



OPEN Radiofrequency radiation-induced changes in Leydig cell function

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Radiofrequency radiation, emitted from commonly used wireless communication devices, has been implicated in disrupting cellular homeostasis; however, its effects on testicular somatic cells such as Leydig cells remain poorly understood. To address this, the present study investigated the frequency- and time-specific effects of RFR on cellular morphology, proliferation, and cell cycle dynamics in TM3 Leydig cells. Cells were exposed to mobile phone radiation and radiofrequency signals at 1800 MHz and 2450 MHz for 15–120 min under non-thermal conditions. Following exposure, morphological alterations were examined using Giemsa staining, while proliferation and cell cycle progression were evaluated by BrdU-ELISA and PI-based flow cytometry. BrdU assays showed a progressive reduction in DNA synthesis across conditions, indicating suppressed proliferative activity. Consistently, cell cycle analysis revealed accumulation of cells in G1 phase with a corresponding decline in S-phase population at longer durations, suggesting checkpoint activation. These changes were supported by morphological alterations such as cell rounding, loss of adherence, and membrane blebbing, features associated with stress-induced antiproliferative responses. Overall, these findings indicate that RFR disrupts cellular morphology, DNA synthesis, and cell cycle progression in a frequency- and time-dependent manner, highlighting Leydig cell vulnerability to prolonged exposure and potential implications for male reproductive health.

Keywords Leydig cells, Radiofrequency electromagnetic radiation (RF-EMR), Cell cycle distribution, DNA synthesis, BrdU assay, Mobile phone radiation

Abbreviations

RFR	Radiofrequency radiation
EMR	Electromagnetic
PI	Propidium iodide

With the widespread adoption of digital and wireless technologies, individuals are now persistently exposed to ambient levels of radiofrequency electromagnetic radiation (RF-EMR) originating from everyday sources including smartphones, Wi-Fi routers, smartwatches, and other smart devices. As a form of non-ionizing radiation, RF-EMR spans frequencies from 30 kHz to 300 GHz and does not possess sufficient photon energy to directly break molecular bonds or ionize atoms¹. However, a growing body of evidence suggests that prolonged or high-frequency RF-EMR exposure can still induce biological effects through non-thermal mechanisms such as oxidative stress, altered gene expression, and membrane disruption^{2,3}. These exposures, which are now virtually unavoidable in modern environments, have raised serious public health concerns, particularly regarding their potential impact on cellular structure, homeostasis and reproductive health^{4,5}.

Among the various tissues potentially influenced by environmental stressors, the testis is particularly vulnerable due to its high metabolic demands and intricate hormonal regulation^{6,7}. Within the testicular interstitium, Leydig cells are essential for synthesizing testosterone, a key hormone responsible for maintaining male reproductive physiology. Disruption of Leydig cell function or proliferation can impair androgen production, compromise spermatogenesis, and ultimately contribute to testicular dysfunction and reduced male fertility^{8,9}. Although numerous studies have implicated RF-EMR exposure in oxidative damage, mitochondrial dysfunction, impaired steroidogenesis, and sperm abnormalities^{10,11}, relatively few have examined its direct effects on Leydig cell proliferation and cell cycle regulation, processes essential for preserving cellular equilibrium

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and supporting testicular endocrine activity. Systematic, comparative studies examining different RF-EMR frequencies and exposure durations are particularly lacking, despite the increasing relevance of such exposures in real-world scenarios.

Proliferation and cell cycle regulation are essential for maintaining cellular equilibrium and genomic stability^{12,13}. DNA synthesis during the S-phase serves as a critical indicator of proliferative capacity, and its quantification has been widely utilized to assess cellular replication under physiological and stress conditions¹⁴. Exposure to RF-EMR has been reported to disrupt these tightly regulated processes. For instance, Zhao et al.¹⁵ demonstrated upregulation of apoptosis-related genes in primary neuronal and astrocyte cultures irradiated to mobile phone radiation, implying interference with proliferation-associated pathways. Similarly, Yao et al.¹⁶ reported RF-EMR-induced damage in lens epithelial cells' DNA, underscoring the genotoxic potential of such exposures. Additional structural cellular damage, including a reduction in neuronal cell counts, has been observed in the cerebellum of rats following 900 MHz RF-EMR exposure¹⁷, suggesting heightened vulnerability of metabolically active tissues. In the reproductive context, RF-EMR has been associated with impaired sperm function⁴, disrupted testicular architecture, and hormonal imbalance in male rodents¹⁰. Developmental studies have further shown that in utero exposure to mobile phone radiation can alter neuronal development and postnatal behaviour in mice¹⁸. These findings collectively support the biological relevance of RF-EMR exposure; however, few studies have systematically explored its influence on proliferation and cell cycle dynamics in testicular cells. Moreover, cellular responses appear to vary depending on both frequency and duration of RF-EMR exposure, emphasizing the need for carefully controlled, comparative investigations.

Recent reviews have explored possible interactions between radiofrequency electromagnetic radiation and reproductive biology, although the overall evidence remains inconclusive. Yadav et al.¹⁹ summarized findings linking radiofrequency exposure with possible alterations in spermatogenesis, sperm motility, and hormonal regulation, yet emphasized that many outcomes depend on exposure duration, dosimetry, and biological model. Kaur et al.²⁰ reviewed studies assessing genotoxic risks to male germ cells and highlighted inconsistencies in experimental design and limited reproducibility of reported effects. In the context of female fertility, Jangid et al.²¹ outlined potential influences of non-ionizing radiation on ovarian and endocrine function, while concluding that further well-controlled investigations are required to establish causal relationships. Overall, these reviews suggest plausible non-thermal mechanisms but collectively emphasize the need for standardized methodologies and cautious interpretation of experimental findings.

Conversely, several investigations have reported no measurable genotoxic, molecular, or cellular alterations following RF-EMR exposure under comparable non-thermal conditions. For example, Lee et al.²² found no evidence of teratogenic or developmental abnormalities in murine fetuses following combined exposure to CDMA (849 MHz) and WCDMA (1.95 GHz) signals throughout gestation, indicating that biological effects of RF-EMR may not be consistently observed under all exposure conditions. Similarly, Speit et al.²³ found no evidence of genotoxicity in human fibroblasts and Chinese hamster cells exposed to 1.8 GHz RF-EMF under comparable non-thermal conditions. Zeni et al.²⁴ also reported no statistically significant differences in DNA damage, chromosomal aberrations, or proliferation indices in human peripheral blood leukocytes exposed in vitro to 900 MHz GSM-modulated radiation compared with sham-exposed controls. Consistent results were obtained by Valbonesi et al.²⁵, who observed no changes in HSP70 expression or DNA integrity in human trophoblast cells exposed to 1.8 GHz amplitude-modulated fields.

Importantly, different RF-EMR frequencies are associated with distinct patterns of energy absorption and tissue penetration, which can influence their biological effects. Lower frequencies such as 900 MHz can penetrate deeper into biological tissues, while higher frequencies like 2450 MHz tend to be absorbed superficially, leading to differential cellular responses²⁶. Although Wi-Fi signals and mobile phones are among the most prevalent RF-EMR sources, it remains uncertain whether these varying frequencies exert different effects on important cellular processes like proliferation and cell cycle progression. Understanding these frequency-dependent effects is critical, particularly in metabolically active or hormone-producing cells, which may be more vulnerable to RF-induced stress. Recent findings suggest that RF-EMR may induce time-dependent cellular responses. For instance, Yadav and Singh²⁷ observed significant modulation of immune parameters in leukemic cells following different durations of mobile phone radiation exposure. Yet, comparative studies investigating how different RF-EMR frequencies and exposure durations influence cellular homeostasis in reproductive somatic cells remains limited.

Although the present study focuses on frequency- and duration-dependent effects, it is important to note that RF-EMR-induced biological responses are influenced by additional physical parameters, including modulation, output power or field strength, duty cycle, polarization, and spatial field homogeneity. Variations in these characteristics can modify the pattern and intensity of energy deposition within biological samples, thereby shaping the magnitude and nature of observed effects. Recognizing these factors provides essential context for interpreting experimental findings and for situating the present work within the broader field of RF dosimetry.

