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Shortwave radiation-induced reproductive organ damage in male rats by enhanced expression of molecules associated with the calpain/Cdk5 pathway and oxidative stress

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ABSTRACT

Shortwave radiation has been reported to have harmful effects on several organs in humans and animals. However, the biological effects of 27 MHz shortwave on the reproductive system are not clear. In this study, we investigated the effects of shortwave whole-body exposure at a frequency of 27 MHz on structural and functional changes in the testis. Male Wistar rats were exposed to 27 MHz continuous shortwaves at average power densities of 0, 5, 10, or 30 mW/cm² for 6 min. The levels of insulin-like factor 3 (INSL3) and anti-sperm antibodies (AsAb) in the peripheral serum, sperm motility, sperm malformation rate, and testicular tissue structure of rats were analyzed. Furthermore, the activity of superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) content, calpain, and Cdk5 expression were analyzed at 1, 7, 14, and 28 days after exposure. We observed that the rats after radiation had decreased serum INSL3 levels ($p < 0.01$), increased AsAb levels ($p < 0.05$), decreased percentage of class A+B sperm ($p < 0.01$ or $p < 0.05$), increased sperm malformation ($p < 0.01$ or $p < 0.05$), injured testicular tissue structure, decreased SOD and CAT activities ($p < 0.01$ or $p < 0.05$), increased MDA content ($p < 0.01$), and testicular tissue expressions of calpain1, calpain2, and Cdk5 were increased ($p < 0.01$ or $p < 0.05$). In conclusion, Shortwave radiation caused functional and structural damage to the reproductive organs of male rats. Furthermore, oxidative stress and key molecules in the calpain/Cdk5 pathway are likely involved in this process.

SUMMARY

Shortwave radiation has been used in communications, medical and military applications, and its damaging effects on several organs of the human body have been reported in the literature. However, the biological effects of shortwave radiation on the male reproductive system are unknown. The present study, by constructing an animal model of short-wave radiation and analyzing the experimental results, revealed that shortwave radiation could cause functional and structural damage to the reproductive organs of male rats, and that oxidative stress and key molecules in the calpain/Cdk5 pathway might be involved in this process. It will provide organizational data for further studies on the mechanisms of male reproductive damage by shortwave radiation.

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Shortwave radiation; testicular; function; structure; oxidative stress; calpain; Cdk5

Introduction

Shortwaves are electromagnetic waves with a frequency of 3–30 MHz and wavelengths of 10–100 m (Yu and Peng 2017). High-frequency shortwave technology has developed rapidly and is widely used in many fields, such as the military, communications, and medical treatments (Altpeter et al. 2006; Israel et al. 2007). Although it adds convenience to human health and lifestyle, it could also have adverse effects. Currently, more scholars are concerned with this issue. Exposed

male reproductive organs have a higher specific absorption rate, making them one of the most vulnerable target organs for electromagnetic radiation injury (Kesari and Behari 2010). Currently, most animal experiments have shown that different wavebands of microwave radiation cause abnormal serum hormones, reduced sperm viability, decreased spermatogenic capacity, and structural damage to testicular tissues, ultimately leading to hypogonadism (Kumar et al. 2013; Ozguner et al. 2005; Saygin et al. 2011). However, the effects of shortwave

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radiation on the structure and function of male reproductive organs remain unknown.

The human body is stimulated by the microwave radiation environment to produce excess free radicals, which in turn reduce the activity of antioxidant enzymes (such as superoxide dismutase [SOD] and catalase [CAT]). The increased lipid peroxidation caused by microwave exposure is one of the critical pathways of electromagnetic radiation-induced damage to the body (Bisht et al. 2017; Moazamian et al. 2015; Valko et al. 2007). Intracellular calcium-dependent cysteine protease, also known as calpain, is associated with sperm fertilization (Aoyama et al. 2001) and can be activated by elevated intracellular Ca^{2+} concentrations and participate in multiple biological pathways, such as cytoskeletal remodeling and regulation of apoptosis. The cell cycle protein-dependent kinase Cdk5, a molecule downstream of the calpain signaling pathway, is present in various tissues. Testicular tissues can regulate the cell cycle and affect cell proliferation and division (Musa et al. 1998; Shu et al. 2018). Currently, we lack studies on oxidative stress in male reproductive organs caused by shortwave radiation and its effect on key molecules in the calpain/Cdk5 pathway.

Therefore, we aimed to establish an animal model of shortwave radiation-induced male reproductive organ injury in rats, to detect changes in testicular tissue function, sperm vitality, and structure, as well as to observe the effects of shortwave radiation on oxidative stress, and changes in the expression of key molecules in the calpain/Cdk5 pathway in rat testicular tissue, which could contribute to exploring the

mechanism of shortwave radiation-induced reproductive injury.

Methods

Animals and groups

Eighty SPF-rated male Wistar rats, weighing 210 ± 20 g, were provided by the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China) and were raised in a standard animal laboratory with free access to food and water. Animal experiments were approved by the Ethical Committee of the Beijing Institute of Radiation Medicine (IACUC-DWZX-2020-632). All the experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animals were randomly divided into the following four groups: 0 mW/cm² exposure group, 5 mW/cm² exposure group, 10 mW/cm² exposure group, and 30 mW/cm² exposure group, with 20 rats *per* group.

Shortwave exposure system and exposure dose

A schematic representation of the exposure setup for shortwave exposure is shown in Figure 1. A shortwave generator (SMF100A, Rohde & Schwarz, Germany) was connected to a power amplifier (BBA100, Rohde & Schwarz, Germany), followed by a dual-directional coupler with a power sensor (NRP-Z91, Rohde & Schwarz, Germany). The forward coupling port transmitted a continuous wave signal with a standard power density (e.g., 10 mW/cm²) at a frequency point of 27 MHz into

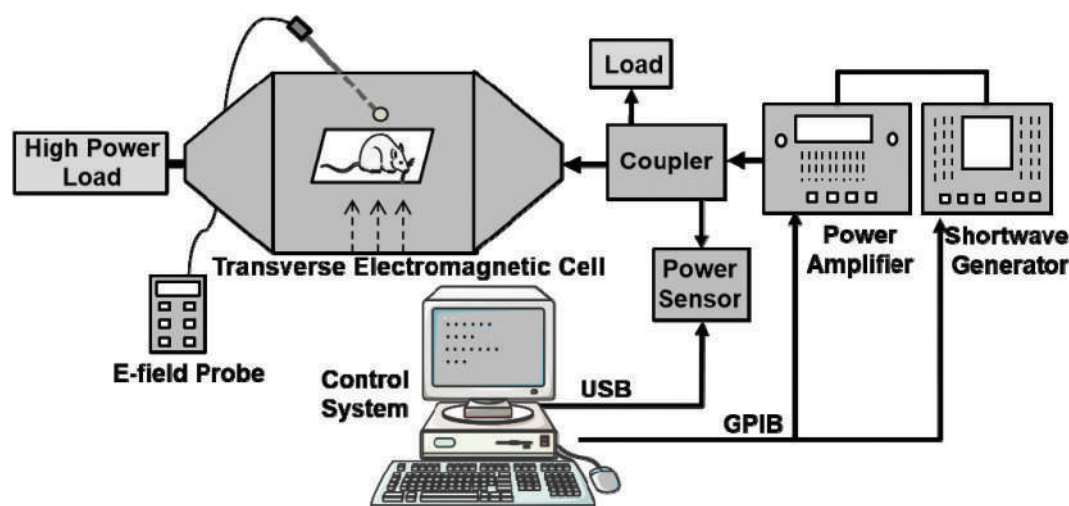


Figure 1. Shortwave radiation device and a schematic diagram of the radiation exposure technique.

