with Harris hematoxylin (Merck) and eosin G (Merck) using a histological stainer (Leica Biosystems, 5020ST).

Immunohistochemical Analysis

Anti-8-Hydroxy-2′-Deoxyguanosine Method: Testicular tissue sections were treated with anti-8-hydroxy-2′-deoxyguanosine 8-OHdG primary antibody (ab48508, Abcam, Cambridge, CB2 0AX, UK) kits. In addition, a Goat Anti-Mouse IgG H&L (HRP, ab205719, Abcam) consistent with the 8-OHdG primary antibody was used. In immunohistochemical analysis, a rotary microtome was used to obtain sections of 2-3 µm from paraffin blocks of testicular tissue, which were then deparaffinized. Using an immunohistochemical stainer (Leica Biosystems, Bond Max, Australia), testicular tissue sections were subjected to a secondary blocking solution to block endogenous peroxidase activity in accordance with the manufacturer's instructions manual. In the next stage, they were incubated with the primary antibody for 60 minutes. After the primary antibody treatment, tissue samples were incubated with the secondary antibody for 60 minutes. The tissues were counterstained with Harris hematoxylin (Merck) and mounted with an appropriate mounting solution.

MAIN POINTS

- The circadian clock is an internal, biological timing system that regulates various physiological and behavioral processes in living organisms, including humans.
- The circadian clock is synchronized with the 24-hour day–night cycle through external cues, primarily light and temperature changes.
- Radiation-induced testicular injury refers to damage to the testes (male reproductive organs) caused by exposure to ionizing radiation, such as x-rays or gamma rays.
- The timing of radiation exposure to the circadian clock can influence the severity of the injury.
- It is crucial to recognize the interplay between the circadian clock and radiation-induced testicular injury, as the timing of radiation exposure can impact the severity of damage and potential long-term consequences.

The Terminal Deoxynucleotide Transferase dUTP Nick End Labeling Method

In order to identify the apoptotic cells in the seminiferous tubules in rat testicular tissue samples, a terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) method-apoptosis detection kit (ab206386, Abcam) was used. After the deparaffinization and dehydration stages of testicular tissue sections, the TUNEL method was performed in accordance with the kit manufacturer's instructions manual. In the next stage, incubation with 3,3′-Diaminobenzidine (DAB) chromogen was followed by counterstaining with methylene green.

Semi-Quantitative Analysis

A testicular damage score (TDS) was calculated considering the Johnsen score, edematous areas, and findings of vascular congestion in testicular tissue sections with respect to previous studies on x-ray irradiation-induced tissue damage.[8,9] The scoring system used for evaluating testicular injury, known as TDS, was implemented according to the following criteria: 0 (mild; ≤5%), 1 (moderate; <25%), 2 (severe; <50%), and 3 (very severe; ≥50%) (Table 1).[9] Thirty randomly selected regions per rat testicular section were evaluated by 2 histopathologists blinded to the study groups.

Cells exhibiting TUNEL and 8-OHdG immunopositivity were evaluated using immunohistochemical techniques and assigned scores based on the extent of staining observed. The scoring system ranged from 0, indicating mild staining affecting ≤5% of cells, to 3, representing severe staining affecting ≥50% of cells. A score of 1 denoted moderate staining affecting less than 25% of cells, while a score of 2 indicated severe staining affecting less than 50% of cells.[8] Two histopathologists evaluated 30 randomly selected regions per rat testicular section. The histopathologists were blinded to the treatment groups.

Statistical Analysis

All data from the histopathological and immunohistochemical analyses of rat testicular tissues were computed using the Statistical Package for Social Science and Statistics software, version 20.0 (IBM Corp., Armonk, NY, USA). Median and 25% and 75% interquartile range values were calculated for the non-parametric data obtained from the analyses. Non-parametric data were analyzed using Mann–Whitney *U*-tests with Bonferroni correction for group differences ($P <$ .05 was accepted as significant). This explorative study performed in line with the Enhancing Quality in Preclinical Data (EQUIPD) guidelines, does not test a null hypothesis and aims to open the door for research questions that may generate new hypotheses.[10] Therefore, the *P*-value calculated in this study should not be interpreted as a hypothesis test but simply as a descriptive value due to the explorative nature of the study.

