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Abstract

The effect of multi-frequency electromagnetic environments on male reproduction has attracted the medical community's interest. Studies have investigated the effects and mechanisms of single-frequency microwave exposure on male reproduction, but comparative research on high-power microwave (HPM) composite and single exposure remains scarce. This study aimed to examine the effects and mechanisms of combined 1.5GHz and 4.3GHz microwave exposure on male reproduction. Male Wistar rats were exposed to 1.5GHz (L-band) and 4.3GHz (C-band) electromagnetic radiation for 15 minutes. The four groups were: sham, 10mW/cm² L-band, 10mW/cm² C-band, and 5mW/cm² L-band and 5mW/cm² C-band compound. Assessments were made on the pathological structures of testes, sperm viability, serum sex hormones, oxidative stress, and energy metabolism levels after radiation. Exposure to 1.5GHz and 4.3GHz microwaves individually resulted in testicular tissue damage and reduced sperm quality. There was little difference between the damage caused by HPM composite and single exposure. The exposed groups showed histological and

ultrastructural changes, with reduced spermatozoa viability, motility parameters, and serum testosterone, luteinizing hormone, follicle-stimulating hormone, and serum inhibin-B on days 1 and 7 after exposure. These tended to recover partially by day 14. Adenosine triphosphate content and lactate dehydrogenase and succinate dehydrogenase activities in the exposed testicular tissue decreased, corresponding to decreased superoxide dismutase activity and increased malondialdehyde content. Both single and combined exposure to L- and C-band HPM affect the male reproductive system. Exposure to single and compound HPM shows no significant difference in risks, with oxidative stress and energy metabolism disturbances playing key roles.
Keywords: high-power microwave; composite exposure; oxidative stress; energy metabolism; male reproduction.

1. Introduction

Microwaves are used in various civil and military fields, including mobile communications, radar satellites and electronic countermeasures. However, organisms exposed to microwaves can have adverse effects. The proliferation of 5G mobile communication technology has increased public concerns about the adverse effects of microwave radiation. Currently, research focuses on microwave radiation in a single frequency band. Yet, in reality, due to the complexity of the electromagnetic environment, the human body is exposed to multiple microwave frequency bands. These include global mobile communication systems, the widely used 4G and 5G cell phones, and commonly used frequency bands including the L-band and C-band [1-3]. Therefore, it is essential to explore the biological effects of combined L- and C-band microwaves exposure.

Testicular tissues are more vulnerable to electromagnetic radiation resulting in a higher Specific Absorption Rate (SAR) because the testicles are protected by only one layer of the scrotum, which offers less shielding than other internal organs. Both ***in vivo*** and ***in vitro*** experimental studies have shown that certain intensities of microwave radiation lead to injuries in the male reproductive system. This includes abnormalities such as reduced testosterone levels, decreased semen quality, damage to testicular tissue and sperm structure, ultimately leading to reduced fertility [4, 5]. However, to date, there have been few reports

published on the effects of simultaneous exposure to different microwave parameters on the male reproductive system.

The mechanisms behind microwave exposure-induced testicular tissue damage are unclear. Elevated levels of oxidative stress and impaired energy metabolism may be important contributors. Studies have shown that oxidative stress is increased both at the onset and during the progression of microwave radiation-induced injuries to the reproductive system. This was evident through an increase in the body's reactive oxygen species (ROS) levels and a decrease in the activity of antioxidant enzymes, which led to the accumulation of the lipid peroxide malondialdehyde (MDA) [6-9]. Oxidative respiration is closely linked with mitochondrial energy metabolism [10]. In eukaryotic cells, the tricarboxylic acid cycle and anaerobic glycolysis are pathways that produce adenosine triphosphate (ATP). Succinate dehydrogenase (SDH) and lactate dehydrogenase (LDH) are key enzymes in these processes. The mitochondria play a critical role in the tricarboxylic acid cycle, which is essential for ATP production. Although antioxidant enzymes such as superoxide dismutase (SOD) provide some protection [11], mitochondrial dysfunction can lead to an increase in unutilized electrons in the oxidative respiratory chain. This excess electron transfer to oxygen results in increased production of reactive oxygen species (ROS), which impairs ATP production. Oxidative stress and impaired energy metabolism are closely linked, yet studies exploring their interplay in causing male reproductive damage from combined microwave exposure are limited.

In our study, male Wistar rats were exposed to 1.5GHz (L-band) and 4.3GHz (C-band) microwaves for 15 minutes, both individually and simultaneously. We primarily investigated the reproductive effects of HPM single and combined exposure in male rats and explored the mechanisms of oxidative stress and energy metabolism disorders, aiming to provide targets for clinically targeted treatment of radiation damage, the foundation for the protection of populations with high microwave exposure, and a basis for developing safety guidelines for the public.

2. Materials and methods

2.1. Animals

We used 80 nine- to eleven-week-old male Wistar rats (Vital River Laboratory Animal Technology, Beijing, China), each weighing approximately 300±20g. These rats were randomly divided into four groups with 20 rats per group: sham-exposed (Sham group), 10mW/cm^2 L-band-exposed (L10 group), 10mW/cm^2 C-band-exposed (C10 group), and combined 5mW/cm^2 L-band and 5mW/cm^2 C-band exposure (LC5 group). All rats were kept in specific pathogen-free (SPF) facilities, following a 12-hour light/dark cycle (7:00 a.m. to 7:00 p.m.) with ambient conditions of 20-24°C temperature and 40%-70% humidity. Each cage housed two rats, which were provided with sterilized feed and autoclaved water, ensuring these essentials were replenished as required to sustain their health.

2.2. Microwave radiation

Our microwave sources operating at 1.5GHz and 4.3GHz were located adjacent to each other in an electromagnetically shielded (EMS) room measuring 7m × 6.5m × 4m. The microwave energy was transmitted through a rectangular waveguide and a 16dB gain horn antenna. The radiation table was designed to rotate uniformly and continuously during microwave exposure to ensure uniform radiation distribution. The EMS room was equipped with an air conditioner to maintain constant temperature levels [12-15]. Plexiglas panels were used to separate 20 individual spaces of equal volume within a transparent resin box with a ventilator, and each rat was placed in one of the individual compartments, where it could move comfortably in small increments. This box was then placed under a horn antenna to ensure precise microwave radiation (Figure 1). Each rat model weighing approximately 300g was developed to analyze the absorption of electromagnetic energy by tissues and the temperature rise using the FDTD-based algorithm and the Pennes bioheat equation, respectively, with the simulation and calculation software SEMCAD 14.8.2. The whole-body averaged SAR, testicular averaged SAR, and the testicular temperature rise of the rats at each of the 20 positions of the resin box were measured, and the maximum temperature rise observed in the testes of the rats was about 0.24°C. 20 rats (n=5/group) were acclimatized by resting in a resin box for 30 minutes before being exposed to microwaves exposure. Subsequently, the anal temperature of rats exposed to HPM for 15 minutes was recorded in real-time using

optical fiber system. This recording clarified that there was no significant difference in temperature changes during HPM exposure in each band.

Rats in the C10 and L10 groups were exposed to 4.3GHz (C-band) and 1.5GHz (L-band) microwaves, respectively. Rats in the LC5 group were simultaneously exposed to microwaves at 4.3GHz and 1.5GHz, with each frequency maintaining an average power density of 5 mW/cm^2. Sham group rats received the same treatment in the resin box but without microwave exposure. Each group of rats was exposed to microwaves for 15 minutes and each rat was acclimatized by resting in a resin box for 30 minutes before microwave exposure. The software package Sim4life v6.0 (ZMT, Zurich, Switzerland) was used to calculate the SAR using the finite-difference time-domain (FDTD) method with the following equation: $SAR = \sigma E^2/\rho$ (W/kg) [16]. In this equation, E represents the electric field strength (V/m), σ(sigma) represents the conductivity (S/m), and ρ(rho) represents the sample density (kg/m^3). Rat models with electric field polarization were calculated for 1.5GHz and 4.3GHz electromagnetic wave exposures that ignore the container and are horizontally polarized. The SAR values were 2.76W/kg (whole-body averaged) and 7.09W/kg (testicle averaged) for the L-band; 2.36W/kg (whole-body averaged) and 3.42W/kg (testicle averaged) for the C-band. For the combined L-band and C-band exposure, the SAR values were 2.56W/kg (whole-body averaged) and 5.26W/kg (testicle averaged). Rats were randomly selected from each group and tested on days 1, 7, and 14 after exposure.