a transverse electromagnetic cell (117486, ETS Lindgren, USA), which was terminated with a high-power load to validate the reflection coefficient. The electric field strength in the electromagnetic cell was measured using an E-field probe (HI-6053, ETS Lindgren, USA) and transferred to the power density unit. The control system was designed by the China Academy of Information and Communications Technology in Beijing, China.

During the exposure, the rats were restrained in a closed Plexiglass cage with holes of 1.5-cm diameter holes to facilitate breathing and placed in the middle of the electromagnetic cell. The entire body of the rats was exposed to shortwave radiation with an average power density of 5, 10, or 30 mW/cm², for 6 min. Correspondingly, the average specific absorption rates (SARs) of the rat testicles were 8×10^{-4} , 1.6×10^{-3} , and 4.8×10^{-3} W/kg, respectively. The SAR distribution was calculated using the finite domain time difference (FDTD) method, reported in a previous study (Li et al. 2015). The 0 mW/cm² exposure group was subjected to the same exposure (with the radiation source turned off and other conditions identical to those of the radiation group).

Detection of peripheral blood biochemical markers

The enzyme marker (PerkinElmer, USA) and ELISA Kit (Huamei Biological Engineering Co., Ltd., Wuhan, China) were used to detect the insulin-like peptide 3 (INSL3) and anti-sperm antibody (AsAb) levels in the rat serum (Niu et al. 2018; Weerakoon et al. 2018). The rats in each group (five rats in each group) were anesthetized using an intraperitoneal injection of 1% sodium pentobarbital (30 mg/kg) at 1, 7, 14, and 28 days after radiation. Furthermore, 1.5 ml of blood was taken from the central abdominal vein to prepare serum, and the related hormone levels were detected using an ELISA Kit as per the manufacturer's instructions.

Determination of sperm quality

In this study, the parameters of sperm motility and malformation rate were detected to assess sperm quality. The rats in each group (five rats in each group) were anesthetized at 14 and 28 days after radiation. The epididymis was cut at the head, and the tissue, the size of a green bean, was placed in an EP tube containing 37°C, 0.4 ml saline for approximately 1 min. A pipette was used to aspirate 20 µl of the sperm suspension dropwise onto a sterilized slide. Sperm motility was measured using the SCA sperm dynamic analysis system (Hirano et al. 2001). Sperm head area, motility, curvilinear velocity (VCL), linear velocity (VSL), average path velocity (VAP), beat-cross frequency (BCF),

and amplitude of lateral movement of sperm heads (ALH) were analyzed by the SCA sperm dynamic analysis system (Microptic, Spain).

The sperm suspensions of each group prepared during the detection of sperm motility were taken on the slides at 1, 7, 14, and 28 days after radiation. They were fixed with a 4% formaldehyde solution and dried, followed by hematoxylin staining, hydrochloric acid ethanol fractionation, eosin staining, gradual dehydration in ethanol, and transparent twice in xylene after neutral resin sealing. Morphological changes in the sperm were observed using an optical microscope. Sperm abnormalities were observed and sperm malformation rates were calculated with the previous reported method (Chen et al. 2019).

Hematoxylin-Eosin (HE) staining

Morphological changes in the testes were observed using HE staining. The rats in each group (five rats per group) were anesthetized on days 1 and 7 after radiation, and testes were fixed in 10% neutral buffered formalin for one week. After dehydration, clearing, wax impregnation, and embedding, the paraffin-embedded tissues were sectioned to a thickness of 3 µm. Following the HE staining, histological sections of the testes were observed and captured using a light microscope (Leica, Germany). Morphological pathology was used to assess the spermatogenic function of the testis by observing changes in the seminiferous tubules, sertoli cells and interstitial cells of the rat testis (Bagheri et al. 2015).

Transmission Electron Microscopy (TEM)

The rats in each group (five rats per group) were anesthetized on days 1 and 7 after radiation. Fresh testicular tissues of 1 mm³ were fixed for 2 h in a 2.5% glutaraldehyde solution and an additional 2 h in a 1% osmium acid, dehydrated with an ethanol gradient, transitioned in acetone, embedded in EPON812, and cut into ultrathin sections and stained with uranyl acetate and lead citrate. The ultrastructural features of the testicular tissue were subsequently observed using TEM (Hitachi, Japan).

Estimation of oxidation index

Testicular tissues from each group of rats (five rats per group) were frozen at -80°C and removed at 1, 7, 14, and 28 days after radiation. The appropriate tissues were weighed and placed in cold saline to prepare for the assay of oxidative stress indicators. The superoxide dismutase (SOD) and catalase (CAT) enzyme activity and

malondialdehyde (MDA) content were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) (Lin et al. 2021). The activity of SOD and CAT and the MDA content were measured using spectrophotometric method. SOD activity was expressed as units per milligrams of protein (U/mg protein). The activity of CAT was expressed as (U/g protein). The MDA content was measured in units of (nmol/mg protein).

Immunohistochemical (IHC) assay for calpain1, calpain2, and Cdk5

The prepared testicular tissue paraffin sections of rats at 1, 7, 14, and 28 days after radiation were dewaxed to water and then incubated in a 3% H₂O₂ solution for 10 min to block endogenous peroxidase, and rinsed in PBS. Subsequently, the testicular tissue was added to a citric acid buffer at a high pressure of 120°C for 15 min, cooled naturally to 25°C, and rinsed with PBS. Next, to block endogenous peroxidase with goat serum for 30 min, added primary antibody, rabbit anti-mouse IgG antibodies calpain1, calpain2, Cdk5 (1:100; Abcam, United Kingdom), incubated overnight at 4°C, rewarmed at 37°C in an incubator, rinsed with PBS, and added dropwise with secondary antibody, goat anti-rabbit IgG (1:200, ZhongShan JinQiao, China), incubated at room temperature for 20 min, rinsed with PBS, and DAB solution was added dropwise. Finally, hematoxylin counterstain was used. The mean optical density (MOD) and integrated optical density (IOD) values of each group were analyzed using Image-Pro Plus 6.0 software. This method has been reported in the study (Huang et al. 2022).