Table 1. Testis Damage Score

Score	Findings
Johnsen score rate (control group/treatment group)	
0	≤1
1	>1
2	≥2
3	≥3
Edema	
0	<5%
1	<25%
2	<50%
3	≥50%
Vascular congestion	
0	<5%
1	<25%
2	<50%
3	≥50%

Results

Histopathological Results

On light microscopic examination of rat testicular tissue sections, the control group had normal cells in the seminiferous tubules, including spermatogonia, primary spermatocytes, and spermatids. In addition, we observed extensive spermatozoa in the lumen of seminiferous tubules and normal Sertoli cells among germinal epithelial cells. Also, there were normal Leydig cells in intertubular spaces (Table 2-3, Figure 1A-B, TDS: 1 (1-2)). Testicular tissue sections of the day x-ray irradiation group showed a reduction in spermatocytes, most primarily spermatozoa and spermatids, along with the formation of edematous areas. In addition, we observed vacuolar accumulation in the cytoplasm of spermatids in the germinal epithelium. In intertubular spaces, necrotic Leydig cells were found (Table 2-3, Figure 1C-D, TDS: 6 (6-7)). On light microscopic examination of the testicular sections of the night x-ray irradiation group, we determined fewer necrotic spermatogenetic cells in the seminiferous tubules compared to the day x-ray group. On the other hand, spermatocytes, primarily spermatozoa and spermatids, had a typical structure. Besides spermatogenic cells, we also observed less necrosis in Sertoli cells and in the Leydig cells in intertubular spaces compared to the day x-ray group (Table 2-3, Figure 1E-F, TDS: 3.5 (3-4)).

Semi-Quantitative Analysis

In the testicular damage scoring, we performed to record the histopathological changes in rat testicular tissue sections, and the histopathological damage score that was measured as 1 (1-2) in the control group was found to increase to 6 (6-7) in the day x-ray irradiation group (Table 3, Figure 1A-D, $P = .001$). In contrast, the TDS that was measured as 6 (6-7) in the day x-ray irradiation group was as low as 3.5 (3-4) in the night x-ray irradiation group (Table 3, Figure 1C-F, TDS: 3.5 (3-4), $P = .001$).

Immunohistochemical Analysis

In order to identify the apoptotic cells in the seminiferous tubules, we marked the testicular tissue sections by applying the TUNEL method. In this context, we observed a greater number of apoptotic spermatocytes showing TUNEL positivity among the spermatogenetic cells of the day x-ray irradiation group (Table 4, Figure 2A-B, $P = .001$, TUNEL positivity score: 1.5 (1-1)). In contrast, the light microscopic

Table 2. Johnsen Testis Injury Score

Group	Johnsen Score Results (median: 25%-75% interquartile range)
Control	10 (10-10)
Day x-ray irradiation	5 (4-5)[a]
Night x-ray irradiation	8 (8-8)[a,b]

[a]$P = .001$ compared to the control group.
[b]$P = .001$ compared to day x-ray irradiation group.
The Mann–Whitney *U*-test with Bonferroni corrections.

Table 3. Testis Damage Score Results

Group	Johnsen Score Rate (Control Group/Treatment Group)	Johnsen Score Rate Score	Edema	Vascular Congestion	TDS
Control	1 (1-1)	**0 (≤1)**	(0-1)	(0-0)	1 (1-2)
Day x-ray irradiation	2 (2-2.5)[a]	**2 (≥2)[a]**	2 (2-2)[a]	2 (2-2)[a]	6 (6-7)[a]
Night x-ray irradiation	1.25 (1.25-1:25)[b]	**1 (>1)[a,b]**	1 (1-1)[a,b]	1 (1-1)[a,b]	3.5 (3-4)[a,b]

[a]*P* = .001compared to the control group.
[b]*P* = .001compared to day x-ray irradiation group.
The Mann–Whitney *U*-test with Bonferroni corrections.

