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ORIGINAL ARTICLE

Immunohistochemistric Investigation on Testicular Tissue after Exposure to High-Frequency Electromagnetic Waves in Rats

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Abstract

The increasing concern over the potential health effects of high-frequency electromagnetic waves (HFEMW) emitted from devices such as mobile phones and modems has prompted this study, which aimed to evaluate the impact of HFEMW exposure on rat testicular tissue. Thirty-five adult male Wistar rats were divided into five groups and exposed to HFEMW (2100–2600 MHz, 1.5 W/kg) for 70 consecutive days, with daily exposure durations of 0, 15, 60, 120, and 180 minutes. Testicular tissue samples were collected for immunohistochemical assessment. Histological examination revealed that short-term exposure (15 minutes) significantly reduced seminiferous tubule atrophy compared to the control group, whereas prolonged exposure caused progressive degenerative changes and severe histopathological damage to testicular tissue. Immunohistochemical analysis demonstrated apoptotic cell death, particularly in groups with longer exposure durations. These findings suggest that chronic and prolonged exposure to HFEMW can negatively affect testicular integrity, emphasizing the need for awareness and preventive measures to mitigate potential reproductive health risks.

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Introduction

A type of electromagnetic radiation that has become a growing concern in recent years is non-ionizing radiation, which includes radio waves, microwaves, and visible light. Although these forms of radiation are generally considered safe at low levels, studies have suggested that excessive exposure to certain frequencies, such as those used in cell phones and wireless networks, may increase the risk of brain cancer and other neurological disorders (1-3).

However, the evidence remains inconclusive, and further research is needed to determine the long-term effects of non-ionizing radiation on human health. Electromagnetic radiation has both positive and negative effects on human health, and these effects depend on the type and intensity of the radiation. While some forms of radiation are essential for daily life and have numerous medical applications, excessive exposure to certain types can lead to adverse health effects. As technology continues to advance, it is crucial to understand the potential risks and benefits of electromagnetic radiation and take appropriate precautions to minimize exposure (4-6).

Moreover, exposure to electromagnetic waves can induce apoptotic death in the tissues of both humans and rats. This programmed cell death process is essential for maintaining tissue homeostasis. Studies have shown that electromagnetic waves from devices such as cell phones disrupt cell function and trigger apoptosis (7, 8). Prolonged exposure to electromagnetic radiation in humans is linked to health issues such as cancer and neurological disorders, partly due to apoptosis induction in tissues. Similarly, studies in rats have demonstrated increased caspase-mediated apoptosis in organs such as the brain and liver due to electromagnetic wave exposure. Understanding how electromagnetic waves induce apoptosis is crucial for mitigating potential health risks and developing strategies to protect human health. Caspases are a family of proteases that play critical roles in the execution phase of apoptosis. They are activated in response to various apoptotic stimuli, including exposure to electromagnetic radiation. Studies have shown that caspase expression is increased in cells exposed to electromagnetic radiation, indicating that this form of radiation triggers apoptotic pathways involving caspase activation. Caspase-3 has been implicated in the apoptotic response to electromagnetic waves, suggesting that it may be a key target for mitigating the adverse effects of electromagnetic radiation. Investigating the role of caspases in the apoptotic response to electromagnetic waves is crucial for developing strategies to protect human and animal health from the potential hazards of prolonged exposure to radiation (9).

In the current study, adult male Wistar rats were exposed to regular daily high-frequency electromagnetic waves (HFEMW) ranging from 2100-2600 MHz (1.5 W/kg) for 70 days to analyze the association between radiation power and exposure duration on the morphology of the testes in exposed rats.

Materials and Methods

Electromagnetic Generator

In this study, an antenna (with an output power of 2000 mW and a lithium-ion battery with a capacity of 127, 2100 mAh) was used to generate HFEMW between 2100 and 2600 MHz frequencies. The output power of the RF source was 2 W, resulting in an estimated power density of approximately 1.6 mW/cm² at the level of the animals, which is within the range commonly used in experimental studies simulating mobile phone exposure.

Experimental Design

Thirty-five adults male Wistar rats (weighing between 300300 and 400 g400 g) were obtained from Mashhad University of Medical Sciences. Prior to the experiment, the animals were housed in the animal facility of the Faculty of Veterinary Medicine in Mashhad for one week to ensure optimal health under standard conditions, including a 12-hour light and 12-hour dark cycle and access to purified water and sufficient food in standard polypropylene cages. The rats were randomly divided into five groups of seven rats each. The sample size was determined based on commonly accepted practices in similar experimental studies. Additionally, a post hoc power analysis was performed to assess the adequacy of the selected sample size. Based on the observed effect size, a significance level of $P < 0.05$, and the number of animals per group (n = 7), the study achieved a statistical power of at least 80%, indicating that the sample size was sufficient to detect meaningful differences between the groups. The rats were exposed daily to electromagnetic waves (2100-2600 MHz) for various exposure periods (0, 15, 60, 120, and 180 min). The RF source was positioned at a fixed distance of 10 cm from the cages to ensure uniform exposure conditions across all experimental groups. The animals were exposed to RF-EMF for a total duration of 70 days. Exposure was applied in a continuous (non-pulsed) manner throughout each daily session. Control animals were kept under identical environmental conditions without RF exposure. Finally, all rats, including those in the control group, were euthanized using Carbon Dioxide (CO_2) to collect testis samples (10).