Statistical analysis

The data were presented as the mean $\pm$ standard deviation (SD), and statistical analyses were performed using SPSS software (version 23.0; IBM, USA). The data were analysed

using a one-way ANOVA followed by an LSD post hoc test. $P < 0.05$ was considered statistically significant.

Result

Shortwave radiation caused abnormalities of active substances in the serum of rats

INSL3 and AsAb are essential active substances in the serum of male rats that affect male reproductive function. The results showed that compared to that in the 0 mW/cm² group, the peripheral blood level of INSL3 in the 10 mW/cm² group was significantly lower on day 1 after radiation. The peripheral blood level of INSL3 in the 30 mW/cm² group was significantly lower on days 1 and 7 after radiation ($p < 0.01$, Figure 2a). Compared with the 0 mW/cm² group, the peripheral serum AsAb level in the 10 mW/cm² group was significantly higher on day 7 after radiation, and the peripheral serum AsAb level in the 30 mW/cm² group was significantly higher on day 7 after radiation. Compared to that in the 0 mW/cm² group, the peripheral serum AsAb levels in the 10 mW/cm² group were significantly increased on day 7 after radiation. The peripheral serum AsAb levels in the 30 mW/cm² group were also significantly increased on days 7 and 14 after radiation ($p < 0.05$, Figure 2b). This indicated that shortwave radiation at 10 and 30 mW/cm² decreased the serum INSL3 levels and increased AsAb levels in rats.

Shortwave radiation decreased sperm quality in rats

Reduction in sperm motility

On day 14 of the shortwave radiation, compared with the 0 mW/cm² group, AR and A+B class sperm were significantly reduced ($p < 0.01$ or $p < 0.05$), and C and D class sperm were significantly increased ($p < 0.01$ or $p < 0.05$, Figure 3a,b) in the 10 and 30 mW/cm² groups. On day 28 after radiation, no significant differences were observed in the proportion of AR, A+B class sperm, and C and

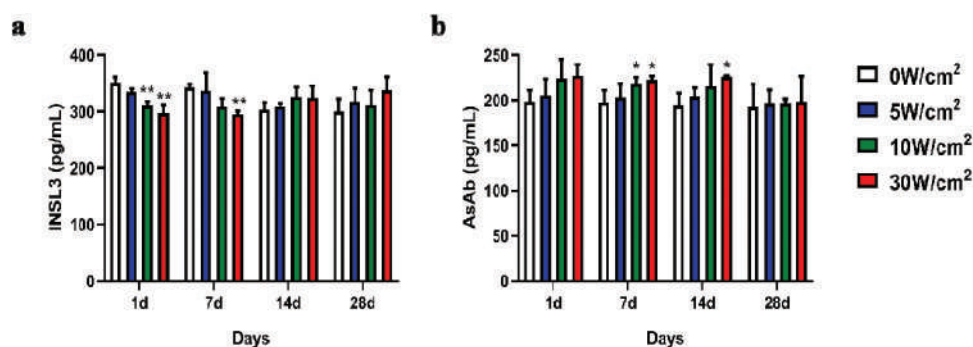


Figure 2. Changes in the INSL3 and AsAb concentrations in the peripheral serum of rats after shortwave radiation. (a) changes in the INSL3 concentration in the peripheral blood were detected 1, 7, 14, and 28 days after radiation. (b) changes in the AsAb concentration in the peripheral blood were detected 1, 7, 14, and 28 days after radiation. * $p < .05$, ** $p < .01$ compared to the 0 mW/cm² group.

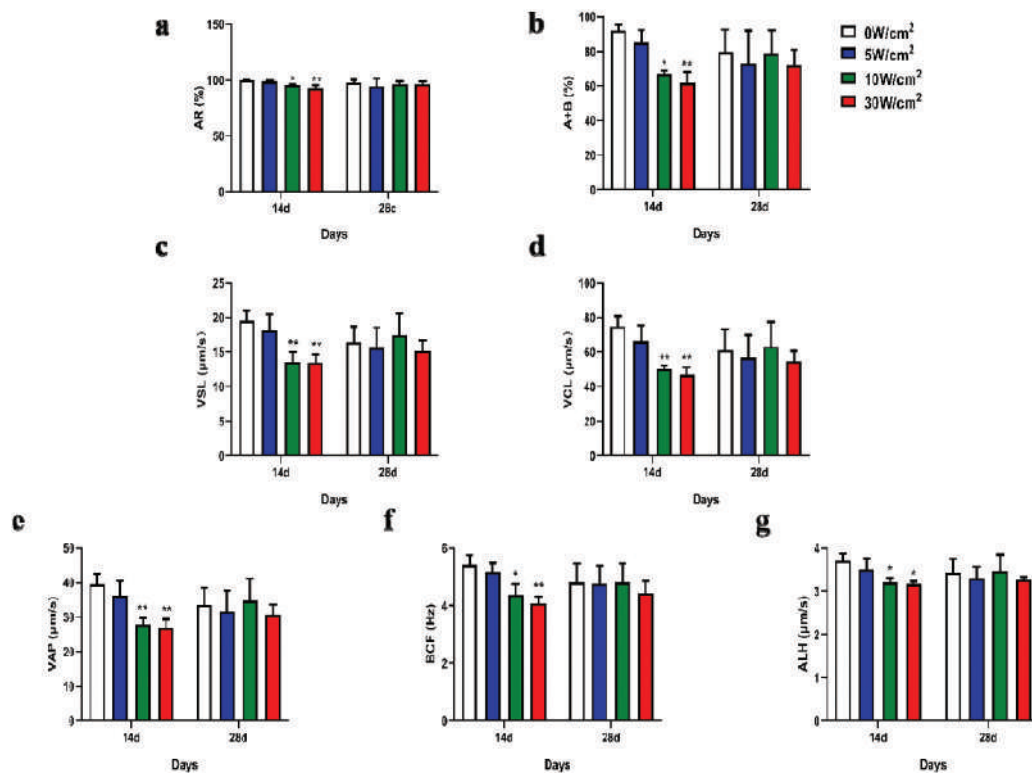


Figure 3. Changes in sperm AR (A+B+C), A+B class sperm ratio, viability parameters, and motility parameters in rats after shortwave radiation. (a-g) Sperm motility (AR, A+B), viability parameters (VCL, VSL, VAP, BCF), and motility parameters (ALH) were measured in rats 14 and 28 days after radiation using the SCA sperm dynamics analysis system. * $p < .05$, ** $p < .01$ compared to the 0 mW/cm² group.