2.3. Sperm viability test

On days 1, 7, and 14 after HPM exposure, rats in the Sham and experimental groups (n=5/group/time point) were anesthetized intraperitoneally with 1% sodium pentobarbital (Foshan Chemical, Foshan, China) at a dose of 30 mg/kg for the indicated groups[13]. Subsequently, the left epididymis was surgically excised for further analysis. An incision of the same size was made at the caudal part of the epididymis, which was immersed in 2 mL of saline at 37°C for 1 minute to dilute the spermatozoa by diffusion [17]. Sperm motility was evaluated, including the proportions of progressive sperm (grades A + B), non-progressive sperm (grade C), and immotile sperm (grade D), using the SCA sperm dynamic analysis system (Microptic, Spain). It is generally accepted that grades A + B represent the optimal quality and fertility. In contrast, a higher percentage of grade D indicates poorer

sperm motility. The assessed sperm motility parameters included curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), and beat/cross frequency (BCF). Additional motion parameters included the linearity index (LIN), straightness index (STR), wobble index (WOB), and amplitude of lateral head displacement (ALH).

2.4. Epididymal sperm malformation observation

Sperm suspension smears of rats in the Sham and experimental groups (n=5/group/time point) from the above experiment were stained with hematoxylin and eosin (H&E) (Leagene Biotechnology, Nanjing, China) to observe microscopic morphological changes with sperm counts of at least 100 for each sample [17], using a classification method for abnormal sperm. The malformed sperm which included various head malformations (e.g., large, small, pointed, double, banana-shaped, unhooked, indeterminate, or headless), broken tails, double tails, or double heads were calculated relative to intact sperm as the spermatozoa deformity rate.

2.5. Rat serum hormone assay

On days 1, 7, and 14 after HPM exposure, rats (n=5/group/time point) were anesthetized intraperitoneally as above. During the designated temporal interval, a blood volume of 3 milliliters was collected through the main abdominal vein of each rat, subsequent to which the serum fraction was meticulously isolated [8]. It was then operated with radioimmunoassay commercial kits of the same batch (20230401, Beijing Furui Runze Biotechnology Co., Ltd., Beijing, China) and a radioimmunoassay system (XH6080, Xi'an Nuclear Instrument Co., Ltd., Xi'an, China). The intra-batch coefficients of variation for these kits were less than 10% for follicle-stimulating hormone (FSH, RK-176), luteinizing hormone (LH, PK-177), inhibin B (INH-B, RK-204), and testosterone (T, PK-179). Additionally, the cross-reactivity rate with analogs was less than 1% for both FSH and LH.

2.6. Testicular histopathology examination

On days 1, 7, and 14 after HPM exposure, the left testes were taken from rats (n=5/group/time point) after anesthesia as described above. These were fixed in 10% buffered formalin for a week, then subjected to graded ethanol dehydration, xylene transparency, paraffin embedding, and slicing by a paraffin sectioning microtome (Thermo Fisher, American) in transverse section at a thickness of 5μm [18]. The tissue sections were stained with H&E (Leagene Biotechnology, Nanjing, China). Subsequently, 5 sections from each group were

observed and photographed using light microscopy (Leica, Wetzler, Germany).

2.7. Testicular tissue ultrastructure observation

On day 1 after HPM exposure, approximately 1 mm^3 of testicular tissue was taken from the right testis of rats (n=5/group) after anesthesia as described above and immediately fixed in 2.5% glutaraldehyde. The tissue samples were sequentially processed with 1% osmium tetroxide fixation, followed by a gradient ethanol and acetone dehydration series, epoxy resin embedding (TAAB Laboratories Equipment, Berks, UK), and subsequent ultrathin sectioning. Sections were double-stained with uranyl acetate and lead citrate (Advanced Technology & Industrial Co., Ltd., Hong Kong, China) and observed and photographed with a HITACHI H7650 type transmission electron microscope (Hitachi, Japan)[8].

2.8. Testis tissue energy metabolism and oxidative stress index detection

On days 1, 7, and 14 after HPM exposure, the right testes were harvested from rats (n=5/group/time point) after anesthesia as described previously and immediately frozen in liquid nitrogen, subsequently stored in the -80℃ refrigerator. Approximately 0.2 g of each testicular tissue was weighed and diluted according to the instructions of the kits (Nanjing Jianjian Bioengineering Research Institute, Nanjing, China) to obtain a 10% tissue homogenate. The homogenate was then centrifuged at 3000 rpm for 5 minutes at 4°C. The supernatant was collected for further analysis. Activities of LDH (A020-2), SDH (A022-1), and SOD (A001-3), as well as ATP (A095-2) and MDA (A003-1) content, were measured using the respective kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.9. statistical analysis

All data were presented as the mean ± standard deviation (SD). SPSS 25.0 software (SPSS Inc.Chicago, IL, USA) was used for statistical analysis. One-way ANOVA with post hoc test was used to analyze the radiation effects between radiation groups and the sham group, including single-frequency radiation effects, compound frequency radiation effects, and frequency-effect relationship. Two-way ANOVA was also employed to investigate the interaction effect of microwave exposure modality and time point. All the statistical graphs were drawn using GraphPad Prism 6.0 software (San Diego, CA, USA). P value < 0.05 was considered to be statistically significant. Comparison with Sham group, * indicates P value < 0.05 and ** indicates P value < 0.01. Comparison with L10 group, # indicates P value < 0.05 and ## indicates P value < 0.01. Comparison with C10 group, $ indicates P value < 0.05

and $$ indicates P value < 0.01.

3. Results

3.1. Effect of microwave exposure on spermatozoa

On day 1 after microwave exposure, the percentages of grades A+B spermatozoa decreased by about 15%, 11%, and 9.5% ($P < 0.01$ or $P < 0.05$) and the percentages of grade D spermatozoa increased by about 5%, 4%, and 2.5% ($P < 0.01$) in L10, C10, and LC5 groups, respectively, compared with the Sham group. On day 7 after exposure, the percentages of grades A+B sperm decreased by about 10.5% while grade D sperm increased by about 3% in the L10 group ($P < 0.01$ or $P < 0.05$), compared with the Sham group. Similarly, the percentages of grade D sperm increased by about 4.5% in the C10 group ($P < 0.01$), compared with the Sham group. On day 14 after exposure, compared with the Sham group, percentages of grades A + B sperm decreased by about 4% while grade D sperm increased by about 0.25% in the L10 group ($P < 0.05$). The changes in sperm motility induced by combined L- and C-band microwave exposure were not statistically significantly different from those induced by single exposure (Figure 2).

The results showed that on day 1 after microwave exposure, the sperm malformation rate increased by approximately 5.5% in the L10 group and by 3% in the C10 group ($P < 0.01$ or $P < 0.05$). Furthermore, there was an observed trend toward elevated sperm malformation rate in the HPM composite exposure (Figure 2). However, no statistically significant disparities were detected between the modifications elicited by the combined versus the single exposures.

The above data show that a certain dose of L-band and C-band microwave exposure, both individually and in combination, can adversely affect spermatozoa, with no significant distinction observed in the degree of damage between single and combined exposures. On day 14 after HPM exposure, the C10 and LC5 groups showed a return to normal levels of D-grade spermatozoa percentage and sperm abnormality rate, indicating the organism's potential self-healing ability. This phenomenon warrants further investigation.