Histological Measurements

Testicular tissues were fixed in 10% formalin for 24 h. The fixed tissues were dehydrated in a graded series of alcohol (70%, 95%, and 100%) to remove water. The tissues were then cleared in xylene to replace the alcohol. The cleared tissues were embedded in paraffin wax for sectioning. Subsequently, the paraffin-embedded tissues were sectioned into thin slices (5-10 µm) using a microtome. Hematoxylin and eosin (H&E) staining was performed: hematoxylin-stained nuclei blue-purple by binding to nucleic acids, while eosin counterstained cytoplasmic proteins and other acidophilic structures pink-orange. Finally, the stained sections were mounted onto glass slides and covered with coverslips using a mounting medium for microscopy analysis. This allowed for the observation of cellular structures, including nuclei, cytoplasm, organelles, and tissue architecture, in the rat testicular samples (11, 12).

Caspase-3 Staining

The tissues were subjected to histological examination following standard immunohistochemistry protocols. Immunohistochemical staining with a Caspase-3 antibody was used to demonstrate apoptosis. The Caspase-3 antibody (100 µL) and secondary antibody Goat Anti-Rabbit IgG H&L (HRP, 1 mg) were purchased from Cell Signaling Technology (code 9661S) and Abcam (code AB6721), respectively. Briefly, the staining process was performed as follows (12):

1- Staining preparation phase:

The samples were placed on poly-L-lysine-linked slides to facilitate the xylene-mediated deparaffinization process. The deparaffinized samples were hydrated using decreasing ethanol concentrations (99% to 70%). The slides were then heated in 10 mM sodium citrate buffer (pH 6) at 98°C for 20 min and incubated for 20 min at 25°C. The samples were washed three times with distilled water and Tris-buffered saline (TBS). Finally, to stop natural peroxidase activity in the cells, the samples were incubated with 3% hydrogen peroxide for 10 min and then washed with distilled water.

2- Staining phase:

The primary antibody was added at a concentration of 1:200 in 100-400 µL and incubated at 4°C for 24 h. Following incubation, the primary antibody solution was removed, and the samples were washed with TBS (1X). The sections were then incubated in the dark at room temperature for 45 min with the secondary antibody and washed with TBS. The samples were then stained with diaminobenzidine (DAB) for 5 min and counterstained with Hematoxylin for 3 min. Finally, the sample dehydration and clearing processes were mediated using ethanol (96% and 100%) and xylene solvents, respectively. The samples were mounted on slides to count all positively stained cells (brownish cells), indicating an antibody-antigen reaction. All cells in the seminiferous tubules were examined and counted using an Olympus BX51 light microscope equipped with an Olympus DP12 digital camera. ImageJ software was used to quantify the apoptotic cell population based on specific color differentiation. The percentage of apoptosis and related statistical analyses were conducted using a one-way ANOVA test.

Data Analysis

Statistical analyses were conducted among the various groups using SPSS version 23 software. The results are expressed as mean ± standard deviation. One-way ANOVA and Tukey's tests were employed to identify significant differences between groups, with *P*-values of < 0.05 considered significant. For graphical representation, Microsoft Excel 2019 was utilized.

Results

Histological Results

All study groups were examined for possible tissue damage. Histological findings (H&E staining) in the testis tissue of the control group demonstrated a normal structure with an organized arrangement of spermatogenic cells, Sertoli cells, and Leydig cells in the interstitial tissue (Figure 1). However, the microscopic results of H&E staining in the experimental groups (1 to 4), following increasing exposure times to HFEMW, revealed irregularity and scattering of spermatogenic tube cells, pyknotic nuclei, and detachment of sperm heads, epithelial thinning, and the depletion of cells within the seminiferous tubules. In experimental group 4 (180 min), fewer mature sperm were observed (Figure 1).

Notably, in experimental group B (Experiment 1, 15 min), disorganization of cells, partial loss of the seminiferous tubules wall, and migration of the layer of cells to the interstitial tissue were observed (Figure 1B). Group C (Experiment 2, 60 min) showed an increase in immature cells and the detachment of sperm heads in the spermatic tubules (Figure 1C). Group D (Experiment 3, 120 min) exhibited an increased number of pyknotic nuclei of cells and immature cells and the detachment of sperm heads in the spermatic tubules. Additionally, atrophy and an increased tubule density in the cross-section (numerical

density) were observed; however, this was solely due to the overall smaller size of the tissue and the compression of the relative tubes, not an actual increase in the number of tubes (Figure 1D). Groups E1 and E2 (Experiment 4, 180 min) showed atrophy of the spermatic tubules and decreased the thickness of the germinal epithelium (Figure 1E).