D class sperm in rats of each dose group ($p > 0.05$, Figure 3a,b). This indicated that 10 and 30 mW/cm² shortwave radiation caused a decrease in sperm motility in rats.

Degradation of sperm motility parameters

The sperm motility parameters, namely VSL, VCL, VAP, and BCF (Figure 3c–f), were significantly lower in the 10 and 30 mW/cm² groups than in the 0 mW/cm² group on day 14 after shortwave radiation ($p < 0.01$ or $p < 0.05$). No statistical differences in the sperm motility parameters VSL, VCL, VAP, and BCF were observed in each dose group on day 28 after radiation ($p > 0.05$). This demonstrated that 10 and 30 mW/cm² shortwave radiation caused a decrease in sperm viability parameters in rats.

The decline in sperm motility manner parameters

The sperm motility parameter ALH (Figure 3g) was significantly decreased in the 10 mW/cm² and 30 mW/cm² groups compared with the 0 mW/cm² group on day 14 after shortwave radiation ($p < 0.05$). On day 28 after shortwave radiation, no statistical difference ($p > 0.05$) was observed in the sperm motility parameters of rats in each dose

group. The results showed that 10 and 30 mW/cm² shortwave radiation caused changes in the sperm motility parameters in rats.

Increase in sperm malformation rate

The results suggested that compared with the 0 mW/cm² group, the sperm malformation rate of rats in the 10 mW/cm² group increased significantly seven days after radiation ($p < 0.01$), and the sperm malformation rate of rats in the 30 mW/cm² group increased significantly on days 1, 7, and 14 after radiation ($p < 0.01$ or $p < 0.05$), with no statistical difference on day 28 ($p > 0.05$). The results are shown in Figure 4. This suggests that 10 and 30 mW/cm² shortwave radiation increased sperm malformation rate in the rats.

Shortwave radiation damaged the histological structure and ultrastructure of rat testes

The HE-stained slices (Figure 5) revealed that the testicular tissue spermatogenic cells were loosely arranged, necrotic and detached, spermatogenic cell structure was lost, luminal spermatozoa were reduced or absent,

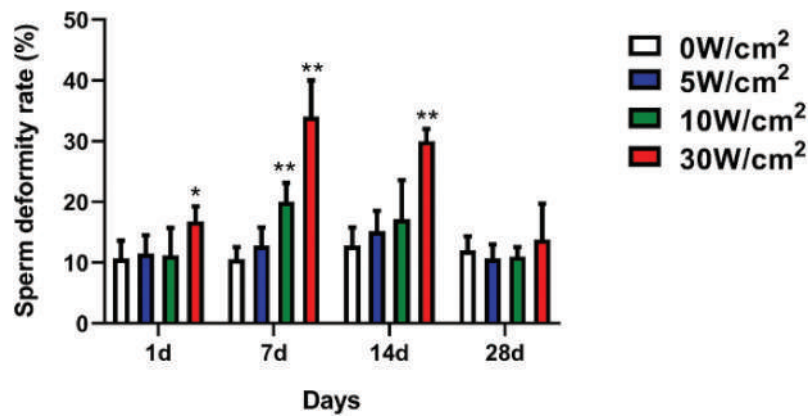


Figure 4. Changes in the sperm malformation rate in rats 1, 7, 14, and 28 days after radiation. * $p < .05$, ** $p < .01$ compared to the 0 mW/cm² group.

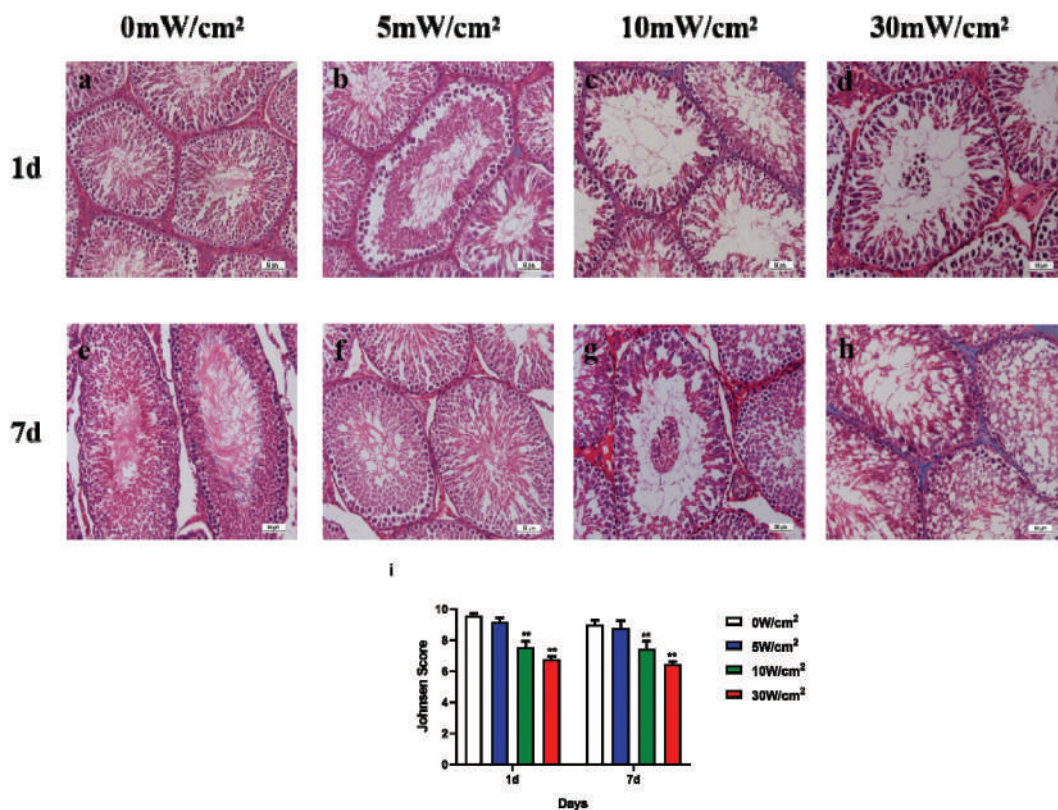


Figure 5. Changes in the morphological structure of rat testis tissue after shortwave radiation (HE staining, scale bar=50µm). (a, e) the testicular tissue in the 0 mW/cm² group. (b, f) the testicular tissue in the 5 mW/cm² group. c, g the testicular tissue in the 10 mW/cm² group. (d, h) the testicular tissue in the 30mW/cm² group. (i) testicular histopathology Johnsen score.

spermatogenic cells degenerated, and interstitial edema was visible in the radiation group, particularly in the 10 mW/cm² group and 30 mW/cm² group at 1 and 7 days after radiation. Normal testicular tissue was observed in the 0 mW/cm² group (Figure 5a,e). The testicular tissue in the 5 mW/cm² group showed no apparent damage (Figure 5b,f). The testicular spermatogenic epithelium in the 10 mW/cm² group was loosely arranged, and shed necrotic spermatogenic cells were observed in the lumen

of the spermatogenic tubules. Spermatozoa in the lumen were reduced or absent (Figure 5c,g). The spermatogenic epithelium in the 30 mW/cm² group was loosely arranged, spermatogenic cells at all levels were disorganized, spermatozoa in the lumen were reduced, and interstitial edema was observed (Figure 5d,h). Compared to the 0 mW/cm² group, there was no significant difference in the Johnsen score of the testis in the 5 mW/cm² group, and the Johnsen score of the testis

in the 10 mW/cm² and 30 mW/cm² groups decreased significantly at 1 and 7 days after shortwave radiation ($p < 0.01$, Figure 5i).