3.2. Effect of microwave exposure on hormone levels

The experimental findings revealed that on day 1 after microwave exposure, testosterone levels had dropped by approximately 2.8ng/ml in L10 and C10 groups compared with the Sham group ($P < 0.01$). Similarly, LH levels decreased by about 1.4mIU/ml and 1.2mIU/ml in L10 and C10 groups compared with Sham group ($P < 0.01$). FSH levels also decreased by about 1.9mIU/ml in L10 and C10 groups compared with Sham group ($P < 0.01$ or $P < 0.05$). Additionally, INHB levels decreased by about 46pg/ml and 58pg/ml in L10 and C10 groups compared with Sham group ($P < 0.01$). The results of the composite-exposed group were consistent with those of the single-exposed groups ($P < 0.01$, Figure 3).

On day 7 after microwave exposure, compared with the Sham group, 0.73 ng/ml in the C10 group testosterone levels dropped by approximately 0.87ng/ml in the L10 group ($P < 0.01$) and by 0.73ng/ml in the C10 group ($P < 0.05$). LH levels decreased by approximately 1.25 mIU/ml in the L10 group, 1.65 mIU/ml in the C10 group, and 1.3 mIU/ml in the LC5 group compared with the Sham group ($P < 0.01$). Similarly, FSH levels decreased by around 0.89 mIU/ml in the L10 group, 1.56 mIU/ml in the C10 group, and 1.11 mIU/ml in the LC5 group compared with the Sham group ($P < 0.01$). INHB levels decreased by about 33pg/ml in the C10 group compared with the Sham group ($P < 0.05$). Moreover, the differences in LH and FSH levels between the L10 and C10 groups on day 7 after microwave exposure were statistically significant ($P < 0.01$ or $P < 0.05$, Figure 3).

On day 14 after HPM exposure, LH decreased by approximately 0.86mIU/ml and 0.68mIU/ml in C10 and LC5 groups compared with the Sham group ($P < 0.05$). Similarly, FSH decreased by approximately 1.3mIU/ml in both the C10 and LC5 groups compared with the Sham group ($P < 0.05$). On day 14 after HPM exposure, there was a tendency for hormone levels to recover (Figure 3). The findings suggest that both individual and combined exposure to L-band and C-band microwaves caused a reduction in sex hormone levels within male rats, subsequently leading to a deterioration in male reproductive capabilities. However, there were no significant differences observed in the decline of sex hormone levels between the HPM compound exposure and the single exposure regimens on day 1 after HPM exposure.

3.3. Effect of microwave exposure on testicular histology

To investigate the effect of microwave exposure on testicular histology, we conducted observations on the testicular tissues of rats at both 1 and 7 days after exposure to HPM. Our findings in the L10 group included vacuolization of spermatogenic cells, spermatogonia shedding in the lumen, and interstitial edema. The C10 group displayed vacuolization of spermatogenic cells, interstitial edema. Our observations in the LC5 group included sparseness and thinning of the spermatogenic epithelium, vacuolization of spermatogonia, denaturation, and necrosis of spermatocytes, spermatogonia shedding in the lumen (Figure 4). The histopathological alterations observed, including vacuolation of the spermatogenic epithelium, degeneration and necrosis of spermatogenic cells, and the shedding of cells into the lumen, exhibited signs of recovery on day 14 after HPM exposure. The damage to the testicular spermatogenic epithelium was consistent across both single and combined microwave exposures, characterized by vacuolation of the epithelium and the detachment of spermatogenic cells from the lumen. Interestingly, interstitial edema was more pronounced in single-band microwave exposure compared to compound exposure. In summary, exposure to a certain dose of L- and C-band microwaves for 15 minutes resulted in certain pathological changes in testicular tissues. Exposure to both L- and C-band microwaves caused similar pathological damage as single exposure in the testicular spermatogenic epithelium (Figure 5A).

3.4. Effect of microwave exposure on testicular microstructure

To investigate the ultrastructural changes in the testis after microwave exposure, our observations revealed that the spermatogonia, spermatocytes, and spermatids in all exposure groups exhibited edema and vacuolization on day 1 after exposure. Additionally, nuclear membranes appeared indistinct, and the chromatin, which had condensed, was unevenly distributed, tending to migrate toward the nuclear periphery. The characteristics of ultrastructural injury were consistent between the L- and C-band microwave-exposed groups. Furthermore, the ultrastructural changes in the group exposed to combined-frequency microwaves paralleled those exposed to single-frequency microwaves (Figure 6).

3.5. Effect of microwave exposure on oxidative stress in testicular tissue

The results showed that on day 1 after HPM exposure, the activity of the antioxidant enzyme SOD in testicular tissues decreased by approximately 46.5 U/ml in the L10 group, 54.2 U/ml in the C10 group, and 41.6 U/ml in the LC5 group, compared to the Sham group ($P < 0.01$). Additionally, the content of malondialdehyde (MDA), an oxidative stress metabolite in testicular tissue, increased by about 0.63 nmol/ml in the L10 group and 0.87 nmol/ml in the C10 group ($P < 0.01$ or $P < 0.05$), and there was also a trend of increased MDA levels in the group exposed to the combined frequencies (Figure 5B). On day 7 after HPM exposure, the activity of the antioxidant enzyme SOD in testicular tissues decreased by roughly 34.4 U/ml in the L10 group, 32.2 U/ml in the C10 group, and 24.3 U/ml in the LC5 group, compared with the Sham group ($P < 0.01$ or $P < 0.05$). Additionally, the MDA content increased by approximately 0.75 nmol/ml in the L10 group and 0.95 nmol/ml in the C10 group ($P < 0.05$). There was also an upward trend in MDA levels in the group exposed to the combined frequencies (Figure 5C). However, on day 14 after microwave exposure, both SOD activity and MDA content exhibited a trend of recovery (Figure 5D). There were no statistically significant differences in SOD activity and MDA content alterations between the HPM composite and the single exposure. After a certain dose of HPM single and compound exposure, the decrease in antioxidant enzyme activity corresponded with the increase in MDA content, suggesting that HPM exposure caused an increase in the level of oxidative stress in testicular tissues.

3.6. Effect of microwave exposure on energy metabolism in testicular tissue

The results indicated that on day 1 after HPM exposure, the activity of the energy-metabolizing enzymes LDH in testicular tissues decreased by approximately 943 U/l in the L10 group, 1089 U/l in the C10 group, and 642 U/l in the LC5 group, compared with the Sham group ($P < 0.01$ or $P < 0.05$). Similarly, the activity of SDH of testicular tissue decreased by about 0.09 U/l in the L10 group and 0.10 U/l in the C10 group, compared with the Sham group ($P < 0.01$ or $P < 0.05$). Additionally, the ATP content decreased by about 6.14 μmol/g in the L10 group, 4.62 μmol/g in the C10 group, and 5.05 μmol/g in the LC5 group, compared with the Sham group ($P < 0.01$ or $P < 0.05$, Figure 7).

On day 7 after HPM exposure, the LDH activity in testicular tissues decreased by

approximately 752 U/l in the L10 group and 688 U/l in the C10 group, compared with the Sham group ($P < 0.01$ or $P < 0.05$). Similarly, the SDH activity of testicular tissue decreased by about 0.09 U/l in the L10 group and 0.07 U/l in the C10 group, compared with the Sham group ($P < 0.05$). Additionally, the ATP content decreased by about 4.20 μmol/g in the L10 group and 4.34 μmol/g in the LC5 group, compared with the Sham group ($P < 0.05$, Figure 7). On day 14 after microwave exposure, there was a noticeable trend of recovery in LDH and SDH enzyme activities, as well as ATP content (Figure 7). No significant differences were observed in LDH activity, SDH activity, and ATP content between HPM compound and single exposure. After HPM single and compound exposure, testicular tissue exhibited decreased SDH activity, diminished oxygen utilization efficiency, and reduced LDH activity. Consequently, both the mitochondrial aerobic and anaerobic energy pathways were obstructed, leading to a decline in ATP production. This suggested that multi-frequency HPM exposure may compromise mitochondrial energy production capabilities, causing an insufficient energy supply in testicular tissue, and potentially leading to damage in the male reproductive system.