The current investigation aimed to evaluate the frequency- and time-dependent effects of non-ionizing RF-EMR on DNA synthesis and cell cycle progression in mouse Leydig (TM3) cells. Cells were exposed to three commonly encountered RF sources: (1) a 4G mobile phone operating in active talk mode, (2) a signal generator emitting at 1800 MHz, and (3) a 2450 MHz frequency. All exposures were conducted under strictly controlled non-thermal conditions to isolate the effects of electromagnetic radiation. BrdU incorporation assay was employed to examine the proliferative activity by measuring the DNA synthesis during the S-phase, whereas flow cytometric analysis was done for evaluating cell cycle phase distribution by using propidium iodide (PI) staining. Through this dual-assay approach, the study aimed to evaluate whether RF-EMR elicits frequency-specific antiproliferative effects and to explore the potential disruption of cell cycle dynamics as a mechanistic basis for RF-induced cellular alterations. In addition, Giemsa staining was employed to assess changes in cellular morphology, providing complementary evidence of cytotoxic or antiproliferative effects.

Our findings add to the accumulating evidence concerning the biological effects of RF-EMR by offering new insights into its frequency- and time-dependent impact on reproductive somatic cells. Specifically, alterations in DNA synthesis and cell cycle dynamics suggest interference with key regulatory processes. Given the essential endocrine functions of Leydig cells and the increasing global dependence on wireless technologies, understanding these effects is critical for accurate risk assessment and for guiding the formulation of science-based public health recommendations.

Methodology

Cell maintenance and culture conditions

TM-3 mouse Leydig cell line (ATCC[®], CRL-1714, American Type Culture Collection, Manassas, USA) was cultured under aseptic conditions using standard procedures. The cells were grown in DMEM/F12 medium (1:1 mixture) containing 1% penicillin–streptomycin solution and enriched with 20% FBS (fetal bovine serum). Cell culture was maintained in 5% CO₂ in a humidified incubator (Thermo Scientific, 371, Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India) at 37 °C. For routine maintenance, cell line was sub cultured in complete medium (18–20 mL) using T-175 flasks. Once cultures approached 80–90% confluence, they were enzymatically detached using 1X Trypsin–EDTA solution (0.25%) for further passaging and then prepared for subsequent experimental assays.

Chemical reagents

All chemicals used were of analytical grade and were primarily obtained from Sigma, Cayman, and Himedia. Propidium iodide (PI) (Invitrogen[™], P3566, Thermo Fisher Scientific, Eugene, USA), RNase (Roche, 10109142001, Merck/Sigma-Aldrich, Mannheim, Germany), Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12) (HiMedia, AL215A, HiMedia Laboratories Pvt. Ltd., Thane, India), Giemsa (Fisher Scientific, 38723, Fisher Scientific India Pvt. Ltd., Mumbai, India), 1% Penicillin–Streptomycin (Sigma-Aldrich, P0781, Sigma-Aldrich, St. Louis, USA), H₂SO₄ (Himedia, RM6244, HiMedia Laboratories Pvt. Ltd., Thane, India), Phosphate Buffered Saline (PBS) (HiMedia, ML023, HiMedia Laboratories Pvt. Ltd., Thane, India), tetramethylbenzidine (TMB) substrate (Abcam, AB171523, Abcam plc, Cambridge, United Kingdom), Heat-inactivated fetal bovine serum (FBS) (Himedia, RM10951, HiMedia Laboratories Pvt. Ltd., Thane, India), dH₂O, Bovine serum albumin fraction-V (BSA) (HiMedia, MB083, HiMedia Laboratories Pvt. Ltd., Thane, India), goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (Abcam, ab6789, Abcam plc, Cambridge, United Kingdom), monoclonal anti-BrdU antibody (Sigma-Aldrich, B8434, Sigma-Aldrich, St. Louis, USA), HCl (Himedia, MB306, HiMedia Laboratories Pvt. Ltd., Thane, India), 0.25% Trypsin–EDTA 1X Solution (HiMedia, TCL007, HiMedia Laboratories Pvt. Ltd., Thane, India), Tween-20 (BIO-RAD, 1706531, Bio-Rad Laboratories Inc., Hercules, USA), Dimethyl sulphoxide (DMSO) (Sigma-Aldrich, D8418, Sigma-Aldrich (Shanghai) Trading Co. Ltd., Shanghai, China), triton X-100 (Qualigens, Q10655, Thermo Fisher Scientific India Pvt. Ltd., Mumbai, India), methanol (Fisher Scientific, 34457, Fisher Scientific India Pvt. Ltd., Mumbai, India), and additional general laboratory chemicals were sourced from Sigma/Merck.

Cell irradiation

Mobile phone irradiation

Radiofrequency radiation (RFR) and sham exposures were conducted in line with the approach detailed by Jangid et al.¹¹ & Yadav et al.²⁷. In short, a 4G smartphone (Xiaomi Note 7), with a power density of 0.224 W/m², was employed to deliver exposure at different time intervals, inside a CO₂ incubator (5% CO₂) maintained at 37 °C. The device operated at a carrier frequency of 2318 MHz, corresponding to the 4G/LTE downlink band. The cell culture suspension (1 × 10⁶ cells/mL) was placed in 15 mL tubes during the procedure and one tube was exposed in each session. The tube was placed vertically, positioned parallel to and at a fixed distance of 1 cm from the mobile phone's radiating surface/Back panel. The antenna region of the mobile phone was aligned at the mid-height of the tube, ensuring uniform exposure along its length. This configuration was used to maintain a consistent and well-defined electromagnetic field around the sample and to eliminate any interference or mutual coupling effects that could arise from multiple tubes.

According to the manufacturer, the specific absorption rate (SAR) of the phone is 0.838 W/kg for the body and 0.962 W/kg for the head (measured at a 15 mm distance). However, in the present setup, the biological samples were positioned at 1 cm from the radiating surface, not 15 mm. The SAR relevant to the cell samples was estimated using the theoretical model described by Yadav et al.²⁷, based on the formula $SAR = \sigma E^2 / \rho$, where σ is the electrical conductivity, ρ is the density, and E is the electric field strength. The calculated SAR at this distance was approximately 0.5 W/kg, representing the actual exposure experienced by the cells.

Calibration of the device was verified using the Narda 520 system by assessing its physical parameters. Over the course of exposure, no variation in the temperature was detected. Background conditions remained the same in both sham and RFR exposure groups.

1800 MHz & 2450 MHz irradiation

The experimental setup for 1800 MHz and 2450 MHz exposure included a signal generator, an incubator (irradiation chamber) (iGene, IG-C160, iGene Labserve Pvt. Ltd., New Delhi, India), and a handheld power meter (Keysight, V3500A, Keysight Technologies, Santa Rosa, USA). A horn antenna & Keysight signal generator (Keysight, N9310A, Keysight Technologies, Santa Rosa, USA) was used to generate electromagnetic waves at 1800 MHz and 2450 MHz, which were transmitted through a horn antenna. The horn-antenna exposures were conducted using a continuous wave (CW) signal generated by the Keysight N9310A system, operating without any external modulation or duty cycle, ensuring a steady, non-modulated RF output throughout the exposure period. Exponentially growing TM3 cells were selected for treatment. The temperature variation between the

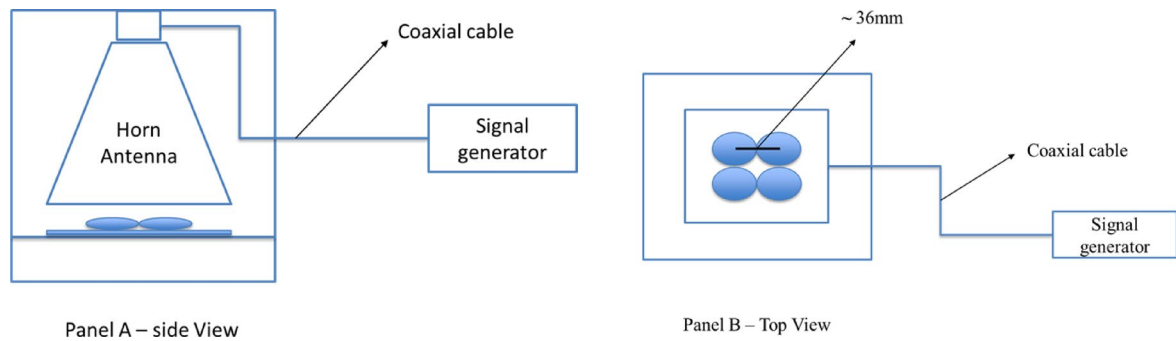


Fig. 1. RF-EMR Exposure System—Schematic Diagram (Panel A: side View and Panel B: Top View).