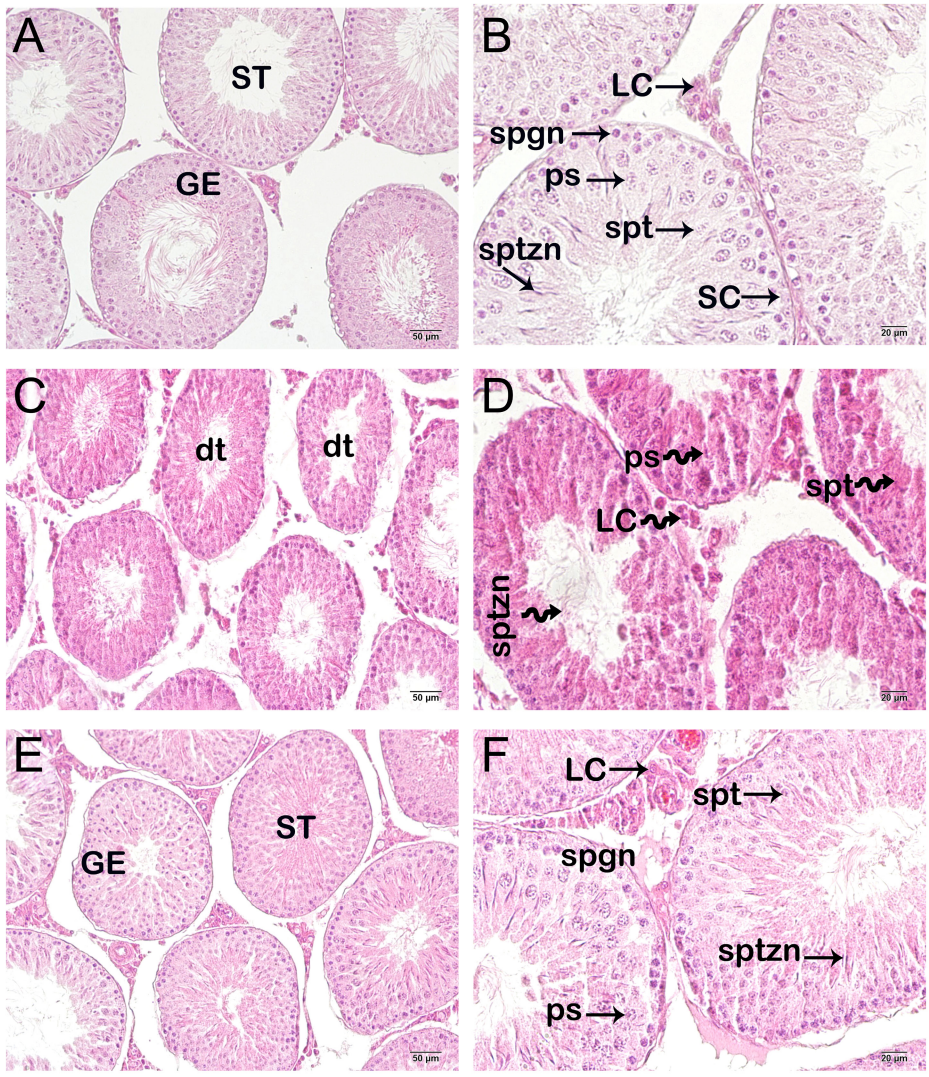

Figure 1. Representative light microscopic images of H+E-stained testicular tissue sections. A(×20)-B(×40) control group: Representative light microscopic screen capture of testicular tissue showing normal seminiferous tubular structures composed of germinal epithelial cells, including spermatogonia, primary spermatocyte, and spermatids. Lumen of seminiferous tubules exhibiting the extensive presence of normal spermatozoa. Also, normal Sertoli cells among germinal epithelial cells and normal Leydig cells in intertubular spaces are observed (testis damage score: 1 (1-2)). C(×20)-D(×40) day x-ray irradiation group: A decrease in spermatocytes, primarily spermatozoa (spiral arrow—sptzn) and spermatids (spiral arrow—spt), was observed in seminiferous tubules. There is also vacuolar accumulation in the cytoplasm of a few necrotic spermatids found in the germinal epithelium. In addition, necrotic Leydig cells are found in the intertubular spaces (spiral arrow—LC) (testis damage score: 6 (6-7)). E(×20)-F(×40) night ×-ray irradiation group: Fewer necrotic spermatogenetic cells are found in the seminiferous tubules. Moreover, spermatocytes, primarily spermatozoa (sptzn) and spermatids, of typical structure are observed. Along with spermatogenetic cells, Sertoli cells and the Leydig cells in intertubular spaces are observed to have a typical structure. GE, germinal epithelium; LC, Leydig cell; ps, primary spermatocytes; SC, Sertoli cell; spgn, spermatogonium; spt, spermatids; sptzn, spermatozoon; ST, seminiferous tubules.