4. Discussion

Due to the unavoidable long-term exposure to microwave radiation in the environment, the potential health risks of microwave radiation have become a focal point of concern for academics worldwide. This issue has been particularly heightened since the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) suggested in 2015 that the health impacts of electromagnetic field (EMF) exposure necessitate long-term regulatory oversight [19]. Studies have demonstrated that the male reproductive system is particularly susceptible to microwave radiation, with different frequencies shown to impair its functionality [6, 20-22]. Epidemiological research has indicated that long-term exposure to microwaves emitted by radar could negatively affect semen quality [23]. Although numerous studies have focused on the effects of specific microwave frequency bands, daily life exposes people to different microwave frequencies and power densities. The studies found that exposure to different microwave frequencies, including S-band, L-band, C-band, and X-band, could damage the nervous, immune, and cardiovascular systems [13, 14, 24, 25]. However, there is a research gap, both nationally and internationally, on the effects of

compound exposure of different microwave bands on the male reproductive system.

The testis, vital for male reproduction, fosters an ideal environment for germ cell survival. Disruption to this delicate balance can interfere with germ cell development, potentially resulting in diminished male fertility or even infertility. Past studies found that 2.4GHz microwave exposure could damage testicular tissues and affect spermatogenesis [21]. There was considerable evidence suggesting that various microwave frequencies adversely affect sperm count, viability, and morphology [26-29]. Indeed, there is ongoing debate within the scientific community about the biological implications of microwave exposure at different frequencies on the male reproductive system. Celik et al. [30] and Dong et al. [31] noted that microwave radiation did not induce substantial pathological alterations in the rat testes. Some scholars believed that microwaves do not significantly damage spermatozoa [32]. The inconsistencies observed in microwave exposure experiments can be traced back to a multitude of factors, such as variations in microwave frequency and modulation, differing experimental approaches, the species, age, and weight of the subjects, along with the length and strength of exposure. To ensure the accuracy and replicability of research findings, it is imperative to have a comprehensive grasp and stringent control over these variables. We found that single exposure to L- and C-band microwaves with an average power density of 10 mW/cm^2 and simultaneous exposure to L- and C-band microwaves with an average power density of 5 mW/cm^2 could damage the structure and function of the testes in rats, which were mainly characterized by decreased sperm quality, and damage to the testicular tissue structure and ultrastructure. The damage caused by single and compound exposure to L- and C-band microwaves showed some similarity in characteristics, such as vacuolization and detachment of spermatogenic cells. By simulating a complex working environment with simultaneous exposure to microwaves of different frequencies, this study explores the reproductive effects of single and compound HPM exposures in adult male rats, charting new territory in the field of research.

Sex hormones play a crucial role in spermatogenesis, driving the conversion of spermatogonia into spermatocytes and facilitating their production and growth. Testosterone is the most active androgen, mainly secreted by testicular interstitial cells. LH stimulates

mesenchymal stromal cells to produce and release testosterone. When FSH binds to its receptors on supporting cells, it enhances the secretion of androgen-binding proteins. These proteins, in synergy with androgens, further stimulate spermatogenesis [33]. INHB is a heterodimeric protein hormone secreted by testicular support cells, which to some extent responds to testicular support cells' function and is closely related to spermatogenesis [34-36]. Previous research indicated that certain doses of microwave radiation could alter sex hormone levels, which could affect male reproductive function. Shahin et al. [4] found that exposing male mice to 2.45GHz microwaves for 2h daily for 15, 30, and 60 days decreased serum testosterone levels and resulted in damage to the spermatogenic epithelium of testicular tissues, a decrease in spermatogonial cells, and a disruption in the arrangement of supporting cells. Furthermore, our study found that rats exposed to 1.5 and 4.3GHz microwaves for 15 minutes showed reductions in serum testosterone and LH levels by approximately 40%-50%, FSH by about 40%, and INHB by about 20%-40% on day 1 after exposure. This indicates that even short-term exposure to high-power microwaves can impact hormone levels in rats. Testosterone, LH, FSH, and INHB levels decreased on days 1 and 7 after L-band and C-band microwave exposure, compared to the control group, and the difference in the degree of reduction in sex hormone levels induced by compound versus single exposure was not significant. Some of the variability observed in our findings may be attributed to the inherent differences among the animal subjects and the intricate feedback mechanisms regulating hormone levels. In contrast to the study by Shahin et al. [4], which focused on the effects of long-term microwave exposure, our research highlights the effect of short-term exposure to HPM.

While many studies demonstrated the adverse effect of different microwave radiation frequencies on the male reproductive system, the underlying mechanisms of combined microwave exposure remain complex. These mechanisms encompass oxidative stress, impaired energy metabolism, overexpression of apoptosis-related genes, abnormal protein expression, and DNA damage, with oxidative stress being one of particular interest to many scholars [6, 37-40]. The intersection of oxidative stress and energy metabolism occurs at the mitochondrial level. Curley et al. [41] found that microwave radiation impairs oxidative respiration by altering the mitochondrial morphology and membrane structure in cancer cells, which consequently leads to an increase in ROS levels. This finding lends support to the

hypothesis that microwave radiation induces mitochondrial-mediated oxidative stress. Similarly, this study revealed that single and combined exposure to HPM induced edema and vacuolization of spermatogenic cells at all levels, and increased vacuolization of some organelles, like mitochondria, further suggesting that HPM can affect mitochondrial function, and thus oxidative stress and energy metabolism. However, other than vacuolization, no significant mitochondrial morphological and structural alterations were observed in the present study, which may be closely related to the study of Curley et al. [41] differing in microwave frequency bands, radiation doses, and radiation targets. De Iuliis et al. [42] found mitochondria as an important ROS source within the male reproductive system, emphasizing that such oxidative stress can cause abnormalities in the mitochondrial electron respiratory chain, thus affecting ATP synthesis. In our study, we found that single and combined exposure to L-band and C-band microwaves decreased SOD activity and increased MDA content in testicular tissues. This was accompanied by enhanced oxidative stress, decreased LDH and SDH activities, and a significant decrease in ATP content after microwave radiation. These observations suggest that exposure to microwave radiation may alter mitochondrial oxidative respiration. In conclusion, oxidative stress and impaired energy metabolism are interconnected, adversely affecting the male reproductive system following microwave exposure. Alterations in mitochondrial structure in testicular tissues of rats after combined HPM exposure warrant further investigation in subsequent studies.

In summary, 1.5GHz and 4.3GHz microwaves could harm the male reproductive system, affecting testicular architecture, sperm morphology and viability, as well as hormone levels. The degree and characteristics of reproductive injury were consistent across both HPM compound exposures and single exposures. Our findings highlighted that enhanced oxidative stress and impaired energy metabolism were important mechanisms behind the damage to the male reproductive system after microwave exposure at 1.5GHz and 4.3GHz. Nevertheless, more in-depth investigation is crucial to fully understand the specific molecular mechanisms and pathways involved. Future research should explore the specific molecular mechanisms of oxidative stress-induced injury in testicular tissues and identify the targets of mitochondrial energy metabolism disorders. This will aid in the effective protection of organisms from the adverse effects of microwave exposure.

Ethical Approval

The animal experiments were approved by the Institutional Animal Care and Use Committee at Beijing Institute of Radiation Medicine (IACUC-DWZX-2020-781).