Exposure condition/ frequency	Average E-field (V/m)	Peak E-field (V/m)	Measurement uncertainty
Mobile phone (2318 MHz)	9.25	9.63	2–3%
1800 MHz	14.69	15.02	2–3%
2450 MHz	13.44	13.82	2–3%

Table 1. Average and peak E-field intensities for different exposure conditions.

exposed and control groups was less than 0.1 °C and the incubator maintained stable conditions of 37 °C and 5% CO₂ throughout the procedure. Cell suspensions (1 × 10⁶ cells/mL) were seeded in Petri dishes (35 mm), and positioned 2 cm away from the horn antenna during exposure.

During each exposure session, four 35 mm Petri dishes were exposed simultaneously. The dishes were arranged adjacently on a flat tray (30 × 20 cm) with a center-to-center spacing of approximately 36 mm. The horn antenna was mounted vertically on the top interior surface of the CO₂ incubator, aligned perpendicular to the sample plane. The tray containing the dishes was centrally positioned within the incubator chamber, maintaining equal spacing from all walls to ensure symmetrical field distribution and minimize reflective losses. Field homogeneity within the exposure zone was evaluated using a Narda 520 system and a close-field probe (Keysight, N9311X-100, Keysight Technologies, Santa Rosa, USA), and the field distribution was found to be approximately uniform across the sample plane. The incubator’s built-in air circulation and CO₂ regulation system maintained stable environmental conditions (37 ± 0.1 °C temperature and 5% CO₂ concentration) throughout the exposure period. To minimize electromagnetic reflections and interference from the incubator’s metallic inner walls, the exposure system was centrally aligned within the chamber, maintaining uniform spacing from all sides. Field mapping using the close-field probe indicated minimal reflection effects, confirming effective confinement of the radiated field within the exposure zone. A schematic illustration of the arrangement of the petri dishes, antenna, and tray positioning is provided in the Fig. 1 (Panel A and Panel B).

E-field measurement The electric-field (E-field) intensity was measured at the position of the biological samples using a calibrated Narda 520 system. Measurements were performed at the sample plane corresponding to the location of the biological samples to ensure accurate field characterization under experimental conditions. Average and peak E-field values for each exposure condition are summarized in Table 1.

Experimental design

TM-3 cells (Mouse Leydig cell line) were divided into different experimental groups during their exponential growth phase:

- 1. Mobile phone
 - Non-irradiated: Cell line was maintained without irradiation.
 - Irradiated: Cell line was subjected to RFR emitted from a mobile phone (4G) in talk mode.
- 2. 1800 MHz
 - Non-irradiated: Cell line was kept under identical conditions without radiation.
 - Irradiated: Cell line was exposed using a signal generator operating at 1800 MHz.
- 3. 2450 MHz
 - Non-irradiated: Cell line was maintained without irradiation.
 - Irradiated: Cell line was exposed using a signal generator operating at 2450 MHz.

Each experiment involved exposure durations of 15, 30, 45, 60, 90, and 120 min. Following irradiation, the treated cells were subjected to downstream assays for further analysis.

In vitro experiments

Morphological analysis: microscopy

TM3 cells (1×10^5 cells/mL) were plated into 6-well culture plates post-irradiation and maintained for 24 h. Following incubation, cells were rinsed with PBS to remove residual media and fixed at room temperature using chilled methanol (100%) for 15 min. After that, methanol was discarded and cells were rinsed with PBS, followed by staining with Giemsa solution (1:20 dilution in distilled water) for 20 min²⁸. Post staining, the cells were again rinsed with PBS two times and imaged under an Olympus microscope at 20X resolution (Eclipse Ts2-FL, Nikon, Japan). Nuclear and cytoplasmic features were evaluated to assess morphological changes.

Proliferation assay: BrDu-ELISA

The method of Hawker Jr, J. R.¹⁴ was employed for the 5-bromo-2-deoxyuridine (BrdU) ELISA. Briefly, following irradiation, cells were plated (50,000 cells/well) in a 96-well plate, and BrdU (100 μ L/well; 10 μ M final concentration, Cayman) was added. Wells without cells or BrdU were included as negative controls to monitor nonspecific reagent binding. The next day, cells were rinsed with PBS (pH 7.4) two times, fixed at room temperature with 70% EtOH (100 μ L/well) for 10 min, followed by another two rounds of washing with PBS and one with dH₂O, permeabilized for 10 min with 0.2% Triton-X-100 in PBS, washed again with PBS and then acid denatured with 0.1 M HCl (100 μ L/well) for 10 min at 37 °C, neutralized with 150 μ L borate buffer per well (pH 9, 0.1 M), rinsed twice with Phosphate-Buffered Saline, and kept in blocking buffer (1% BSA in PBS; 100 μ L/well) for 30 min at RT. Monoclonal anti-BrdU antibody (100 μ L/well; 1:3000 dilution; Sigma) was then added to the wells at room temperature for 60 min, followed by rinsing of wells with PBST (PBS + 0.1% Tween-20) to rinse away unbound antibody, then treated with 100 μ L of HRP (goat anti-mouse IgG horseradish peroxidase) conjugate per well (1:3000 dilution; Abcam) for 30 min at RT. Wells were again rinsed with PBST, and then with dH₂O, and subsequently prepared for detection. 100 μ L tetramethylbenzidine (TMB) substrate (Abcam) was then added to the wells for 25–30 min to allow the development of a blue chromogenic signal. Wells were quenched thereafter with 1N H₂SO₄ and the reading was taken at 450 nm by an ELISA plate reader (Bio-Rad). The intensity of the developed colour, reflected in the absorbance values, is directly proportional to DNA synthesis and cell proliferation.

Cell cycle analysis: flow cytometry

Cell cycle analysis was adapted from the method described by Schoene et al.²⁹, with minor modifications. TM3 Leydig cells were plated in 6-well plates (1×10^6 cells/well) after exposure to RF-EMR and maintained for 24 h under standard culture parameters (37 °C, 5% CO₂). Post-incubation, cells were rinsed with PBS and redispersed in chilled 70% ethanol and incubated overnight at –20 °C. Following this, fixed cells were rinsed with PBS to remove residual ethanol, and stained with a propidium iodide (PI) staining solution containing Triton X-100 (0.1%), RNase A (25 μ g/mL), and PI (50 μ g/mL). The cells were kept at room temperature with this staining cocktail for 40 min in the dark. Flow cytometric analysis was conducted using the BD FACSAria™ Fusion flow cytometer (BD Biosciences, USA). Prior to sample acquisition, instrument performance was verified through daily quality control (QC) using BD FACSDiva™ Cytometer Setup and Tracking (CS&T) Research Beads. A total of 30,000 events per sample were captured for analysis. Data analysis was performed using FlowJo™ software.

Statistics

Each experimental procedure was conducted independently on at least three different instances, and every parameter was measured in three technical replicates. The resulting datasets ($n = 3$) were subjected to statistical analysis using GraphPad Prism version 8 (GraphPad Software, USA) and Microsoft Excel 2013. Differences among groups were analysed via ordinary one-way and 2way ANOVA along with Dunnett's multiple comparisons test. The data of the study are summarized as mean $\pm$ SEM for each group, significance levels were represented as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****), and significance was defined at $p < 0.05$.

Results

Electric-field (E-field) intensity at the position of the biological samples was measured using a calibrated Narda 520 system. The E-field was 14.69 V/m for 1800 MHz and 13.44 V/m for 2450 MHz. The fields were verified to be stable throughout the exposure period, with variations remaining within the measurement accuracy of the probe.

Effect of radiofrequency radiation on morphology of Leydig cells

To assess the impact of radiofrequency radiation on cellular morphology, Phase-contrast microscopy was used. TM3 Leydig cells were irradiated to mobile phone, 1800 MHz, and 2450 MHz radiation for 15–120 min (Fig. 2). Control cells displayed spindle-shaped morphology with a flattened cytoplasm and intact nuclei, indicative of healthy cell adherence and growth.

In contrast, exposure to mobile phone radiation induced morphological alterations. Cells exhibited early signs of cytoplasmic shrinkage and nuclear condensation at 60 min, which became more evident at 90 and 120 min, where cell rounding, detachment, and reduced density were pronounced, suggestive of cell death in the culture.

Cells exposed to 1800 MHz radiation displayed relatively mild morphological changes maintaining structural integrity similar to controls. However, with longer exposures (90 and 120 min), significant alterations emerged, including cell rounding, detachment, and membrane blebbing, indicating that extended exposure is required for visible structural damage at this frequency.