evaluation of the sections of the night x-ray irradiation group determined relatively fewer apoptotic spermatogenetic cells showing TUNEL positivity in the seminiferous tubules compared to the day x-ray irradiation group (Table 4, Figure 2B-C, $P = .001$, TUNEL positivity score: 0 (0-1)).

Upon the incubation of the spermatogenetic cells found in the seminiferous tubules of rat testicular tissue with 8-OHdG primary antibody, we observed a greater number of 8-OHdG-positive spermatocytes among the spermatogenetic cells of the day x-ray irradiation group (Table 4, Figure 3A-B, $P = .001$, 8-OHdG positivity score: 2.5 (2-3)). In contrast, the light microscopic evaluation of the sections of the night x-ray irradiation group determined relatively fewer apoptotic spermatogenetic cells showing 8-OHDG positivity in the seminiferous tubules compared to the day x-ray irradiation group (Table 4, Figure 2B-C, $P = .001$, 8-OHdG positivity score: 1 (0-1)).

Discussion

Given the susceptibility of the testicles to radiation-induced damage, the protection of male reproductive health against exposure to ionizing radiation is a highly important matter. The present study highlights the potential benefits of chronoradiotherapy in ameliorating x-ray ionizing radiation-induced testicular damage by reducing apoptosis and inflammation.

Ionizing radiation, an example of which is x-rays, can release high-energy particles that can penetrate through cells, disrupt their genetic materials, and trigger stages of response.[11] Among susceptible targets are the male testicles, which are critical for fertility and house the germ cells with a high division rate.[4,12] Since the cellular consequences of radiation exposure, including apoptosis (programmed cell death) and oxidative stress, have been widely researched, a new dimension concerning the interaction between these effects and the circadian clock has come to light.[13] Apoptosis, a fundamental process for cell development and maintenance, is reinforced by radiation stress and generally results in significant tissue damage.[14] At the same time, oxidative stress, characterized by an imbalance of reactive oxygen species and cellular antioxidants, contributes to DNA damage, inflammation, and, ultimately, organ dysfunction.[15] At the center of this process lies the 8-OHdG molecule, which is a marker of oxidative DNA damage that serves as an indicator of genetic instability.[16] This study demonstrates x-ray ionizing radiation-induced apoptosis and oxidative stress in testicular tissues immunohistochemically via an increase in TUNEL and 8-OHdG immunopositivity.

Table 4. Immunohistochemical Positivity Results

Group	TUNEL Positivity Score	8-OHdG Positivity Score
Control	0 (0-0)	0 (0-0)
Day x-ray irradiation	1.5 (1-1)[a]	2.5 (2-3)[a]
Night x-ray irradiation	0 (0-1)[b]	1 (0-1)[b,c]

Median: 25-75% interquartile range. 8-OHdG, 8-hydroxy-2′-deoxyguanosine; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.
[a]$P = .001$compared to the control group.
[b]$P = .001$compared to day x-ray irradiation group.
[c]$P = .001$compared to the control group.
Mann–Whitney *U*-test with Bonferroni corrections.