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Informed Consent

Not applicable.

Disclosure Statement

No potential conflict of interest was reported by the authors.

Data Availability

The datasets used and/or analyzed during the current study are available from the author on reasonable request.

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Acknowledgements

B.Y. and R.P. conceived the project and drafted the manuscript. B.Y. performed the experiments. Y.L. analyzed the data, interpreted experimental results, and prepared figures. J.M., Y.P., J.G., Y.B., H.W., J.Z., L.Z., X.X., J.D., and C.L. helped to perform experiments and analyze data. All authors read and agreed to the published version of the manuscript.

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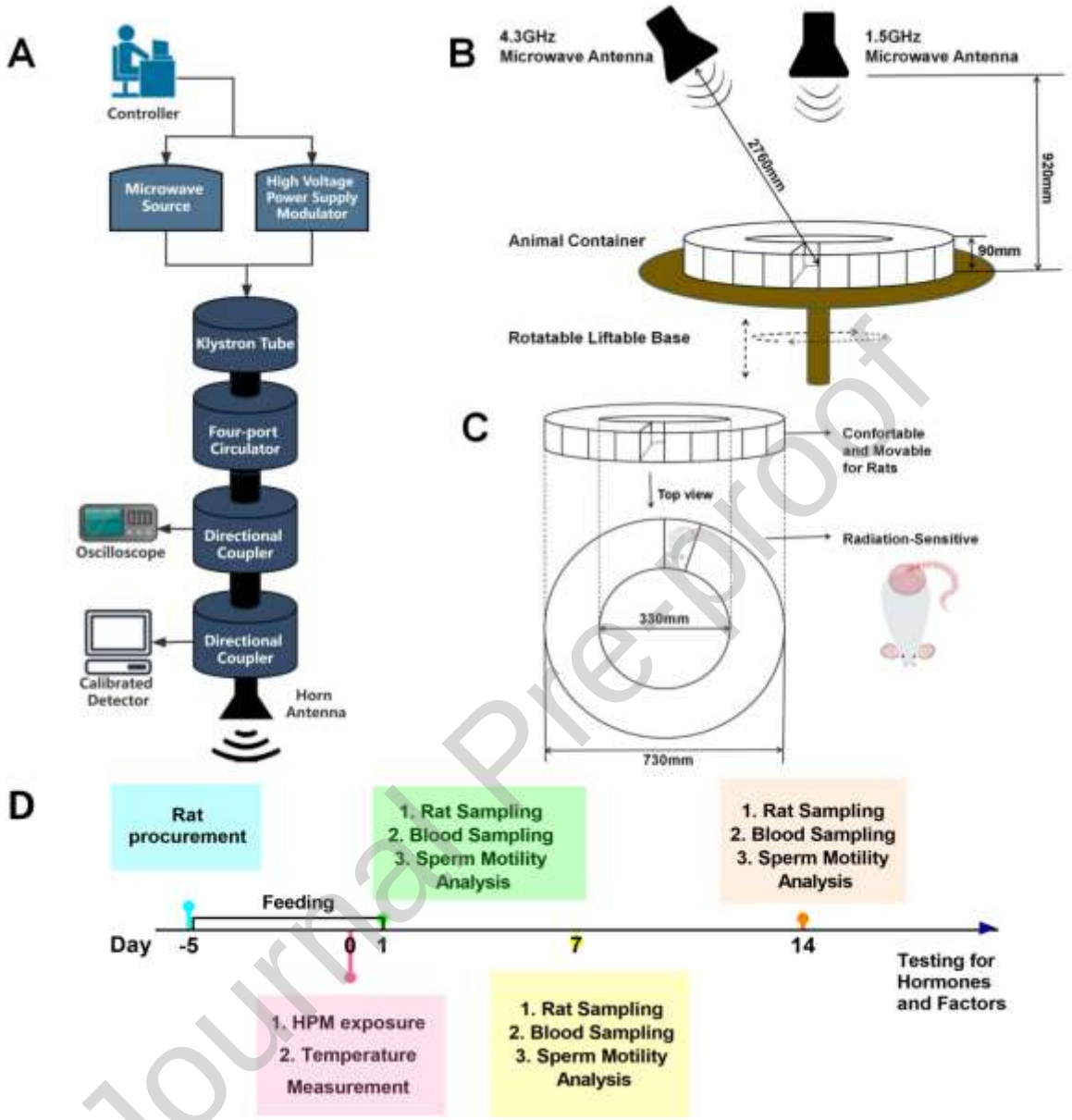

Figure 1. Schematic diagram of the radiation apparatus and rats experiments timeline. A: Schematic diagram of microwave radiation source structure and principle. B:

Microwave radiation device and experimental design. C: Structure diagram of microwave radiation container. D: Schematic diagram of the whole experiment.

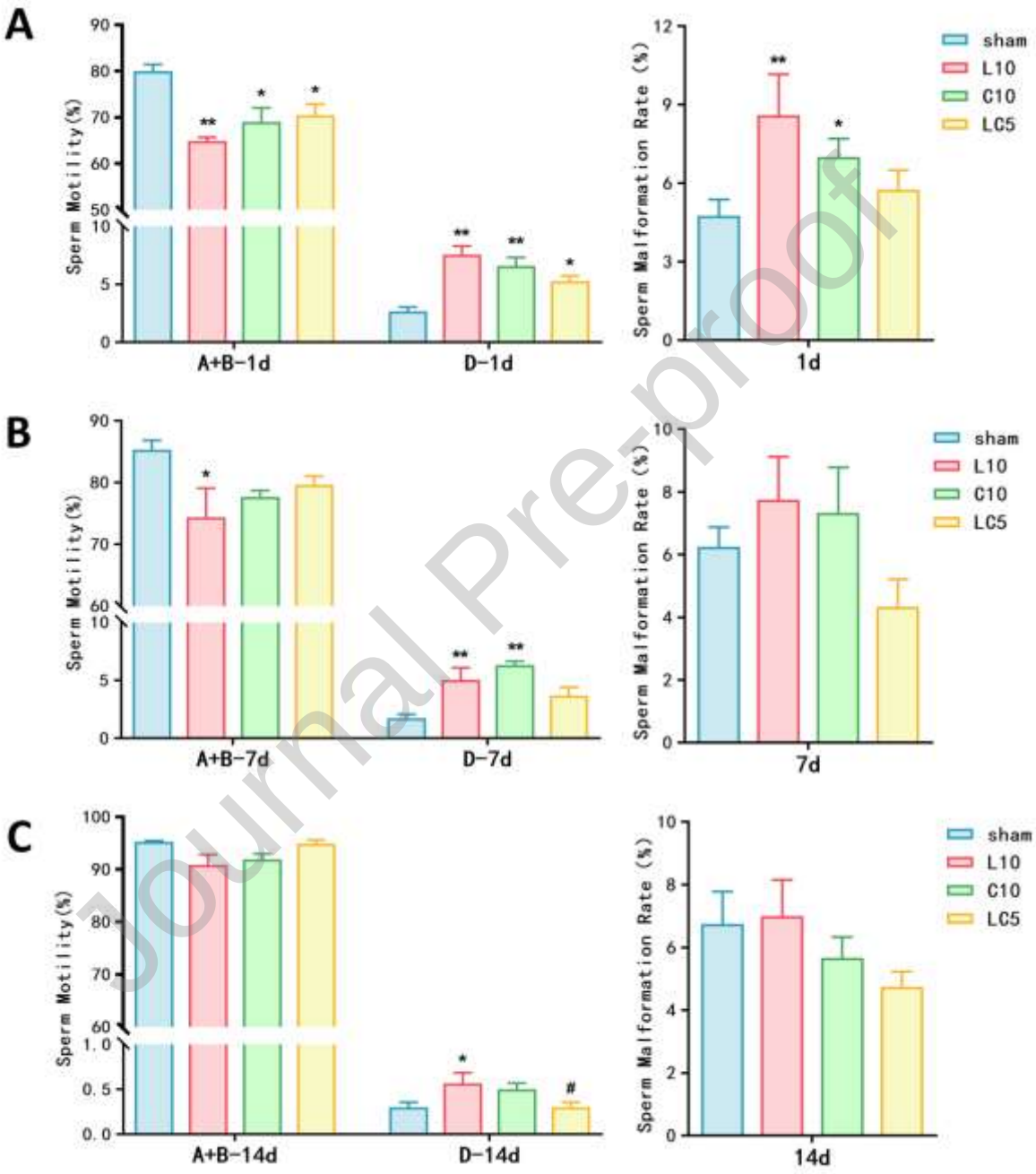

Figure 2. Changes in sperm motility and sperm malformation rate in rats after 1.5GHz and 4.3GHz HPM exposure. A: on day 1 after exposure; B: on day 7 after exposure; C: on day 14 after exposure. Sperm motility was analyzed using the SCA sperm dynamic