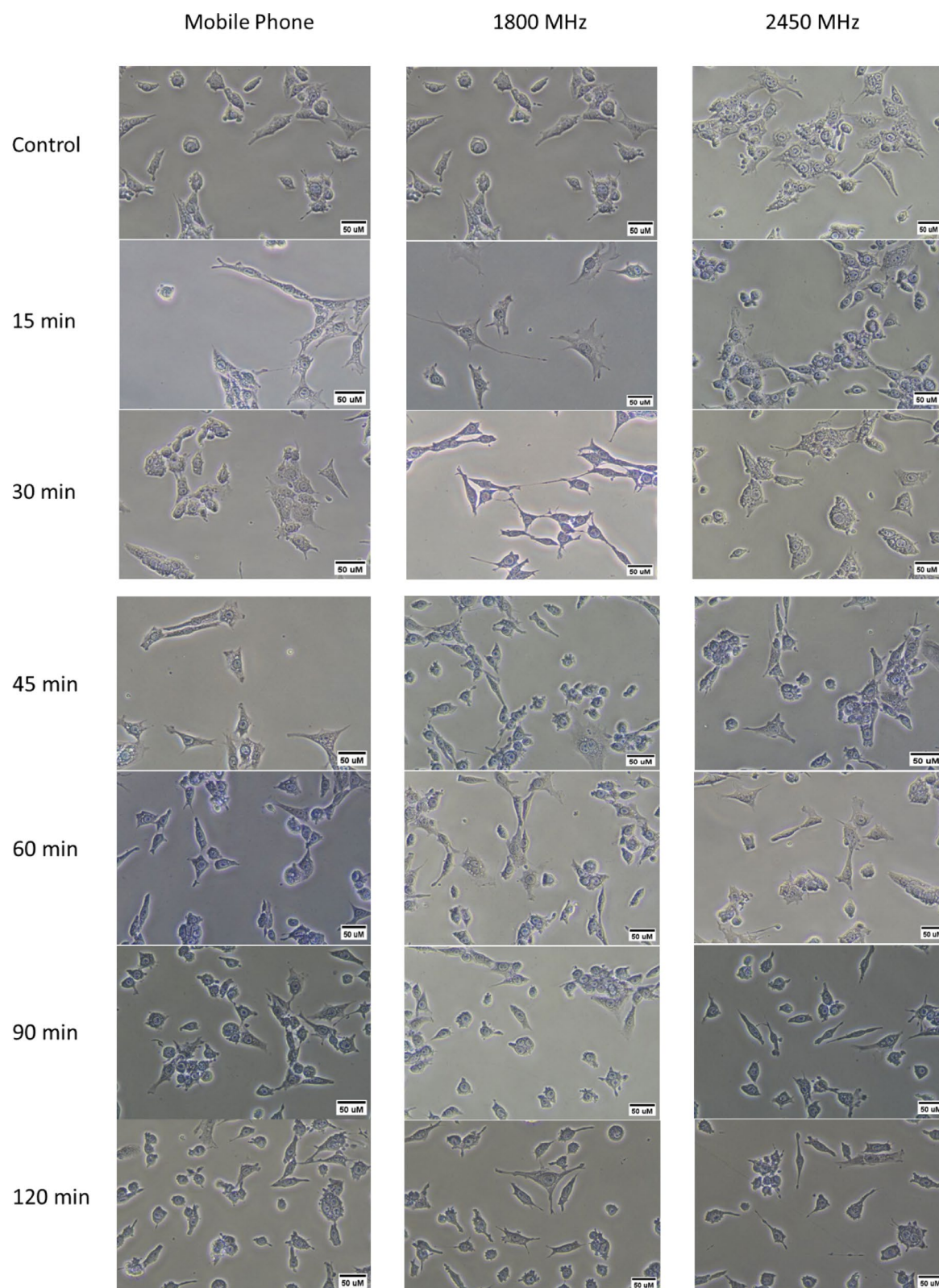


Fig. 2. Representative phase-contrast micrographs showing the effect of radiofrequency radiation on TM-3 Leydig cells.

Exposure to 2450 MHz radiation elicited the most severe morphological stress responses. Cells demonstrated membrane blebbing, and clustering after exposure. Prolonged exposure (≥ 60 min) resulted in marked cell shrinkage, loss of adherence, and increased numbers of rounded cells, consistent with stress-induced cytotoxicity.

Collectively, these findings suggest that RFR induces morphological changes in TM3 Leydig cells in a frequency- and time-specific manner. Among the tested conditions, an increased response by the cells to longer exposure of RFR was observed, as more dead cells detached from the culture dishes after the duration of time was increased to 120 min. 2450 MHz radiation triggered the most prominent alterations followed by mobile phone radiation, while 1800 MHz required prolonged exposure to exert similar effects. These morphological disruptions support the hypothesis that RFR exposure compromises Leydig cell integrity, potentially impairing their physiological function.

Mobile phone radiation, 1800 MHz radiation, and 2450 MHz radiation on TM3 Leydig cell morphology at different exposure durations (15–120 min). Control cells displayed normal morphology with intact nuclei. Exposed cells exhibited frequency- and time-dependent alterations, including cytoplasmic shrinkage, rounding, detachment, and nuclear condensation. Scale bar: 50 μm .

Effect of radiofrequency radiation on DNA Synthesis as an indicator of proliferation in TM3 Leydig cells

BrdU incorporation assay was conducted to examine the impact of RFR on DNA synthesis in TM3 cells. As a thymidine analogue, BrdU becomes integrated into DNA synthesized during the S-phase, making its detection a reliable marker of cell proliferation. Cells were irradiated to RFR emitted from a mobile phone, as well as to 1800 MHz and 2450 MHz RFR from a signal generator, for durations ranging from 15 to 120 min. BrdU incorporation was quantified and expressed relative to the control group. Statistical analysis revealed time-specific effects of RFR on DNA synthesis.

In response to mobile phone radiation exposure, TM3 cells exhibited a progressive decline in BrdU incorporation with increasing exposure time. Although a slight reduction was noted at 30 min, statistical significance emerged at 45 min ($p < 0.01$), became more prominent at 60 min ($p < 0.001$), and was most pronounced at 90 and 120 min ($p < 0.0001$), suggesting an inhibitory effect of prolonged exposure on S-phase entry and DNA replication.

On the other hand, exposure to 1800 MHz radiation did not significantly alter BrdU incorporation at shorter time points (15–45 min), suggesting a delayed cellular response to this frequency. Interestingly, a mild but statistically significant decrease in DNA synthesis was noted at 60 min ($p < 0.05$), indicating the onset of cellular response to 1800 MHz exposure. However, this was followed by a sharp and statistically significant decline at 90 and 120 min ($p < 0.0001$), suggesting that extended exposure duration is required for this frequency to exert a measurable antiproliferative effect on TM3 Leydig cells.

Exposure to 2450 MHz radiation also induced a similar downward trend in BrdU incorporation, starting from early time points (15–60 min), although these changes were not statistically significant ($p > 0.05$). However, a significant antiproliferative response emerged at 90, and 120 min ($p < 0.0001$), suggesting that this frequency also progressively reduces proliferation as the exposure duration increases. Thus, both frequencies demonstrated a comparable pattern, ultimately culminating in a strong antiproliferative effect at extended exposure durations.

Collectively, the BrdU assay results (Fig. 3) indicate that the degree and timing of proliferative suppression in TM3 Leydig cells are both the frequency and exposure duration specific. The strongest and earliest effects were observed with mobile phone radiation, followed by 1800 MHz radiation, while the 2450 MHz radiation group displayed a delayed yet significant decline in proliferation. Notably, the 120 min exposure condition consistently produced the greatest inhibition across all radiation types, suggesting that it may represent a critical threshold for RFR-induced antiproliferative effects in Leydig cells. Additionally, to ensure comprehensive comparison, direct between-group comparisons among the three exposure types at matched durations were also performed (Supplementary Figure S1 a–f). Between-group differences were generally non-significant, except at 90 min and 120 min, where statistically significant variations in proliferation were detected. These observations collectively suggest that prolonged RFR exposure may interfere with DNA replication processes, thereby compromising cellular proliferative capacity.

Mobile phone exposure, 1800 MHz exposure, and 2450 MHz exposure.

Cells were exposed to the indicated radiation sources for 15, 30, 45, 60, 90, and 120 min. DNA synthesis was quantified through BrdU incorporation and expressed as relative cell proliferation percentage compared to the control group. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparison test. Asterisks indicate levels of significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Results are presented as the mean $\pm$ standard error of the mean (SEM) from one independent experiment ($n = 3$ for each group).