Ultrastructural changes in rat testes were observed using TEM. According to the HE results, damage to the testicular tissue structure was most significant on day 7 after radiation. Therefore, we chose seven days after radiation as the observation time for changes in the ultrastructure of the testicular tissue in each group over the different doses of shortwave radiation. In the 0 mW/cm² group, normal testicular tissue ultrastructure was observed, with normal spermatogonia (Figure 6a), spermatocytes (Figure 6e), and spermatozoa (Figure 6i). In the 5 mW/cm² group, edematous spermatogonia (Figure 6b) and spermatocytes (Figure 6f), and approximately normal spermatozoa (Figure 6j) were observed. After 10 mW/cm² and 30 mW/cm² shortwave radiation exposure, the basic ultrastructural lesions of rat testes tissues mainly showed abnormal condensation and side shift of spermatogonia and spermatocytes chromatin (Figure 6c, g), necrosis of spermatogonia and spermatocytes (Figure 6d, h), edema of the spermatozoa head (Figure 6k), the nuclear concentration of spermatozoa head, abnormal increase of chromatin, and abnormal development of acrosome (Figure 6l). The lesions in the radiation

groups indicated that the morphological structure and ultrastructure of the testicular tissue were damaged by the shortwave radiation.

Shortwave radiation-induced changes on oxidative stress-related indicators in rat testicular tissue

Reduction in SOD activity

Compared to that in the 0 mW/cm² group, no change was observed in SOD activity caused by 5 mW/cm² shortwave radiation; SOD activity was significantly reduced on day 7 after 10 mW/cm² shortwave radiation ($p < 0.01$) and days 1, 7 and 14 after 30 mW/cm² shortwave radiation ($p < 0.01$ or $p < 0.05$, Figure 7a).

Decrease in CAT activity

Compared to that in the 0 mW/cm² group, no change was observed in CAT activity caused by 5 mW/cm² shortwave radiation. CAT activity was significantly reduced on days 1 and 7 after 10 mW/cm² shortwave radiation ($p < 0.01$) and on days 1, 7, and 14 after 30 mW/cm² shortwave radiation ($p < 0.01$ or $p < 0.05$, Figure 7b).

Increase in MDA content

Compared to that in the 0 mW/cm² group, no statistical difference was observed in the MDA content of the 5

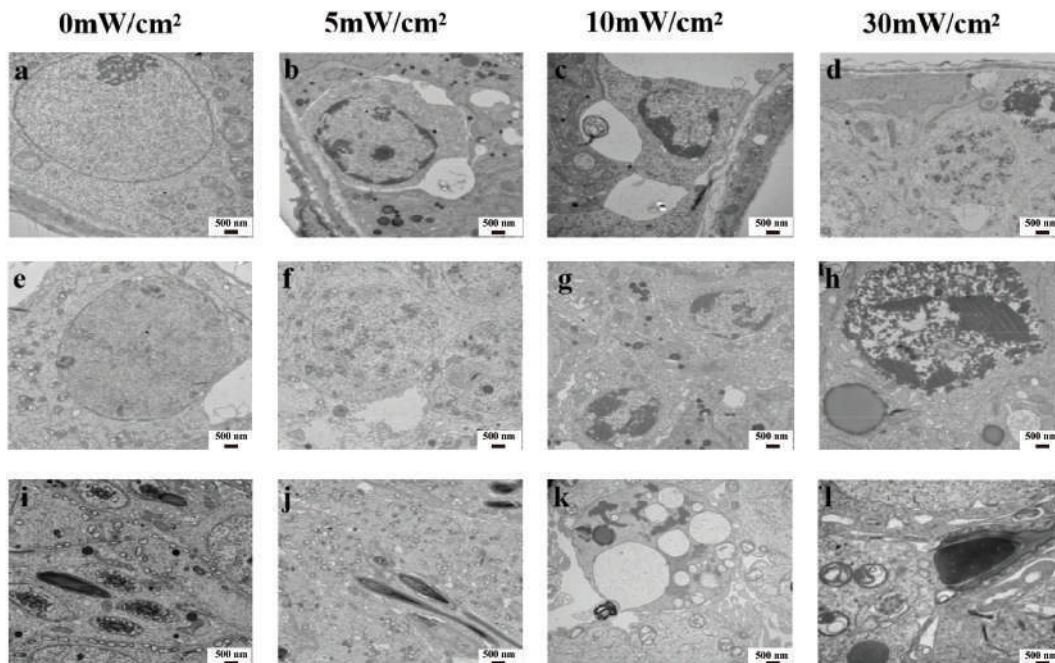


Figure 6. Ultrastructural changes in rat testis on day 7 after shortwave radiation (TEM, scale bar=500 nm). (a) the normal spermatogonia in the 0 mW/cm² group. (b-d) gradual increase of spermatogonia damage and vacuolation of cells in the 5, 10, and 30 mW/cm² groups. (e) the normal spermatocytes in the 0 mW/cm² group. (f-h) gradual increase of spermatocyte damage in the 5, 10, and 30 mW/cm² group. (i) the normal sperm cell and spermatozoa in the 0 mW/cm² group. (j-l) progressive increase in spermatocyte damage in the 5, 10, and 30 mW/cm² groups.