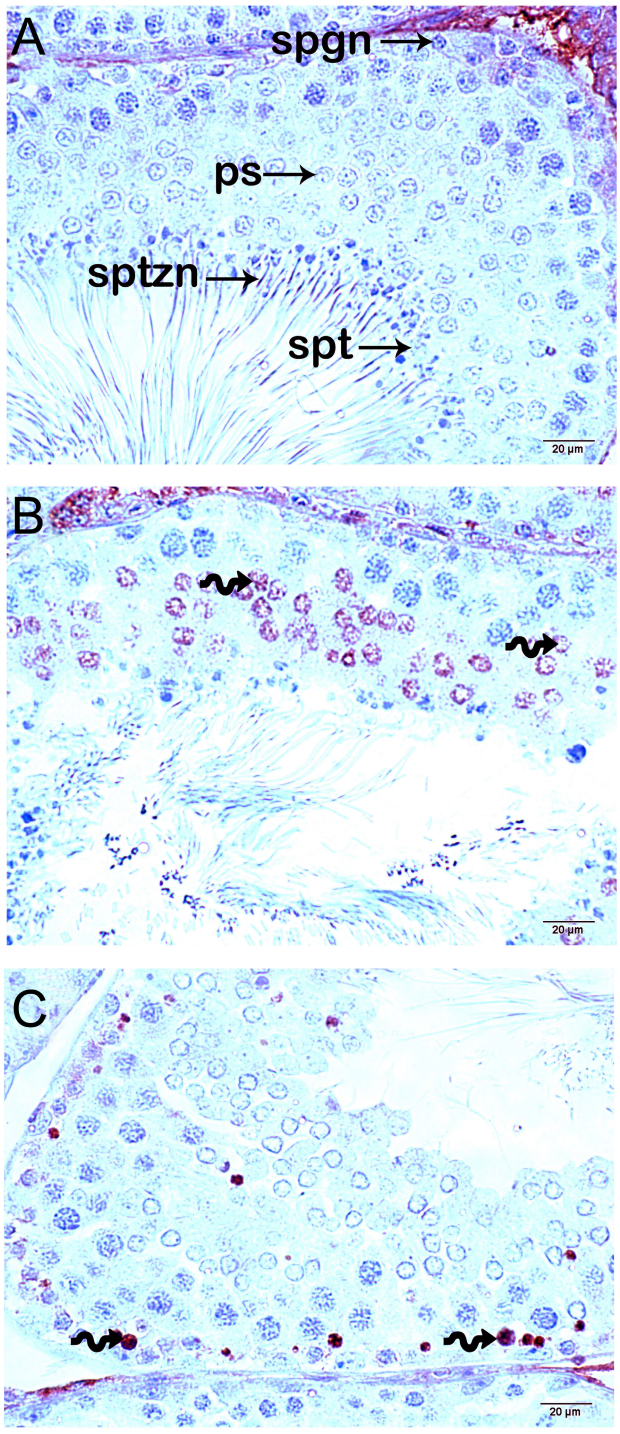

Figure 2. Representative light microscopic images of apoptotic cells marked by the TUNEL method. A(×40) control group: Representative light microscopic screen capture of testicular tissue showing normal spermatogonia, primary spermatocytes, spermatids, and spermatozoa that are immunonegative (TUNEL positivity score: 0 (0-0)). B(×40) day x-ray irradiation group: Spermatogenetic cells show apoptotic spermatocytes that exhibit TUNEL positivity (TUNEL positivity score: 1.5 (1-1)). C(×40) night x-ray irradiation group: Fewer apoptotic spermatogenetic cells showing TUNEL positivity are found in the seminiferous tubules (TUNEL positivity score: 0 (0-1)). ps, primary spermatocytes; spgn, spermatogonium; spt, spermatids; sptzn, spermatozoon.