Effect of radiofrequency radiation on cell cycle progression in TM3 Leydig cells

To assess whether radiofrequency radiation modulates cell cycle distribution, TM3 cells were subjected to flow cytometry analysis following exposure to mobile phone radiation, 1800 MHz, and 2450 MHz frequencies for varying durations (15–120 min). Cell distribution across the G1, S, and G2/M phases was quantified and compared against unexposed controls (Fig. 4, Gating strategy for cell cycle distribution in TM-3 cells).

Mobile phone irradiation resulted in a time-specific increase in the G1-phase population, which became statistically significant from 45 min onward ($p < 0.05$). A decline in S-phase cells was observed, reaching significance at 45 min ($p < 0.01$), with further reductions at 60, 90, and 120 min also remaining statistically significant. The G2/M population remained largely unchanged across all exposure durations, with no statistically significant alterations observed. These shifts indicate a G1-phase arrest and a reduction in S-phase entry, consistent with impaired DNA replication under prolonged mobile phone exposure.

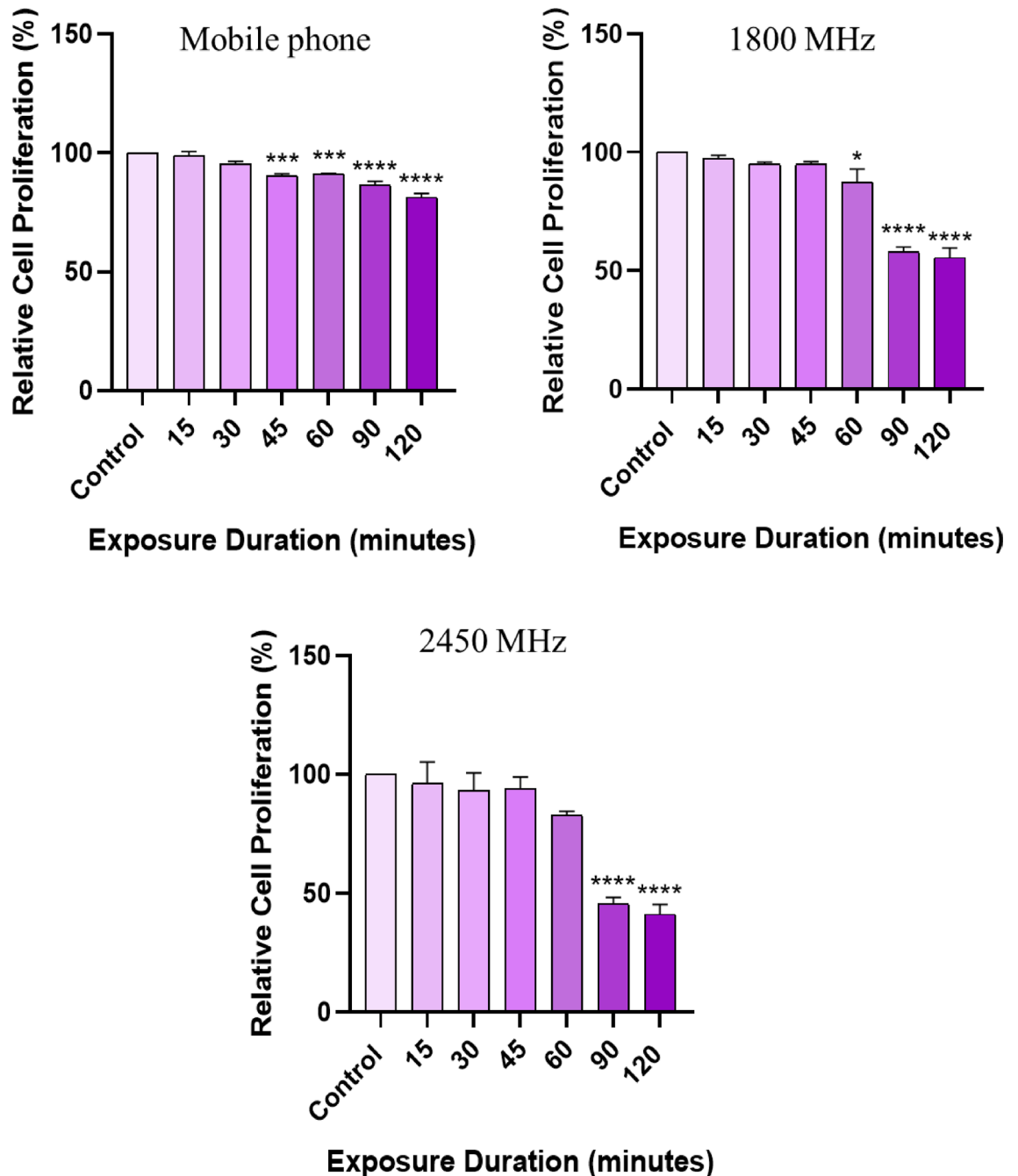


Fig. 3. Effects of radiofrequency radiation on proliferation of TM3 Leydig cells.

In the 1800 MHz-exposed group, the G1-phase population showed a significant increase at 60 min ($p < 0.05$), 90 min ($p < 0.01$) and 120 min ($p < 0.01$) of exposure. In contrast, the S-phase population demonstrated a marked reduction beginning at 60 min ($p < 0.01$), persisting at 90 min ($p < 0.01$) and becoming highly significant at 120 min ($p < 0.001$). Meanwhile, the G2/M population remained relatively stable, with no statistically significant alterations observed across the exposure durations. These results indicate that 1800 MHz radiation disrupts cell cycle progression by inducing G1 arrest and by suppressing S-phase entry, suggesting activation of cell cycle checkpoints under prolonged exposure.

In the 2450 MHz-exposed group, cell cycle changes became evident only after prolonged exposure. We observed an increase in G1 cell population at 90 min ($p < 0.01$) and 120 min ($p < 0.05$) of exposure, suggesting delayed cell cycle progression. The S-phase population, however, showed a gradual decline over time, reaching significance at 120 min ($p < 0.01$). The G2/M-phase percentage remained largely unchanged across all durations, with no statistically significant changes observed.

These findings correspond well with the BrdU assay results, where a consistent reduction in DNA synthesis was observed across all exposure durations, becoming statistically significant at 90 and 120 min. While BrdU

Gating strategy for cell cycle distribution in TM-3 cell

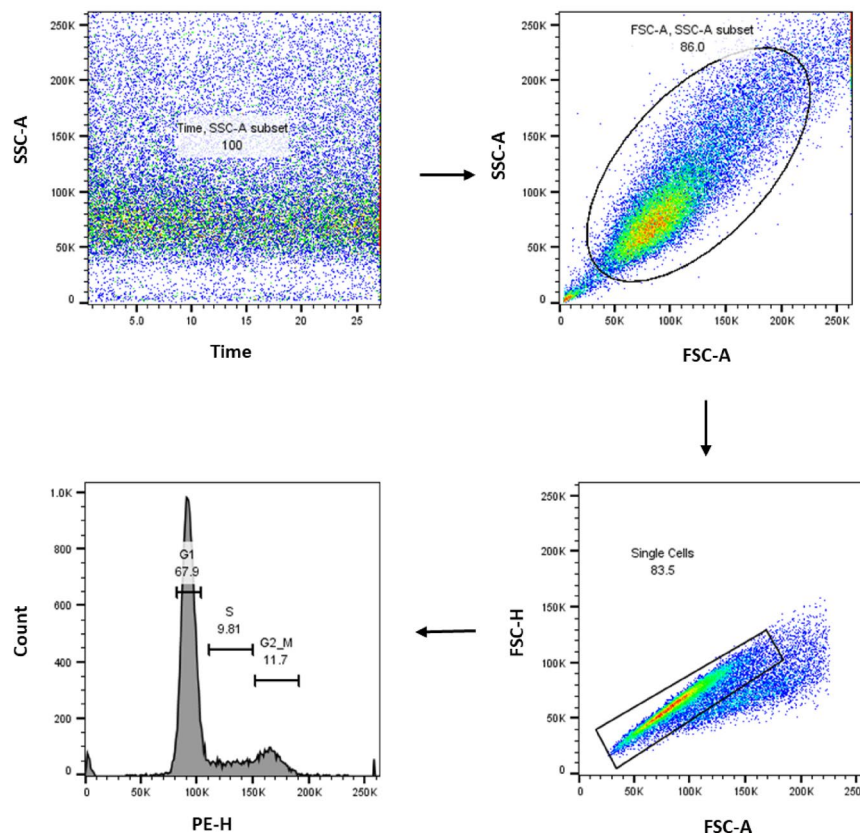


Fig. 4. Effect of radiofrequency radiation on cell cycle distribution in TM3 Leydig cells.

incorporation showed significant suppression of DNA synthesis at 90 and 120 min, the cell cycle analysis revealed a corresponding reduction in S-phase population that reached significance at 120 min. These results together indicate that both assays reflect impaired S-phase progression, with BrdU capturing functional changes in replication and cell cycle analysis confirming the structural redistribution of cells under prolonged 2450 MHz exposure. Together, these findings suggest that 2450 MHz radiation disrupts cell proliferation by interfering with G1 progression, eventually leading to measurable shifts in cell cycle dynamics.