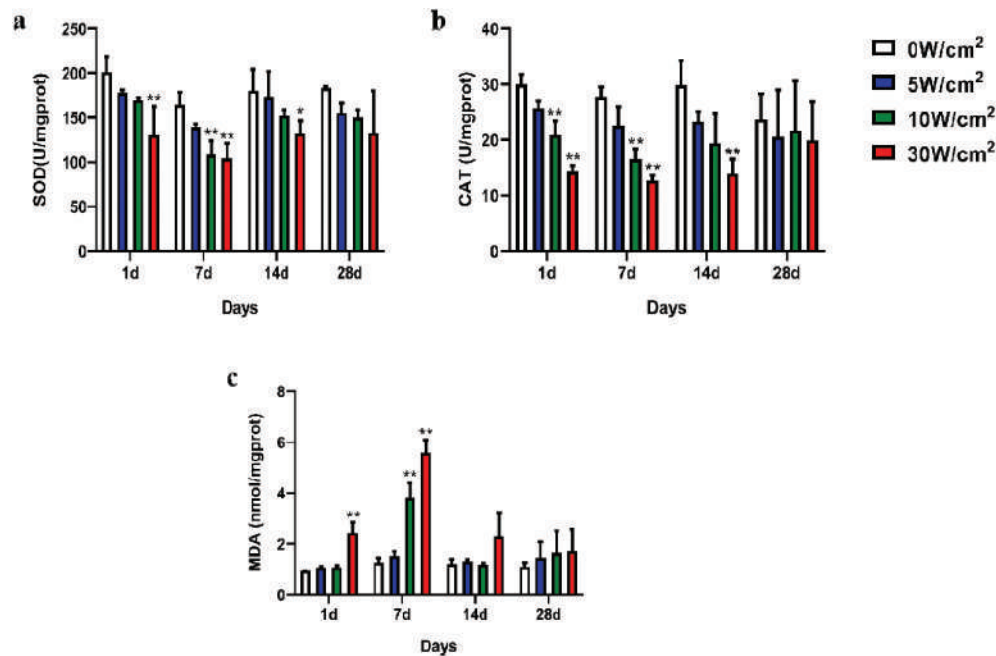


Figure 7. Changes of oxidative stress indicators in rat testis testicular tissue after short-wave radiation. (a) changes in testicular tissue SOD activity were measured 1, 7, 14, and 28 days after radiation. (b) Changes in testicular tissue CAT activity were measured 1, 7, 14, and 28 days after radiation. (c) changes in testicular tissue MDA content were measured 1, 7, 14, and 28 days after radiation. * $p < .05$, ** $p < .01$ compared to the 0 mW/cm² group.

mW/cm² group ($p > 0.05$); the content of MDA increased significantly on day 7 after 10 mW/cm² shortwave radiation ($p < 0.01$) and days 1 and 7 after 30 mW/cm² short-wave radiation ($p < 0.01$, Figure 7c).

Shortwave radiation increases the expression of calpain1, calpain2, and Cdk5 in rat testis tissue

The 0 mW/cm² group calpain1 (Figure 8a,c,e,g), calpain2 (Figure 9a,c,e,g), and Cdk5 (Figure 10a,c,e,g) were expressed in small amounts in all levels of germ cells and the cytoplasm was brownish-yellow positive at 1, 7, 14, and 28 days after shortwave radiation; the 30 mW/cm² group calpain1 (Figure 8b,d,f,h), calpain2 (Figure 9b,d,f,h), and Cdk5 (Figure 10b,d,f,h) were expressed in the cytoplasm of germ cells as a dark brown-yellow strong positive. The number of positive cells were significantly increased. The results of the analysis revealed that the calpain1, calpain2, and Cdk5 expression in testicular tissues were significantly increased in the 30 mW/cm² group compared to that in the 0 mW/cm² MOD group on days 1, 7, 14, and 28 after shortwave radiation ($p < 0.01$ or $p < 0.05$, Figures 8i–10i).

Discussion

The testis is a male spermatogenic organ, and damage to reproductive organs affects the structure and function of

spermatogenic cells and sperm biogenesis. Moreover, it may even affect the health of offspring through the transfer of damaged genetic material (Dasdag et al. 2015; Houston et al. 2016). Epidemiological survey results have shown that practitioners exposed to long-term shortwave radiation suffer from headaches, dizziness, insomnia (Altpeter et al. 2006; Wang et al. 2011), sinus arrhythmias (Chen et al. 2013), abnormal reproductive function (Ping et al. 2002), and endocrine dysfunction (Altpeter et al. 2006). Animal experiments have demonstrated that shortwave radiation can cause abnormal synthesis and release of neurotransmitters and disturbance of electrical activity in rats (Yu et al. 2019), damage to heart function (Zhang et al. 2020), and decrease the secretion of adrenaline (Dogaru and Craciun 2015). These results suggest that shortwave radiation could damage multiple organ systems. Male reproductive organs play a critical role in biological survival and continuation. The damaging effects of shortwave radiation on the function and structure of rat testicular tissue have not been reported in recent years. Therefore, we investigated the characteristics of reproductive function and structural damage in male rats after shortwave radiation at different power densities and elucidated the underlying mechanism.

INSL3 is a member of the insulin-like hormone superfamily, also known as testicular mesenchymal cell-derived insulin-like peptide because of its high

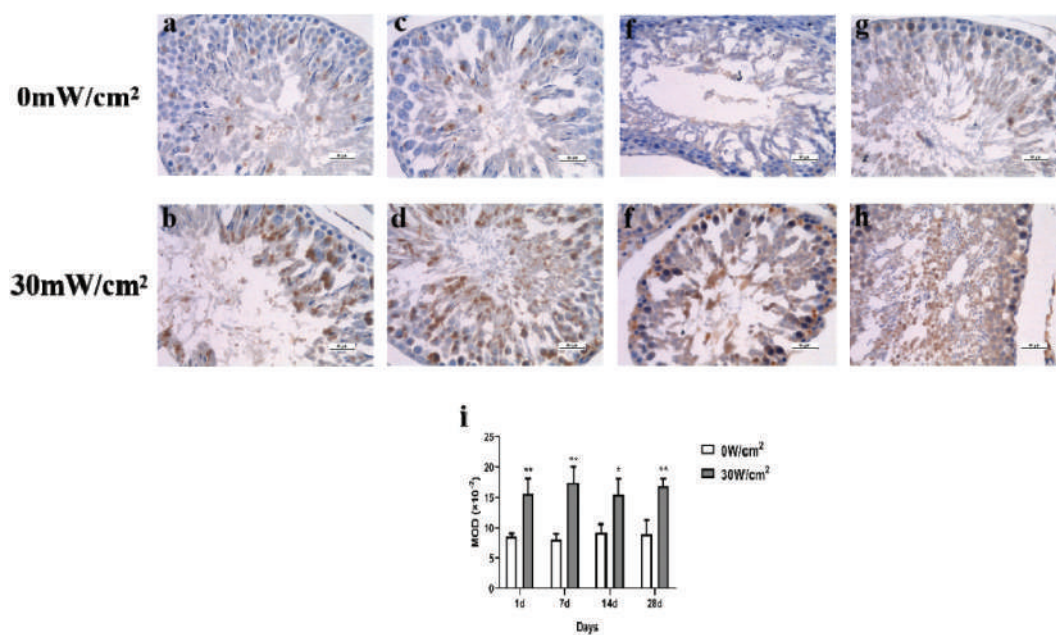


Figure 8. Changes of calpain1 expression (ultrasensitive two-step method, scale Bars=30 μ m) in rat testicular tissue after shortwave radiation. (a, c, e, g) 0 mW/cm² group showed slight positivity in spermatogonia, spermatocytes, and spermatocytes cytoplasm on 1, 7, 14, and 28 days after radiation. (b, d, f, h) 30 mW/cm² group showed positive results in spermatogonia, spermatocytes, and spermatocytes cytoplasm 1, 7, 14, and 28 days after radiation. (i) quantitative analysis of MOD. * $p < .05$, ** $p < .01$ compared to the 0 mW/cm² group.