analysis system, including the proportions of progressive sperm (grades A + B) and immotile sperm (grade D) at each time point after HPM exposure. H&E staining was utilized to quantify malformed and normal spermatozoa within the microscopic field, calculating the sperm malformation rate at each time point after HPM exposure. Data are shown as mean ± SD. *, $P < 0.05$, **, $P < 0.01$ vs. Sham group; #, $P < 0.05$ vs. L10 group; $, $P < 0.05$ vs. C10 group.

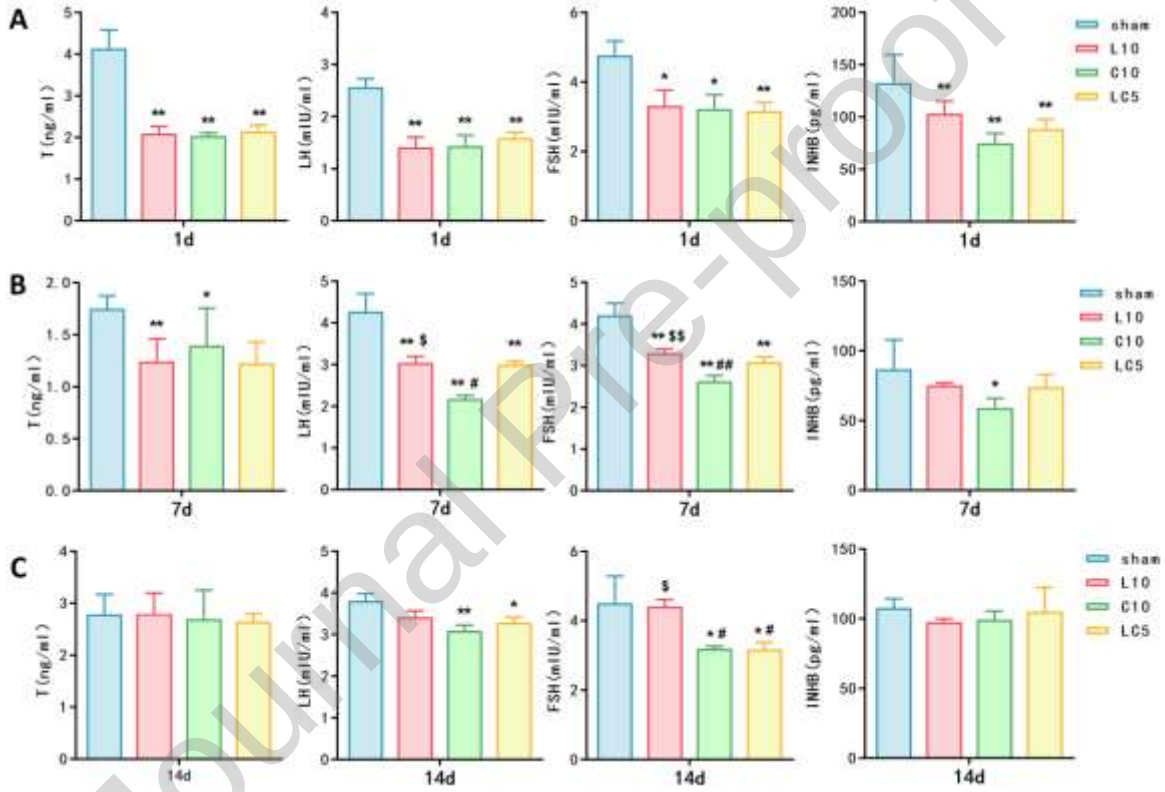

Figure 3. Effects of 1.5GHz and 4.3GHz HPM on serum hormones in rats. A: on day 1 after exposure; B: on day 7 after exposure; C: on day 14 after exposure. At each time point after HPM exposure, venous blood was drawn from rats, and serum was extracted by centrifugation. The levels of T, LH, FSH, and INHB in the serum were measured using radioimmunoassay. Data are shown as mean ± SD. *, $P < 0.05$, **, $P < 0.01$ vs.

Sham group; #, P < 0.05, ##, P < 0.01 vs. L10 group; $, P < 0.05, $$, P < 0.01 vs. C10 group.

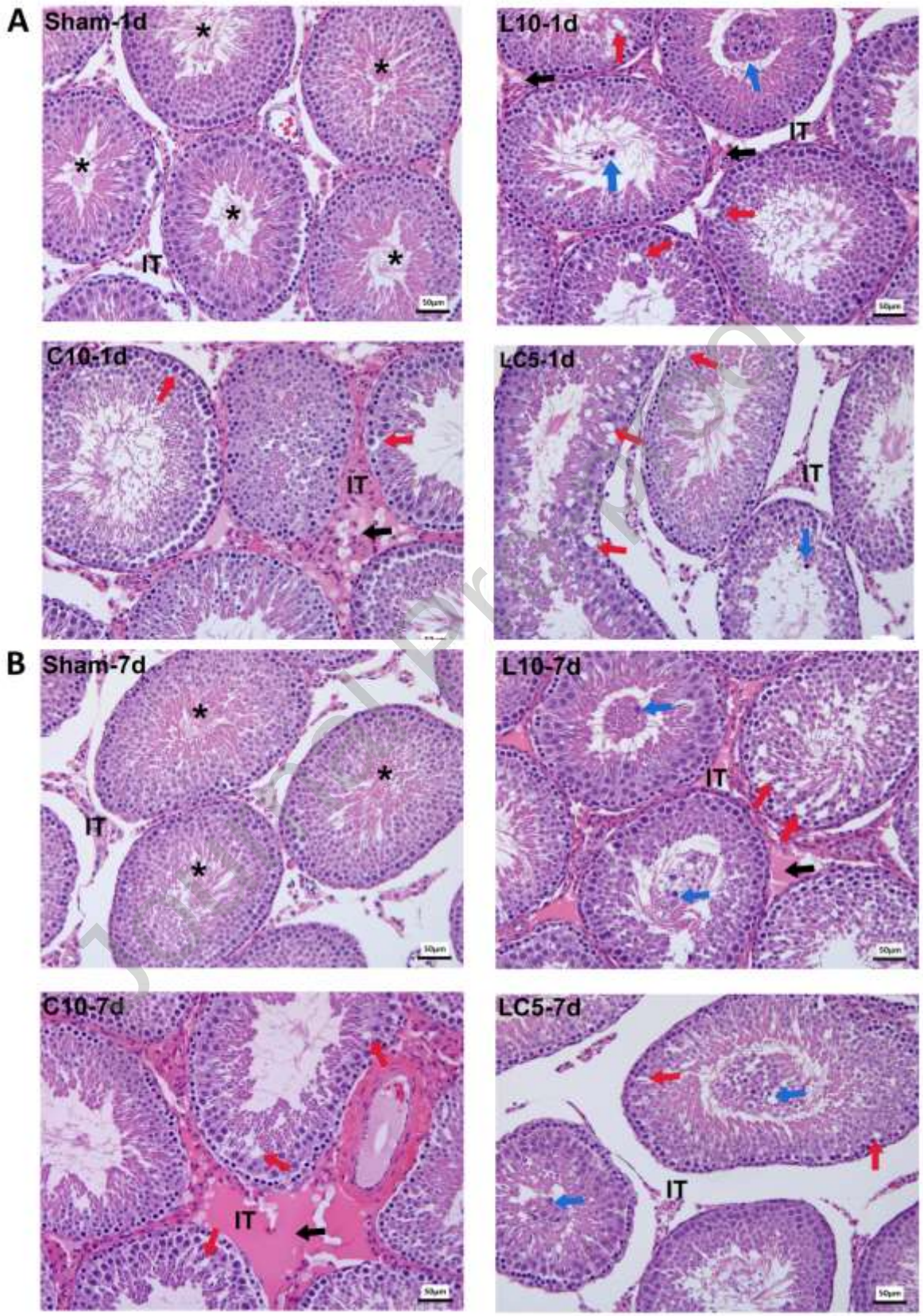
A
Sham-1d
L10-1d
C10-1d
LC5-1d
B
Sham-7d
L10-7d
C10-7d
LC5-7d
IT