In addition to the within-group analyses presented above, direct comparisons among mobile phone, 1800 MHz, and 2450 MHz exposure groups were performed at matched exposure durations (Supplementary Figure S2 a–f). Significant alterations in G₁ and S-phase distributions were observed at most durations, whereas G₂/M phase differences were evident at 15, 60, and 120 min. Collectively, these comparisons revealed variations in cell cycle phase distribution among the three exposure types, indicating that the extent of change varied with both frequency and exposure duration, and underscore the vulnerability of cell cycle checkpoints to RFR exposure, with potential consequences for testicular function and Leydig cell proliferation.

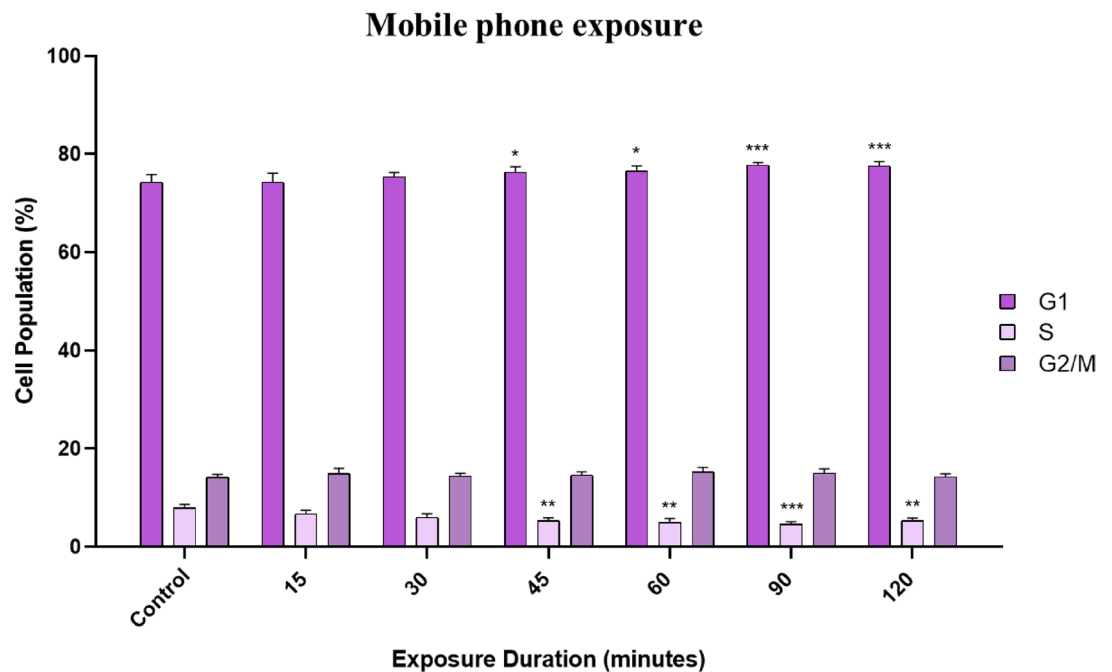
Gating strategy for cell cycle distribution in TM-3 cell, Mobile phone exposure, MFI of Mobile phone exposure, 1800 MHz exposure, MFI of 1800 MHz exposure, 2450 MHz exposure, and MFI of 2450 MHz exposure. TM3 cells were exposed for 15, 30, 45, 60, 90, and 120 min. Post-exposure, PI staining was used to quantify cell distribution across the G₁, S, and G₂/M phases of the cell cycle using flow cytometry. Data are presented as mean ± SEM (n = 3 independent experiments). Statistical significance was determined by 2way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test against the respective control group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

A schematic summary of the RF-EMR exposure conditions and assessed cellular endpoints is provided in Fig. 5.

TM3 mouse Leydig cells were exposed to RFR emitted from a mobile phone, as well as to 1800 MHz and 2450 MHz RFR from a signal generator for 15–120 min. Post exposure analyses included cell cycle distribution, cellular morphology, and cell proliferation/DNA synthesis. The figure summarizes the exposure conditions and experimental endpoints evaluated in this study.

Discussion

Our findings demonstrate that exposure to radiofrequency radiation disrupts TM3 Leydig cell morphology, proliferation, and cell cycle dynamics in a frequency- and time-dependent manner. Morphological assessment



MFI of Mobile phone exposure

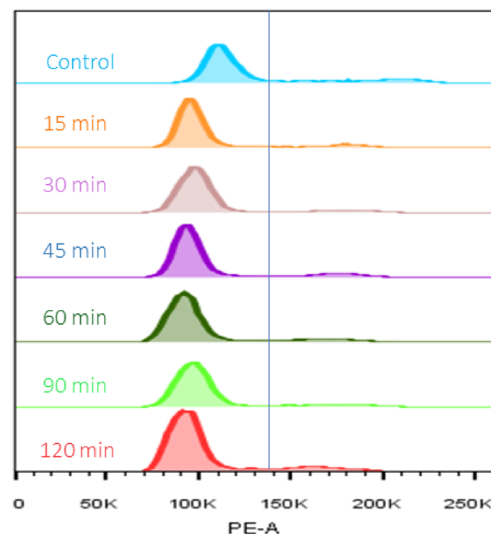
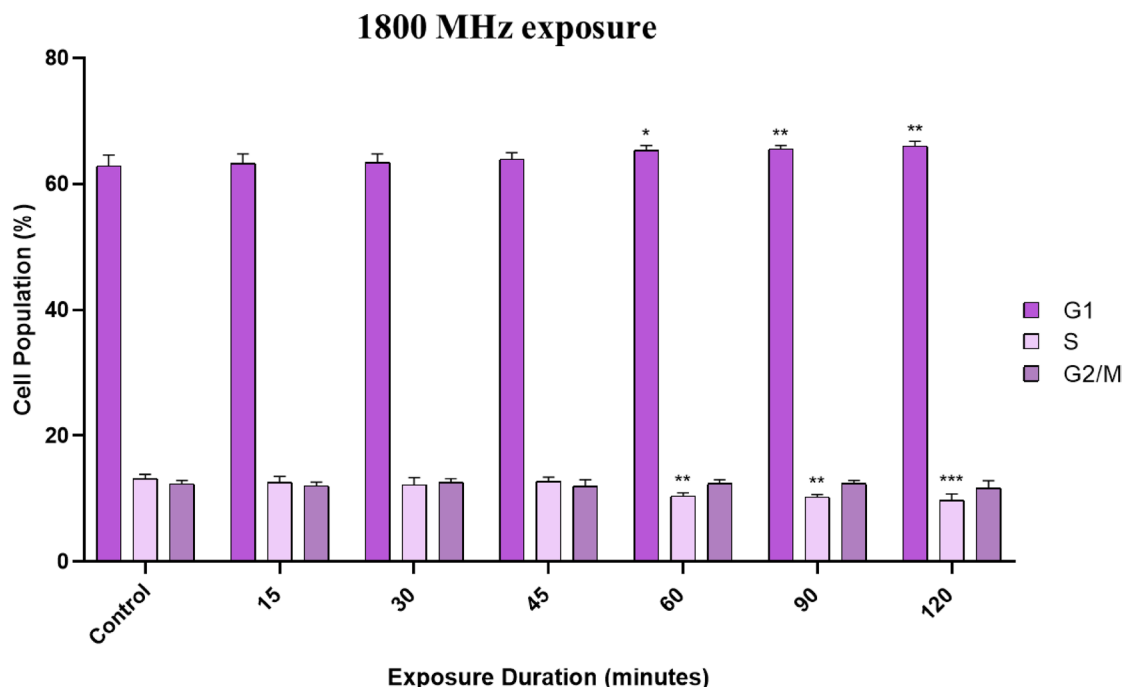


Fig. 4. (continued)

revealed structural alterations in irradiated cells, including reduced cell density, loss of cellular adherence, and an overall stressed appearance. Such structural impairments are consistent with earlier reports of RFR-induced cytoskeletal and membrane perturbations in reproductive and somatic cells^{3,30,31}. These visual changes confirm that cellular integrity is compromised under RFR exposure, complementing the functional evidence generated from BrdU incorporation and cell cycle assays in this study.