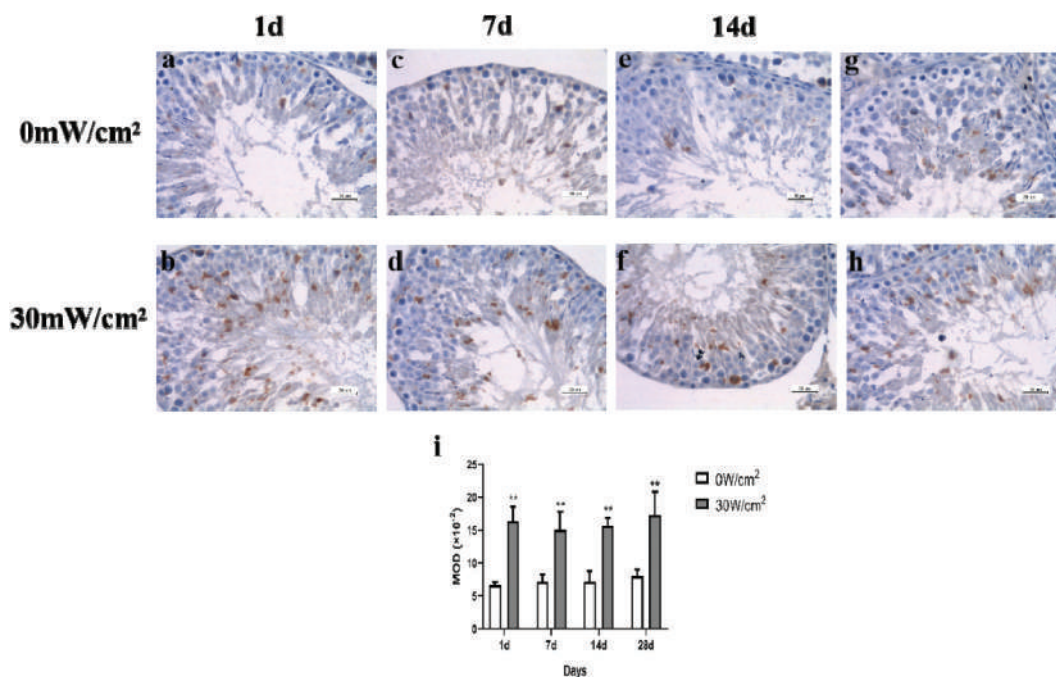


Figure 9. Changes of calpain2 expression (ultrasensitive two-step method, scale Bars=30 μ m) in rat testicular tissue after shortwave radiation. (a, c, e, g) 0 mW/cm² group showed slight positivity in spermatogonia, spermatocytes, and spermatocytes cytoplasm 1, 7, 14, and 28 days after radiation. (b, d, f, h) 30 mW/cm² group showed positive results in spermatogonia, spermatocytes, and spermatocytes cytoplasm 1, 7, 14, and 28 days after radiation. (i) quantitative analysis of MOD. * $p < .05$, ** $p < .01$ compared to the 0 mW/cm² group.

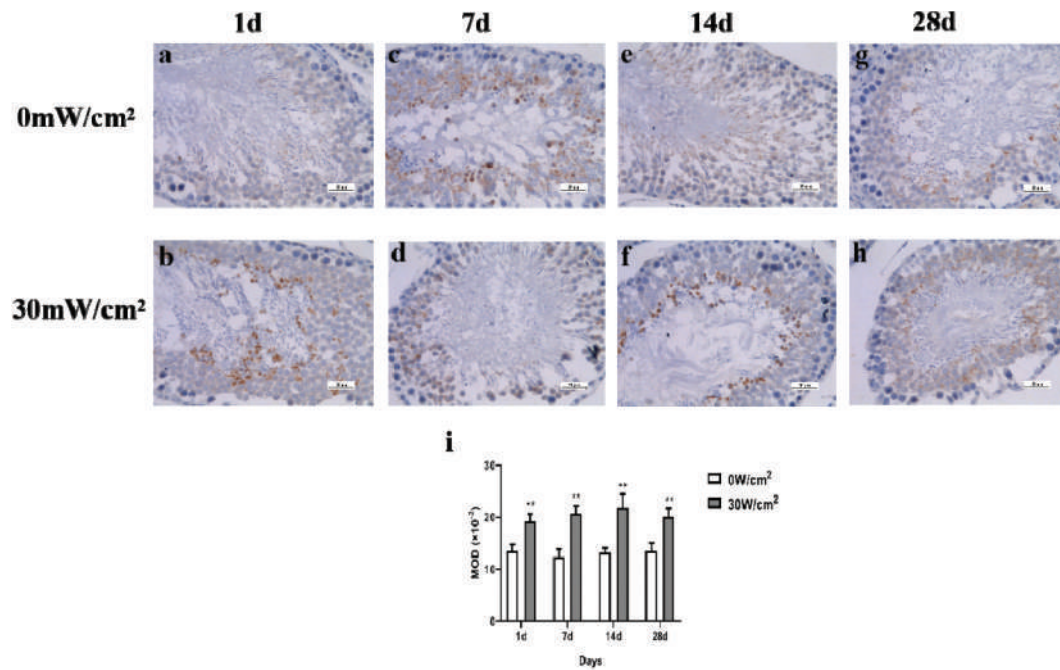


Figure 10. Changes of Cdk5 expression (ultrasensitive two-step method, scale Bars=30 μ m) in rat testicular tissue after shortwave radiation. (a,c,e,g) 0 mW/cm² group showed slight positivity in spermatogonia, spermatocytes, and spermatocytes cytoplasm 1, 7, 14, and 28 days after radiation. (b,d,f,h) 30 mW/cm² group showed positive results in spermatogonia, spermatocytes, and spermatocytes cytoplasm 1, 7, 14, and 28 days after radiation. (i) quantitative analysis of MOD. * $p < .05$, ** $p < .01$ compared to the 0 mW/cm² group.

expression in testicular mesenchymal cells, namely Leydig (Bathgate et al. 2013), which begins to rise gradually during pubertal development, peaks during sexual maturation, and gradually declines after hypogonadism (Shirnesan et al. 2008). It is a marker of differentiated maturation of testicular mesenchymal cells (Ivell et al. 2017). AsAb is an autoantibody in males that decreases sperm survival and viability, which leads to male sterility (Li et al. 2021; Zhang et al. 2018). Consequently, alterations in INSL3 and AsAb could affect male reproductive endocrine function and fertilization process and are important indicators for evaluating reproductive function impairment in male rats. It carries male genetic information, and its viability, number, and morphology are related to the production, differentiation, and maturation with the release, which directly responds to the spermatogenic capacity of the testes. Studies have reported a reduction in germ cell death by INSL3 through apoptosis in rat and boar testis cells (Minagawa et al. 2018; Sagata et al. 2015) and that microwave radiation reduced sperm count and sperm motility parameters (Liu et al. 2015; Zalata et al. 2015). Similar results were found in this study, where shortwave radiation of a specific intensity caused a decrease in serum INSL3, an increase in AsAb levels, a decrease

in sperm viability, and an increase in malformation rate in rats. The most significant damage was observed at a radiation dose of 30 mW/cm². Therefore, the present study analyzed testicular function after shortwave radiation and clarified its damaging effect on testicular function, starting from sensitive blood biochemical indices and sperm vitality.