Figure 4. Effects of 1.5GHz and 4.3GHz HPM on Testicular morphology of rats (H&E, bar = 50μm). A: on day 1 after exposure; B: on day 7 after exposure. The light microscopic examination was performed on H&E-stained sections of rat testicular tissue to ascertain the histological alterations. (⋆) = normal seminiferous tubules. (IT) = interstitial tissue. Red arrows mark the vacuolization of the seminiferous epithelium. Black arrows mark interstitial

tissue edema. Blue arrows mark spermatogenic cells shedding into the seminiferous tubule lumen.

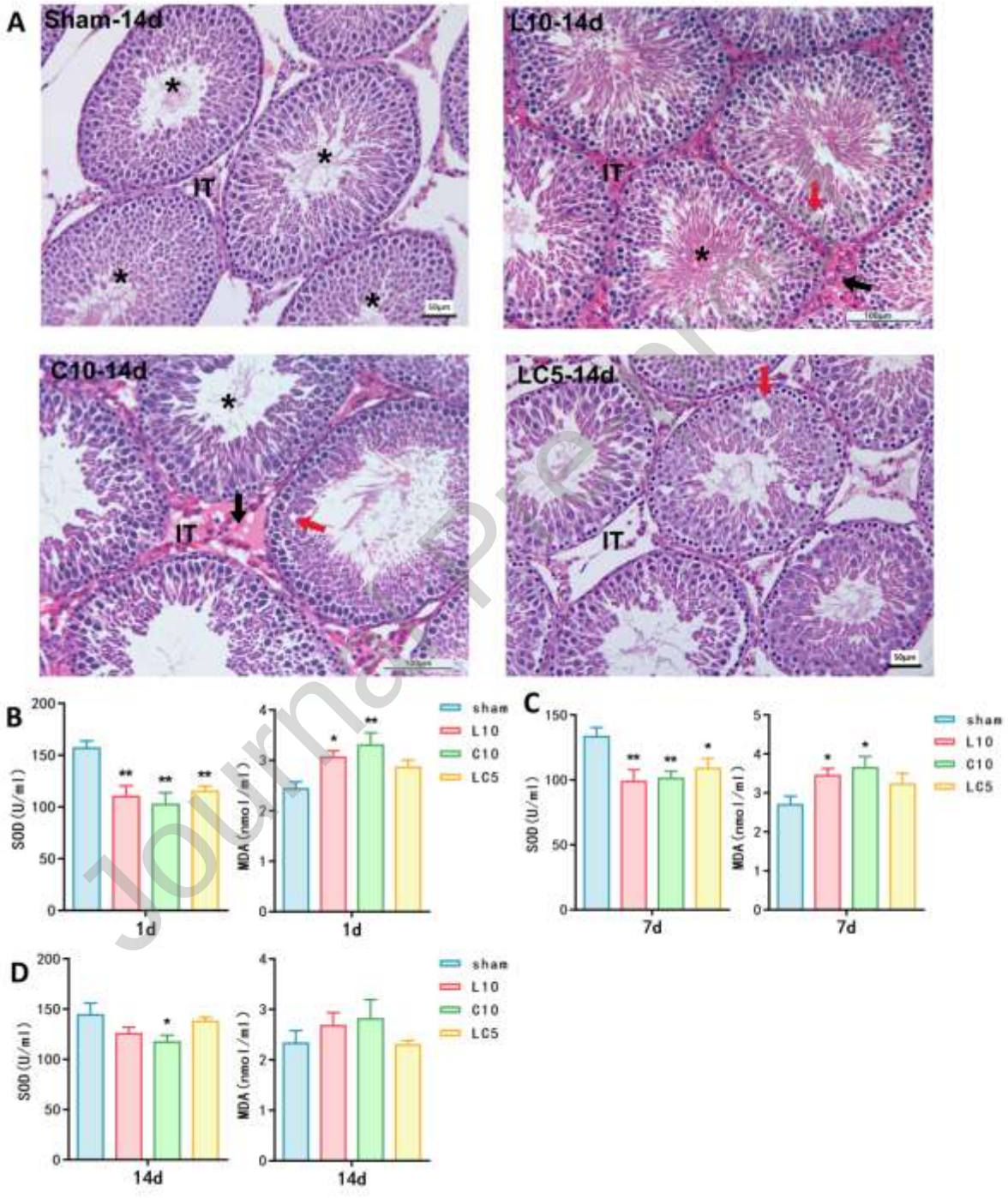

Figure 5. A: Effects of 1.5GHz and 4.3GHz HPM on Testicular morphology of rats on day 14 after exposure(H&E, L10-14d and C10-14d: bar = 100μm; the others: bar =

50μm). (*) = normal seminiferous tubules. (IT) = interstitial tissue. Red arrows mark the vacuolization of the seminiferous epithelium. Black arrows mark interstitial tissue edema. B-D: Effects of 1.5GHz and 4.3GHz on oxidative stress indices in testicular tissues of rats. B: on day 1 after exposure; C: on day 7 after exposure; D: on day 14 after exposure. At each time point after HPM exposure, venous blood was drawn from rats, and serum was extracted by centrifugation. The levels of SOD and MDA in the

serum were measured using radioimmunoassay. Data are shown as mean ± SD. *, $P < 0.05$, **, $P < 0.01$ vs. Sham group.