Notably, while BrdU incorporation showed a continuous decline in DNA synthesis, cell cycle analysis revealed a reduction in S-phase population. This suggests that RFR may impair DNA replication at the functional level, which is then followed by structural shifts in cell cycle phase distribution. The coherence between these two assays highlights the progressive nature of RF-EMR-induced replication stress. Such sequential disruption, functional inhibition of DNA synthesis preceding measurable cell cycle redistribution, is consistent with mechanistic models of replication stress described in broader EMF literature^{32,33}.



MFI of 1800 MHz exposure

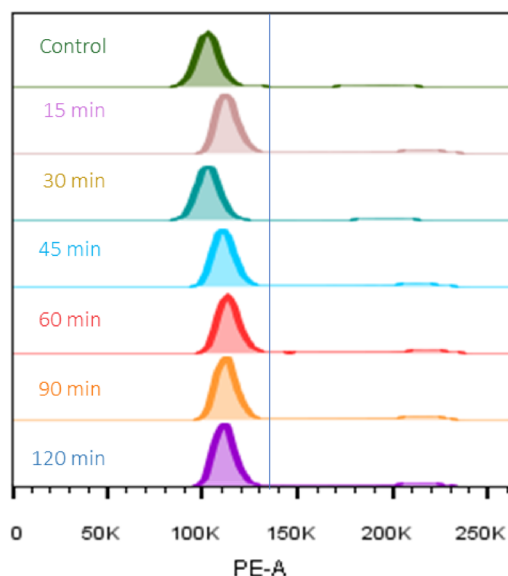


Fig. 4. (continued)

Our results also align with our previous study¹¹, in which RFR exposure impaired Leydig cell function by reducing proliferation and testosterone production while inducing significant increases in intracellular ROS. The disruptions observed in that work parallel the present findings of DNA synthesis suppression and altered cell cycle distribution. In particular, the decline in testosterone production reported earlier may reflect the downstream consequence of the replication stress and proliferative impairment observed here, since reduced Leydig cell proliferation and checkpoint arrest are expected to compromise both steroidogenic activity and cellular support for spermatogenesis. Together, these studies underscore oxidative stress-driven replication stress as a mechanistic link between cellular dysfunction and endocrine disruption in RFR-exposed Leydig cells.

Previous investigations have similarly linked RFR to DNA and proliferative damage in testicular cells. Aitken et al.³⁴ demonstrated DNA fragmentation in the male germline, supporting our observation that rapidly dividing testicular cells are especially vulnerable to non-ionizing radiation. Pandey et al.³¹ further reported that 900 MHz-induced damage in the germ cells' DNA and cause cell cycle arrest, in line with our findings of suppressed

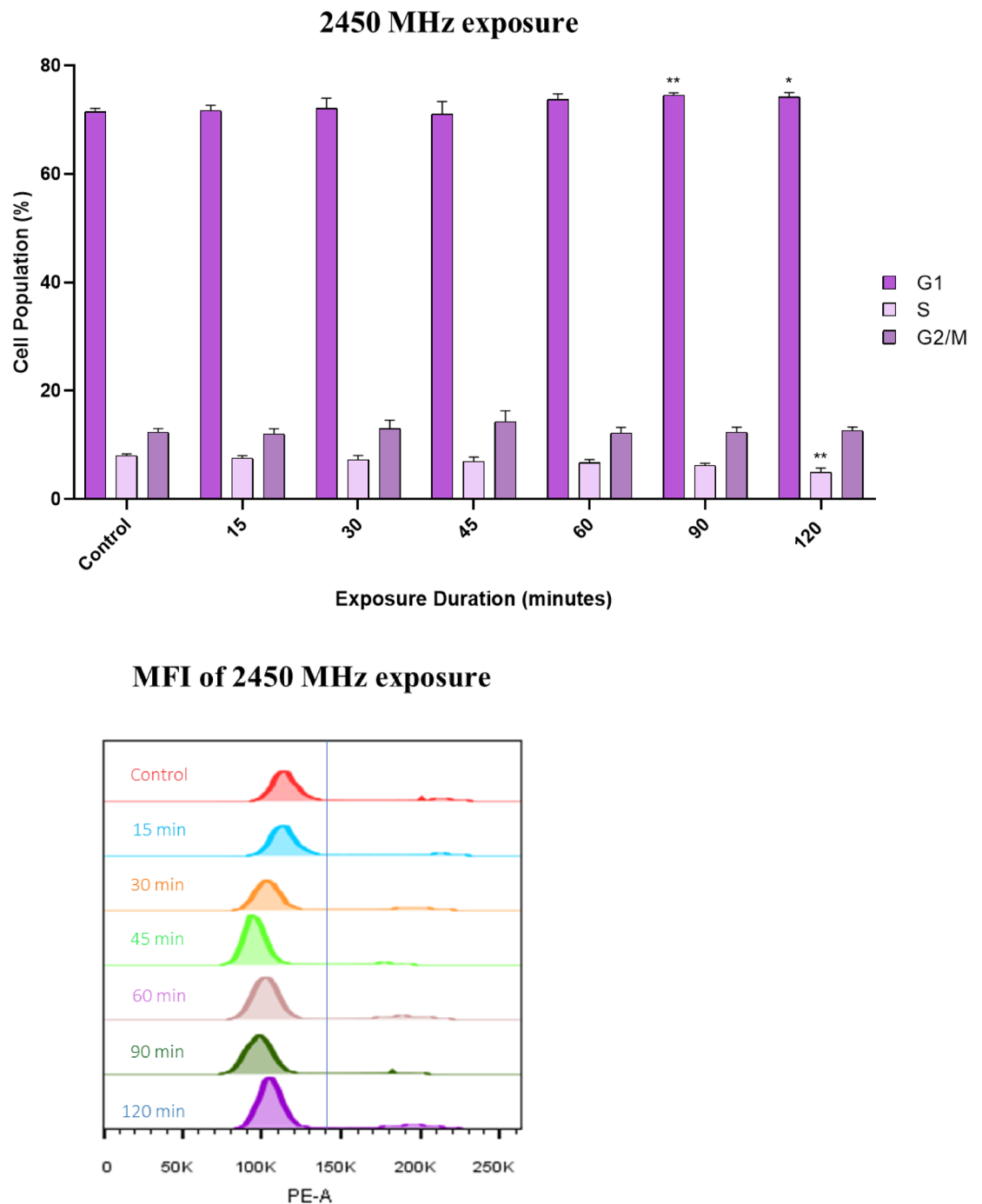


Fig. 4. (continued)

DNA synthesis and altered cell cycle progression in TM3 cells. Similarly, Kesari & Behari³⁰ and Kesari, Kumar & Behari³⁵ described reproductive dysfunction in male rats exposed to RFR, underscoring the reproductive toxicity of these exposures.

While the present study shows that RF-EMR can influence Leydig cell proliferation and cell cycle regulation in a frequency- and time-dependent manner, findings across the wider literature are not entirely consistent. Several studies conducted under comparable exposure conditions have reported little or no measurable biological changes, indicating that RF-EMR effects are not universally observed across biological systems. A few experimental studies have likewise reported no significant genotoxic or proliferative alterations under comparable non-thermal exposure conditions, suggesting that the biological impact of RF-EMR may vary depending on cellular context and methodological design^{23,24}. Recently, Joushomme et al.³⁶ explored multiple stress-related signaling pathways in live fibroblasts and keratinocytes exposed to 5G-modulated 3.5 GHz signals and found no consistent evidence of molecular activation or cellular stress, reinforcing the notion that measurable effects