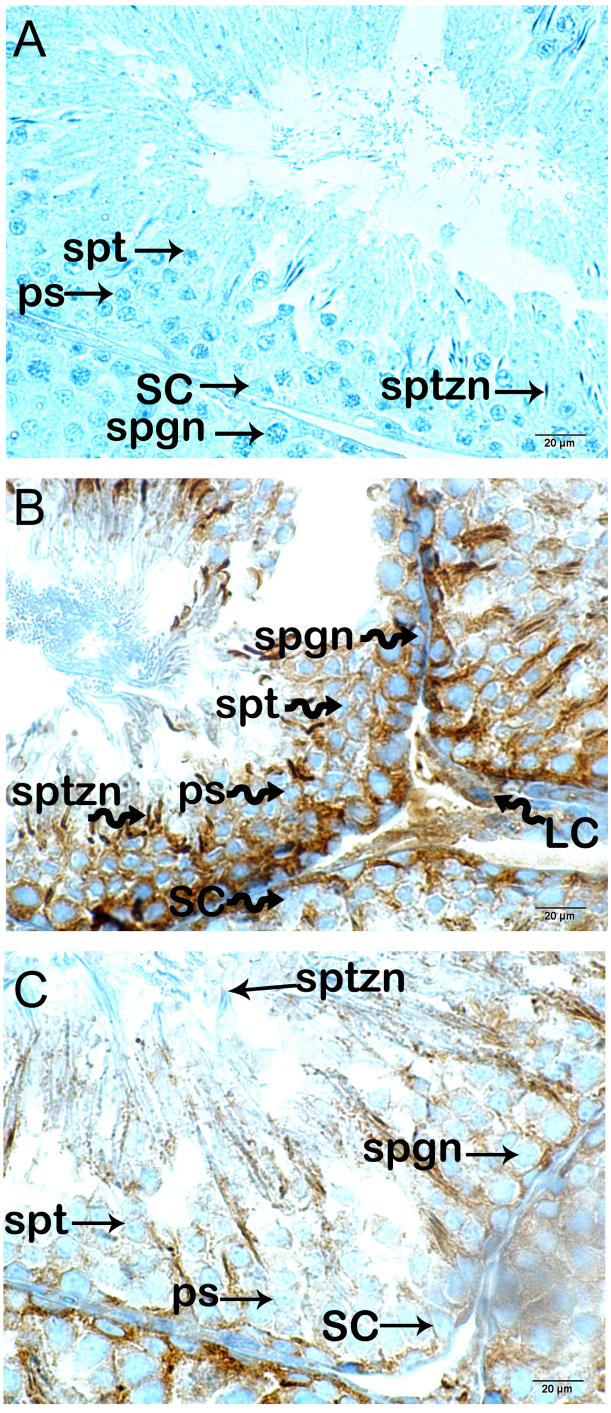

Figure 3. Representative light microscopic images of apoptotic cells marked by the 8-OHdG method. A(×40) control group: Representative light microscopic screen capture of testicular tissue showing normal spermatogonia, primary spermatocytes, spermatids, and spermatozoa that are 8-OHdG negative (8-OHdG positivity score: 0 (0-0)). B(×40) day x-ray irradiation group: Spermatogenetic cells in the seminiferous tubules show spermatocytes that exhibit strong 8-OHdG positivity (8-OHdG positivity score: 2.5 (2-3)). C(×40) night x-ray irradiation group: Among spermatogenetic cells in the seminiferous tubules, fewer cells showing strong 8-OHdG positivity are found (8-OHdG positivity score: 1 (0-1)). LC, Leydig cell; SC, Sertoli cell; spt, spermatids; ps, primary spermatocytes; spgn, spermatogonium; sptzn, spermatozoon.

The circadian clock, an endogenous biological rhythm regulating various physiological processes over 24 hours, has gained importance in recent research due to its role in the protection of homeostasis and the optimization of responses to environmental stress factors.[17] Certain studies have indicated that the circadian clock could significantly protect testicular tissues against radiation-induced damage.[4,18] In a study by Smith et al[19], the effects of x-ray ionizing radiation on the testicles of mice with a disrupted circadian rhythm due to a clock gene mutation were investigated. Strikingly, clock-mutant mice exhibited a higher susceptibility to radiation-induced testicular damage than wild-type mice. This suggested that a functional circadian clock could protect against radiation-induced damage. Studies have supported these findings by showing that the expression of DNA repair genes, which play a key role in reducing radiation-induced damage, is associated with circadian secretion.[4,20] The circadian clock disruption endangered the repair of radiation-induced DNA damage by impairing the secretion associated with these genes.