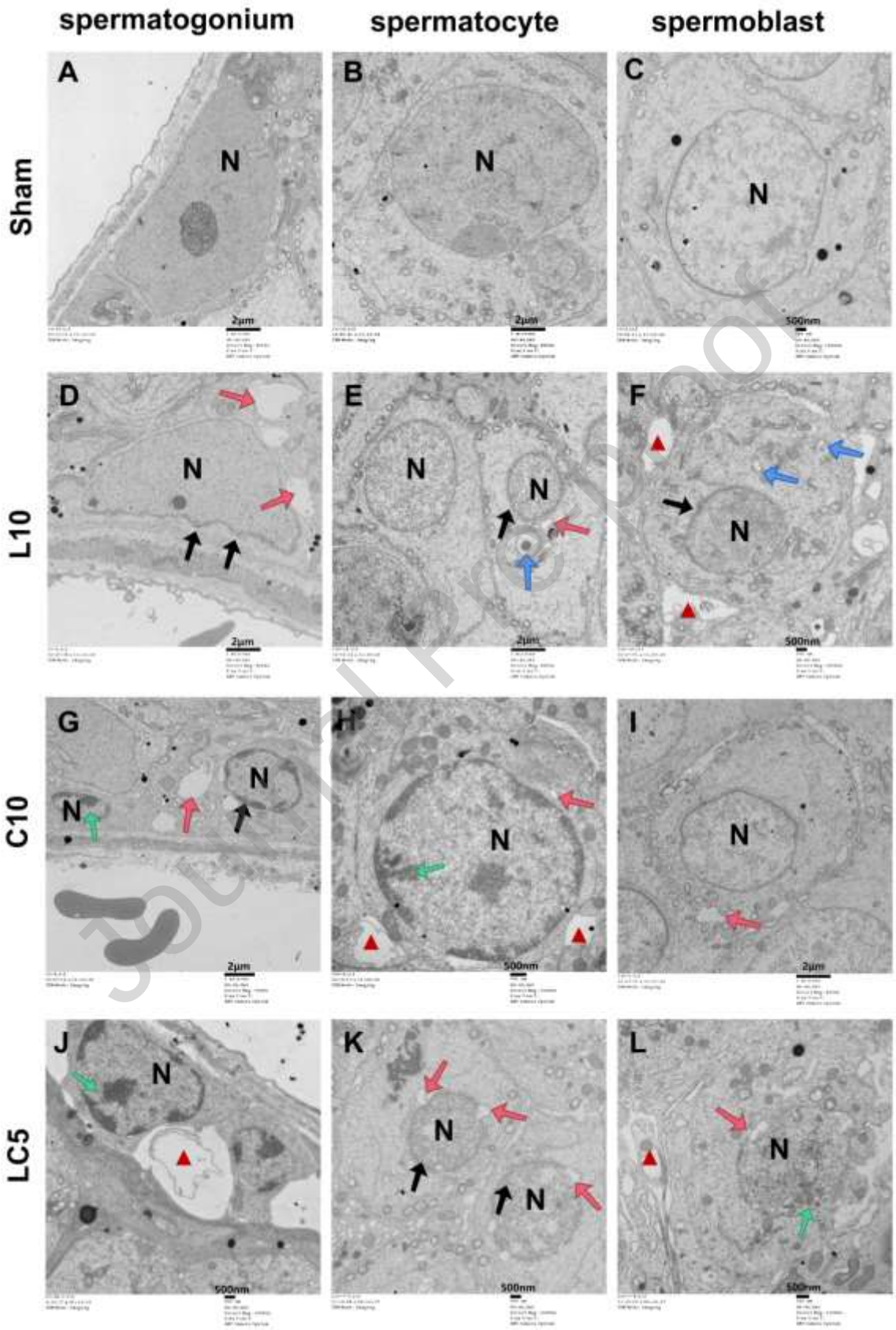

Figure 6. Effect of 1.5GHz and 4.3GHz microwave radiation on the ultrastructure of rat testis(TEM, A-B, D-E, G, and I: bar = 2μm; C, F, H, and J-L: bar = 500nm). Representative transmission electron micrograph of rat testicular tissue on day 1 after HPM exposure. (**N**) = cell nuclei. (▲) = intercellular edema. Red arrows mark the vacuolization of cells. Black arrows mark indistinct nuclear membranes. Blue arrows mark the vacuolization of organelles. Green arrows mark chromatin condensation, uneven distribution, and migration toward the nuclear edge.

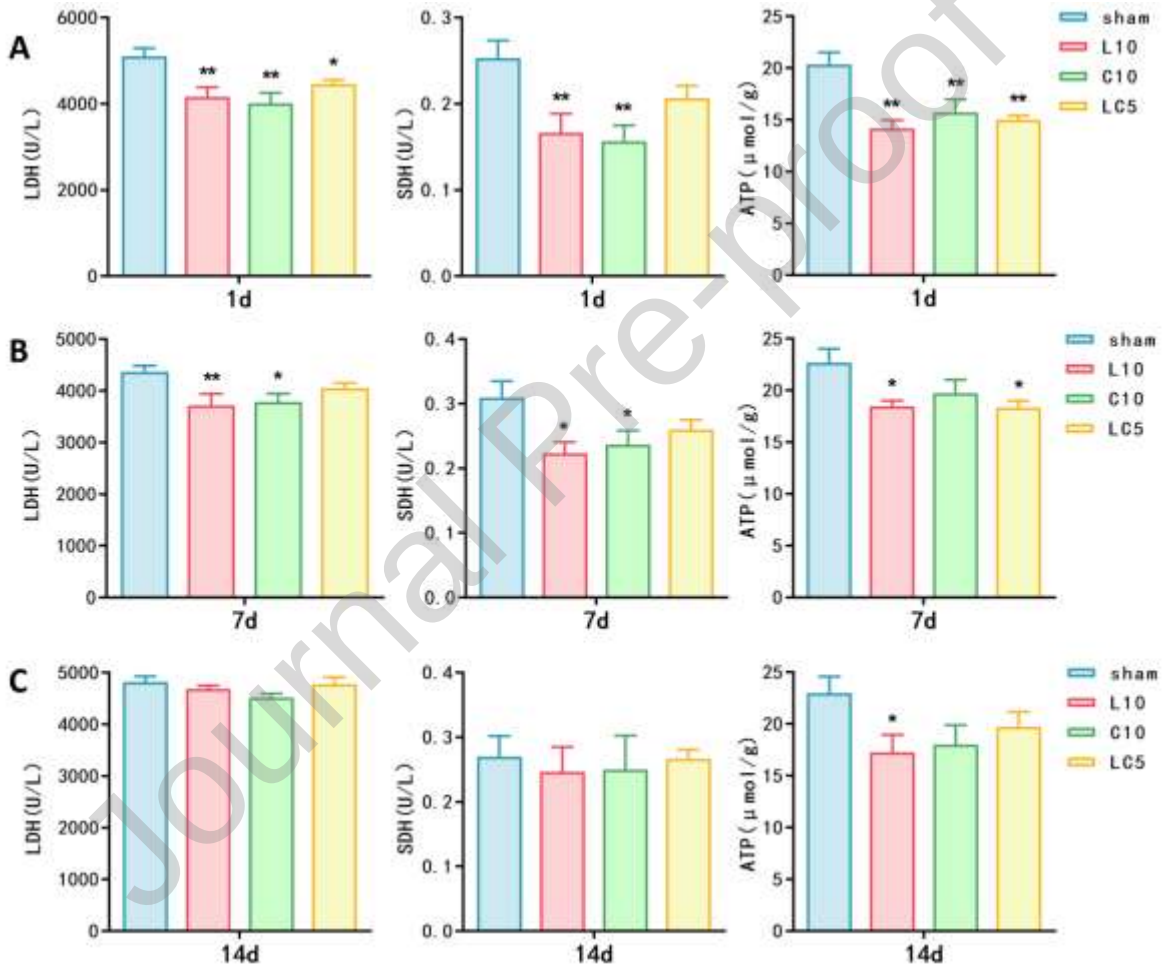

Figure 7. Effects of 1.5GHz and 4.3GHz on energy metabolism indexes in testicular tissues of rats. A: on day 1 after exposure; B: on day 7 after exposure; C: on day 14 after exposure. At each time point after HPM exposure, venous blood was drawn from rats, and serum was extracted by centrifugation. The levels of LDH, SDH, and MDA

in the serum were measured using radioimmunoassay. Data are shown as mean ± SD. *, $P < 0.05$, **, $P < 0.01$ vs. Sham group.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Highlights

1. Multi-frequency HPM combined exposure imitated human daily microwave exposure.
2. Exposure to 1.5 and 4.3 GHz HPM induced structural damage to the testicular tissue, compromised sperm quality, and disrupted the normal levels of sex hormones in rats.
3. Male reproductive damage from HPM single and combined exposures was similar.
4. Oxidative stress and energy metabolism effect male reproduction after HPM exposure.
5. Oxidative stress and impaired energy metabolism are mutually causal mechanisms.