The testis is a vital organ of the male reproductive system, and its abnormal function causes structural changes. We explored the significance and mechanisms from testicular tissue to germ cells and the subcellular level of germ cells, corresponding to their functional changes. A certain intensity of microwave radiation leads to disorganization of germ cells, swelling and vacuolization of cells, degeneration and necrosis of spermatogenic cells in the germinal tubules, and ultrastructure of chromatin agglutination, nuclear consolidation and lysis, and vacuolization of mitochondria (Shokri et al. 2020; Tenorio et al. 2012). However, studies on damage to testicular tissue structure and ultrastructure by shortwave radiation have not been reported. To this end, we observed the effects of different doses of shortwave radiation on the testicular tissue structure and ultrastructure in rats. Our results revealed that the epithelium of the seminiferous tubules was loosened, the arrangement of spermatocytes at all levels was disturbed, and the spermatozoa in the lumen of the seminiferous tubules were reduced, the spermatocytes were necrotic

and shed. Furthermore, the interstitial cells were reduced, the edema fluid was accumulated, the nuclear chromatin was agglutinated and shifted, necrosis occurred, the perinuclear gap was widened, and the mitochondria were swollen and cavitated after shortwave radiation at different power densities. Shortwave radiation can cause structural damage to rat testes, which may lead to reproductive dysfunction, especially when the radiation dose is 30 mW/cm². Therefore, these findings provide a basis for further exploration of the reproductive damage effects of shortwave radiation. To further explore the mechanism of structural and functional damage in rat testes, we analyzed the expression of oxidative stress indicators and key molecules in the calpain/Cdk5 pathway.

Oxidative stress is a major cause of sperm dysfunction (Raeeszadeh et al. 2022), as sperm requires a small number of free radicals for energy (O'Flaherty and Matsushita-Fournier 2017), whereas the accumulation of a large number of free radicals decreases the activity of antioxidant enzymes. Thus, the resulting increase in lipid peroxidation damages the sperm plasma membrane and reduces sperm viability and ability to fuse with the oocyte (Bisht et al. 2017). Microwave radiation at different power densities increases ROS production, decreases SOD and CAT activity, increases MDA content, alters the testicular tissue structure, disrupts the arrangement of spermatogenic cells, and sections of the cells degenerate (Bin-Meferij and El-Kott 2015; Esmekaya et al. 2011). Therefore, in the current study, the ability of testicular tissue to scavenge oxygen free radicals was determined by SOD and CAT activity, and the antioxidant capacity of testicular tissue was determined by changes in MDA content to clarify the antioxidant capacity of rat testicular tissue and the degree of oxidative damage after shortwave radiation. We observed that short-wave radiation could decrease the SOD and CAT activity and increase the MDA content in rat testis tissue, with a certain dose-effect relationship. This suggests that oxidative stress occurred in the rat testis tissue after short-wave radiation, damaging the testicular structure and eventually affecting the reproductive function of rats.

Based on the above experimental results, we could conclude that testicular tissue damage was worst at a radiation dose of 30 mW/cm². Thus, we chose this dose for investigating the mechanism. The calpain/Cdk5 pathway is mainly triggered by intracellular Ca²⁺ overload, which causes calpain to be activated by different concentrations of Ca²⁺, which can further activate Cdk5 to participate in the cell cycle and apoptotic necrosis processes (Hagen et al. 2011). Calpain is a neutral cysteine sulfhydryl endopeptidase with 15 members in the family (Goll et al. 2003). Calpain 1 and calpain 2 are expressed in the testes and

play a crucial role in processes such as spermatogenesis and acrosome reaction (Aoyama et al. 2001). According to previous reports, calpain induces apoptosis and causes cell damage, while oxidative stress activates calpain, triggering apoptosis (Letavernier et al. 2012; Squier et al. 1994). Cdk5 is a proline-directed serine/threonine kinase expressed in all tissues through phosphorylation of the cytosolic protein backbone, which is involved in apoptosis (Nie et al. 2022). Cdk5 and p35 contribute to the density of sperm tail fibers and regulate sperm motility through differential phosphorylation of substrates (Rosales et al. 2004). Therefore, changes in the expression of calpain1, calpain2, and Cdk5 in rat testicular tissue after shortwave radiation were observed using immunohistochemical techniques.

In conclusion, this study demonstrated that shortwave radiation impaired the structure and function of testicular tissue in male rats, and this abnormality was accompanied by a series of dose-dependent pathophysiological changes, including decreased serum hormone INSL3, increased AsAb, increased sperm malformation, decreased sperm viability, and damaged testicular tissue structure in rats. Furthermore, the data indicated that shortwave radiation caused oxidative stress and increased intracellular Ca²⁺ concentration in testicular tissues, inducing high expression of calpain1 and calpain2 in testicular tissues, which in turn activated Cdk5 to increase its expression, leading to increased germ cell apoptosis and ultimately to structural and functional disorders in testicular tissues. These findings imply that the mechanism of damage to reproductive organs by shortwave radiation might be related to oxidative stress and the calpain/Cdk5 signaling pathway. However, the available data could not confirm this, and more intensive experimental results are still needed to prove this. Nonetheless, the results of this study will provide tissue data to support future in-depth studies on the underlying damage mechanism.

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Author contributions

Conceptualization, Binwei Yao and Junqi Men; Investigation, Shuchen Liu, Yanxin Bai and Chao Yu; Methodology, Yabing Gao, Xinpeng Xu, Li Zhao; Validation, Jing Zhang, Hui Wang and Yanyang Li; Writing – original draft, Junqi Men;

Writing – review & editing, Binwei Yao and Ruiyun Peng;
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Authorship clarified

All authors agreed with the content and that all gave explicit consent to participate and publish.

Data availability statement

The authors would like to share the detailed/raw data privately with interested researchers.

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