The molecular bases of circadian protection against radiation-induced testicular damage involve complex signaling pathways.[21] The circadian clock is tightly intertwined with the DNA damage response (DDR) pathway.[22] Core clock genes were demonstrated to directly regulate the expression of DDR genes in testicular cells.[4] This regulation maximizes the efficiency of damage repair by allowing optimal synchronization of DNA repair processes with daily fluctuations in radiation exposure. In addition, the circadian clock is known to modulate the cell metabolism and antioxidant defense systems, which play highly significant roles in reducing radiation-induced oxidative stress.[23] Circadian disruption reduced antioxidant enzyme expression and elevated oxidative damage in testicular tissues after radiation exposure.[23] This emphasizes the importance of the circadian clock in the protection of redox homeostasis and the minimization of oxidative damage.

The regulation of DNA damage repair pathways is a noteworthy mechanism through which the circadian clock may exert protection against testicular damage.[24] Circadian release in DNA repair gene expression has been observed in various tissues, indicating a rhythmic tendency for DNA damage repair.[18] Studies have shown that disrupting the circadian clock through genetic manipulation or environmental perturbations may threaten the effectiveness of DNA repair mechanisms in response to ionizing radiation.[2] In contrast, maintaining consistent circadian rhythmicity increased the fidelity of DNA repair processes, thus reducing the accumulation of DNA lesions and the risk of genetic mutation.[17,25] Furthermore, the effects of the circadian clock on cell proliferation and apoptosis may also contribute to its protective role against ionizing radiation-induced testicular damage.[26] Cell cycle progression and the rhythmic regulation of apoptosis affect the germ cell cycle rate and alter the fate of damaged cells.[22] The ability of the circadian clock to synchronize the cell cycle control points and apoptotic cells with the timing of radiation exposure may contribute to the reduction of radiation-induced testicular damage.

Additionally, the effects of the circadian clock appear to extend to the modulation of inflammatory and immune responses.[1] Ionizing radiation triggers a series of proinflammatory cytokines and immune mediators that may exacerbate tissue damage.[27] Studies have shown that disrupting circadian rhythmicity may lead to abnormal immune responses, while maintaining a consistent circadian function may reduce excess inflammation.[28] Considering the complicated interrelation between inflammation and radiation-induced tissue damage, the

regulatory role of the circadian clock in immune homeostasis may contribute to its protective effect against testicular damage.

Studies have shown that the susceptibility of testicular cells to radiation varies throughout the day due to the rhythmic expression of the genes involved in DNA damage repair.[4] Melatonin, a hormone mainly secreted by the pineal gland in the dark stage of the circadian cycle, possesses remarkable antioxidant and anti-inflammatory properties.[14] Its ability to scavenge reactive oxygen species and reduce oxidative stress has been comprehensively studied.[14] Oxidative stress plays an important role in radiation-induced cell damage including that observed in the testicles.[8] The circadian clock influences endogenous melatonin production, and its rhythmic secretion corresponds to the natural nocturnal rest period of the body.[29] This temporal correspondence presents an interesting connection between the protective effects of melatonin and the time-dependent susceptibility of testicular cells to radiation-induced damage. The potential synergy between chronoradiotherapy and endogenous melatonin relies on their common impact on circadian rhythms and oxidative stress.[30] The temporal coordination of radiation exposure with the optimal DNA repair stages, as facilitated by chronoradiotherapy, is consistent with the antioxidant and anti-inflammatory capacities created by endogenous melatonin during the night. This synchronization may effectively reinforce the testicular ability to repair DNA lesions and reduce the harmful effects of radiation-induced oxidative stress. In our study, 8-OHdG immunopositivity, which is a marker of oxidative stress-related DNA damage, was significantly lower in rats exposed to ionizing radiation at night.