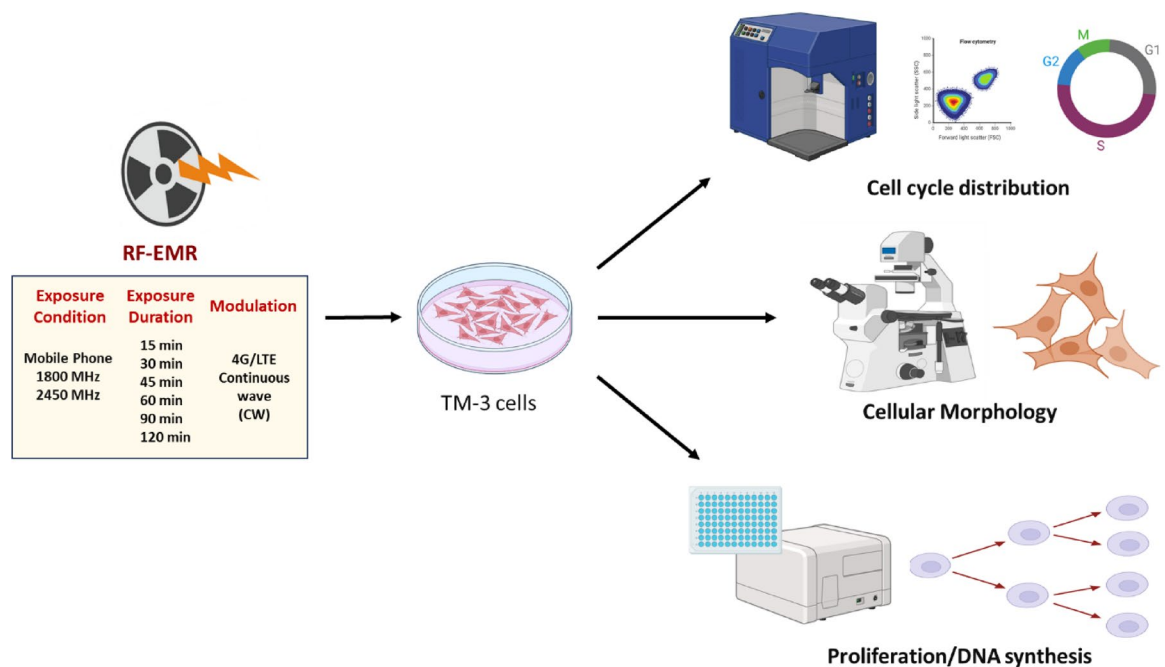


Fig. 5. Schematic overview of RF-EMR exposure conditions and assessed cellular endpoints in TM3 Leydig cells.

are not always observed even under prolonged exposure. Other investigations have suggested that outcomes may vary depending on modulation scheme or exposure configuration. For instance, Valbonesi et al.³⁷ reported increased HSP70 gene expression in neuronal-like cells exposed to GSM-modulated 1.8 GHz fields, but no associated changes in protein levels or signaling pathways, implying a limited or transient cellular response. Likewise, Yan et al.³⁸ observed no significant disruption in testicular structure, sperm quality, or fertility in male mice following long-term 2.0 GHz exposure. Meta-analyses and systematic reviews have also highlighted that studies employing stringent dosimetric validation, sham controls, and blinded analyses often report null or inconsistent findings^{39,40}. Such variability across the literature likely reflects differences in exposure accuracy, modulation patterns, duty cycles, and biological sensitivity among models. Collectively, these observations suggest that RF-induced cellular responses are highly context dependent and should be interpreted with careful consideration of both methodological and biological diversity across studies.

According to Jangid et al.⁴¹, oxidative stress, mitochondrial dysfunction, and DNA instability represent plausible mediators of RF-EMR-induced testicular alterations, although the extent and consistency of these effects remain variable across studies. Oxidative stress has been strongly implicated as a unifying mechanism in RFR biology. Yakymenko et al.³ and Dasdag & Akdag⁴² both emphasized oxidative imbalance as a driver of genotoxicity and impaired proliferation. In our earlier work¹¹, we confirmed significant ROS accumulation in RFR-exposed Leydig cells, and the current BrdU and cell cycle results suggests a possible role of ROS as a mediator of replication stress and checkpoint activation. This mechanism integrates well with reviews linking oxidative stress to impaired replication fork progression and checkpoint signaling⁴³, providing a plausible pathway from oxidative imbalance to suppressed DNA synthesis, proliferative arrest, and reduced testosterone biosynthesis.

At the reproductive level, our results complement experimental and clinical evidence that RFR compromises male fertility. Human studies have consistently reported reduced sperm motility, viability, and acrosome function after RF exposure^{4,44,45}. Gorpichenko et al.⁴⁶ further demonstrated decreased sperm quality following direct mobile phone radiation, while Panagopoulos et al.⁴⁷ observed reduced reproductive capacity in *Drosophila melanogaster*. These findings collectively suggest that RFR exerts multifaceted reproductive toxicity, impairing both germ cells and other reproductive cells, thereby weakening endocrine support and proliferative capacity. Reviews on radiation and male fertility^{4,43} emphasize this compounded risk, which our current findings reinforce.

Our data also connect with emerging transcriptomic evidence. Tuysuz et al.⁴⁸ reported time-dependent gene expression changes and apoptosis in glioblastoma cells exposed to RFR, demonstrating modulation of survival pathways, while Jyoti et al.⁴⁹ found no significant alterations in 5G-exposed skin cells. These contrasting outcomes emphasize that the biological consequences of RFR are context-dependent, with proliferative and reproductive cells appearing more vulnerable than terminally differentiated somatic cells.

In summary, our study establishes that RFR disrupts TM3 Leydig cell function through a sequence of morphological alterations, suppression of DNA synthesis, and redistribution of cell cycle phases. Integrated with our previous work¹¹ and other experimental evidence, the data support a mechanistic model in which oxidative stress induces replication stress, leading to checkpoint activation, proliferative impairment, and reduced testosterone synthesis. Future studies incorporating oxidative stress markers, mitochondrial function

assays, and transcriptomic analyses will be essential to delineate the molecular underpinnings and long-term reproductive consequences of RFR exposure.

Limitations

While this study provides valuable insights into the biological effects of controlled electromagnetic exposure, several experimental and interpretational limitations persist. First, although measures were taken to minimize reflections and improve field uniformity during exposures, field uniformity was evaluated using localized probe measurements and found to vary within $\pm 10\%$. However, detailed three-dimensional field mapping was not performed, and therefore minor spatial variations in field strength inside the incubator cannot be ruled out, which may have contributed to variability in the biological responses observed. Second, during mobile phone exposure experiments, the 15 mL tube was oriented vertically, and the cells were distributed along the height of the tube. This geometry could produce a field-strength gradient along the tube's vertical axis, resulting in different local SARs between the bottom, middle, and top regions of the suspension. Such variation could introduce heterogeneity in cellular exposure, potentially influencing observed differences in cell proliferation and cell-cycle outcomes. Third, although SAR values were determined for both 15 mL tubes and 35 mm petri dishes, these values represent averaged estimates under the defined experimental conditions. Variations in sample geometry, medium depth, and the dielectric properties of the culture medium can influence local energy absorption. As a result, while the reported SARs provide realistic exposure estimates, they may not fully capture spatial variations in absorbed power across the sample volume. Additionally, the use of a single cell line (TM3) limits the broader generalizability of the findings across other testicular or somatic cell types.

Furthermore, the present study did not systematically evaluate other exposure-related physical parameters, such as modulation, duty cycle, polarization, or waveform complexity, which are recognized as influential in shaping RF-EMR bioeffects and may contribute to variability in the observed outcomes. Recognizing these factors is important for situating the present findings within the broader context of RF dosimetry and biological response variability. Accordingly, future studies integrating computational dosimetry simulations, 3D field mapping, and multi-point SAR validation would help overcome these limitations and support more precise quantification of exposure conditions.

Conclusion

Our findings demonstrate that non-thermal exposure to radiofrequency electromagnetic radiation disrupts morphology, proliferation, and cell cycle regulation in TM3 Leydig cells in a frequency- and time-dependent manner. These results highlight frequency-specific vulnerabilities of Leydig cells and points towards replication stress and checkpoint activation as potential mechanisms. Given the central role of Leydig cells in testosterone synthesis and male reproductive health, such impairments may carry significant physiological implications. Considering the ubiquity of wireless devices and unavoidable RFR exposure, our study underscores the need for further mechanistic investigations and long-term in vivo evaluations to better assess reproductive risks and inform public health policies.

Data availability

All data generated and/or analyzed during the current study are included in this article and its supplementary information files. Additional datasets supporting the conclusions of this study are available from the corresponding author on reasonable request.

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Author contributions

Pooja Jangid: Investigation, Methodology, Writing—Original draft preparation, Writing—Reviewing and Editing, Formal analysis. Umesh Rai: Writing—Reviewing and Editing. Jayesh Kumar Sevak: Resources. Ravi Ranjan: Resources. Sanjay Singh: Writing—Reviewing and Editing. Rajeev Singh: Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing—Reviewing and Editing, Writing—Original draft preparation.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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