Apoptosis, a strictly regulated, programmed cell death process, plays an important role in protecting tissue homeostasis and removing damaged cells.[31] Ionizing radiation may lead to abnormal apoptosis and potential tissue damage by disrupting the sensitive balance between proapoptotic and antiapoptotic signals.[19] Both chronoradiotherapy and endogenous melatonin regulate apoptosis in the context of radiation-induced testicular damage.[6] The temporal modulation of radiation exposure in chronoradiotherapy is consistent with the circadian rhythms of apoptosis-related factors. Studies have shown that certain times in the day are associated with higher apoptotic thresholds and potentially allow normal cells to avoid apoptosis related to excessive radiation.[13] This may contribute to the protection of the integrity of testicular tissue during radiation exposure. The antiapoptotic properties of endogenous melatonin were attributed to its ability to resist radiation-induced oxidative stress and modulate apoptotic signaling pathways.[32] Melatonin was shown to increase cell survival under conditions of radiation stress by downregulating proapoptotic factors and upregulating antiapoptotic factors.[33] The synchronization of melatonin secretion throughout the night aligns with the body's natural repair processes and thus helps suppress excessive apoptosis.[12] In our immunohistochemical analysis performed with the TUNEL method, which is a marker of apoptotic cells, the lower immunopositivity observed in rats exposed to x-ray ionizing radiation at night is likely associated with the synergistic effects of the time of radiation exposure and endogenous melatonin.

Inflammation is a complicated process involving the activation of immune responses to resist tissue damage and initiate repair. However, uncontrolled inflammation may exacerbate radiation-induced damage by promoting tissue damage and dysfunction.[12] Both chronoradiotherapy and endogenous melatonin possess anti-inflammatory properties that may potentially reduce the harmful effects of radiation-induced inflammation in the testicles.[34] The optimization of radiation transmission according to circadian rhythms by chronoradiotherapy extends to the regulation of immune responses.[5] Circadian clock genes influence the intensity and duration of inflammation by affecting immune cell activity and cytokine release.[18] Chronoradiotherapy may limit the extent of radiation-induced inflammation in the testicles by aligning radiation exposure with periods of low inflammatory activity.

The anti-inflammatory effects of endogenous melatonin arise from its capacity to suppress the production of pro-inflammatory cytokines and reduce immune cell infiltration.[16] The rhythmic nocturnal secretion of melatonin corresponds to the natural decrease in inflammatory processes in the body and further promotes a coherent balance between the immune response and tissue protection.[14]

Our study should be interpreted in consideration of certain limitations. Firstly, this is an animal model study. Therefore, the sleep–wake cycles cannot be considered to represent those of humans exactly. Our study only included histological and immunohistochemical analyses. Also, the focus was solely on the effects of chronoradiotherapy on testicular tissue. Therefore, the circadian rhythm may lead to different cellular cycles and effects for each cell or tissue type. The most important question that begs clarification is how the circadian rhythm affects a cancerous cell. In addition, this study investigated the effects of the circadian rhythm on x-ray ionizing radiation-induced testicular damage in the acute period. Further studies are needed to explore long-term effects.

In conclusion, this study indicates a relationship between the circadian clock and protection against x-ray ionizing radiation-induced testicular damage. Our results demonstrate that, besides ionizing radiation, the time period during which radiation exposure occurs may also significantly affect the testicles. The interaction between chronoradiotherapy and endogenous melatonin involves the modulation of apoptosis and inflammatory pathways to reduce ionizing radiation-induced testicular damage.[35] The circadian regulation of DNA repair, cell cycle progression, apoptosis, and immune responses may have a collective contribution to a regulated defense mechanism that reduces the harmful effects of radiation on testicular function. More studies are needed to unravel the molecular bases of this protective phenomenon and to completely elucidate the complex signaling pathways and the interactions of the specific molecular pathways through which the antioxidant effects of melatonin might create synergy with a chronoradiotherapy regimen. Also, clinical studies on the combined effects of chronoradiotherapy and endogenous melatonin in protecting testicular function during radiation therapy are needed to confirm the translational potential. We can discover strategies to minimize the negative effects of radiation exposure on testicular tissue by taking advantage of circadian rhythms and endogenous hormonal rhythms. The information obtained from these kinds of studies provides promising consequences for the development of chronotherapy-based strategies that aim to minimize radiation-induced testicular damage and protect male reproductive health.

Availability of Data and Materials: The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Committee Approval: This experimental study was performed with approval from Recep Tayyip Erdoğan University Animal Research Ethics Committee (approval number: 2023/23, date: 30/06/2022).

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