It should be noted that further changes can be observed in testicular tissue samples through histological and immunohistochemical techniques. Using immunohistochemical techniques, abnormal tubules were identified at various stages of sperm development in the groups exposed to electromagnetic waves.

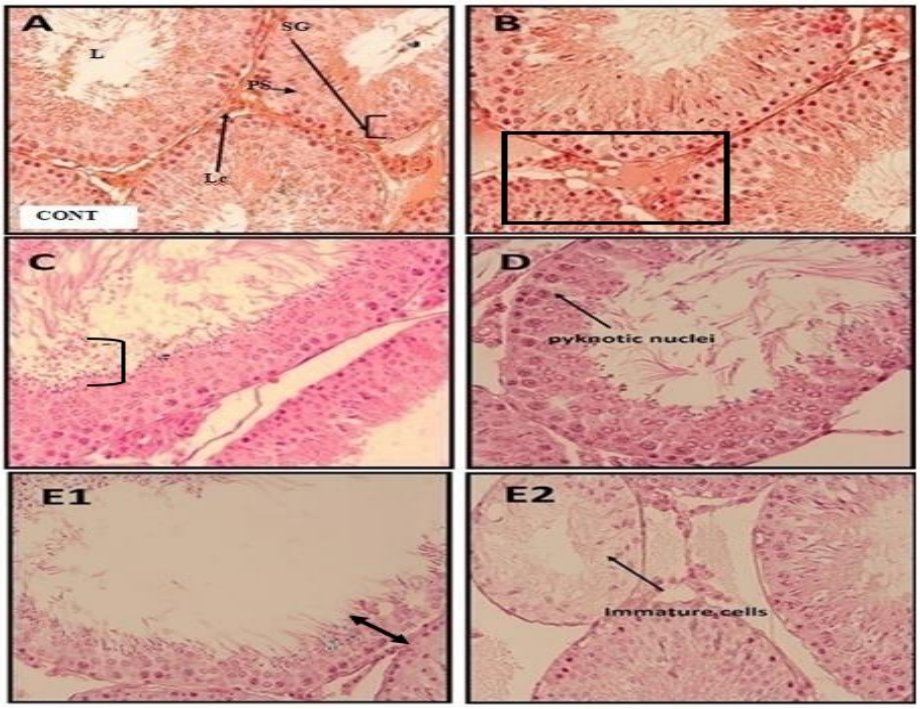

Figure 1. The testicular tissue is under the influence of HFEMW in different groups. A) The control group, B) The rats exposed for 15 minutes; the area marked with a square indicates partial loss of the wall of the seminiferous tubules, as well as the migration of the layer of cells to the interstitial tissue. C) The rats exposed for 60 minutes; the right bracket shows an increase in immature cells and the detachment of sperm heads in the spermatic tubules D) Group D, after 120 min of exposure to electromagnetic waves, exhibited a higher number of pyknotic nuclei in the germ cells of the seminiferous tubules (arrows).Group E (E1 and E2), after 180 min of exposure, showed a significant reduction in the thickness of the germ epithelium lining the seminiferous tubules, accompanied by tubular atrophy. The double-headed arrow highlights the reduction in epithelial thickness in these groups. (H&E 200x). The arrows represent L: Lumen, LC: Leading cell, SG: spermatogonia, PS: primary spermatocytes, CONT= Control, Magnification: ×400.

Immunohistochemistry Results

The results of the immunohistochemical studies of testicular tissue exposed to HFEMW in the control group showed the natural structure and morphology of the testicular tissue, with several layers of cells in the seminiferous tubules from the periphery toward the lumen, including spermatogonia, spermatocytes, spermatids, and sperm (Figure 2A). In contrast, in the experimental groups, increasing exposure time to HFEMW was associated with an increased density of tubes in the cross-section" (numerical density); this was due solely to the overall smaller size of the tissue and the compression of the tubes, not an actual increase in tube number. However, most samples exhibited atrophy, a decrease in the diameter of the seminiferous tubules, an increase in the lumen of seminiferous tubules compared with controls, a decrease in the thickness of the epithelium of seminiferous tubules, and in several cases, vacuolization and disorganization of the epithelium, pyknotic nuclei of cells (chromatin condensation and nuclear shrinkage), and displacement of cellular layers were observed (Figure 2B-D). In some cases, no spermatogenic stages were observed in experimental group 4 (three hours exposure), and only a limited number of cells were observed in the walls of some seminiferous tubules that were not identifiable (Figure 2E). In addition, as a result of changes in cell morphology, apoptotic cells appeared, and an increase in apoptosis was observed during the increasing exposure time of the groups under the influence of HFEMW. Moreover, the measurement of the testicular tissue area presenting apoptotic cells indicated a

significant increase in the apoptotic cell counts in response to prolonged exposure ($P < 0.001$) (Figure 2F).

Moreover, the average count of apoptotic cells in exposed rats increased significantly ($P \leq 0.001$) with longer exposure durations (Figure 3).

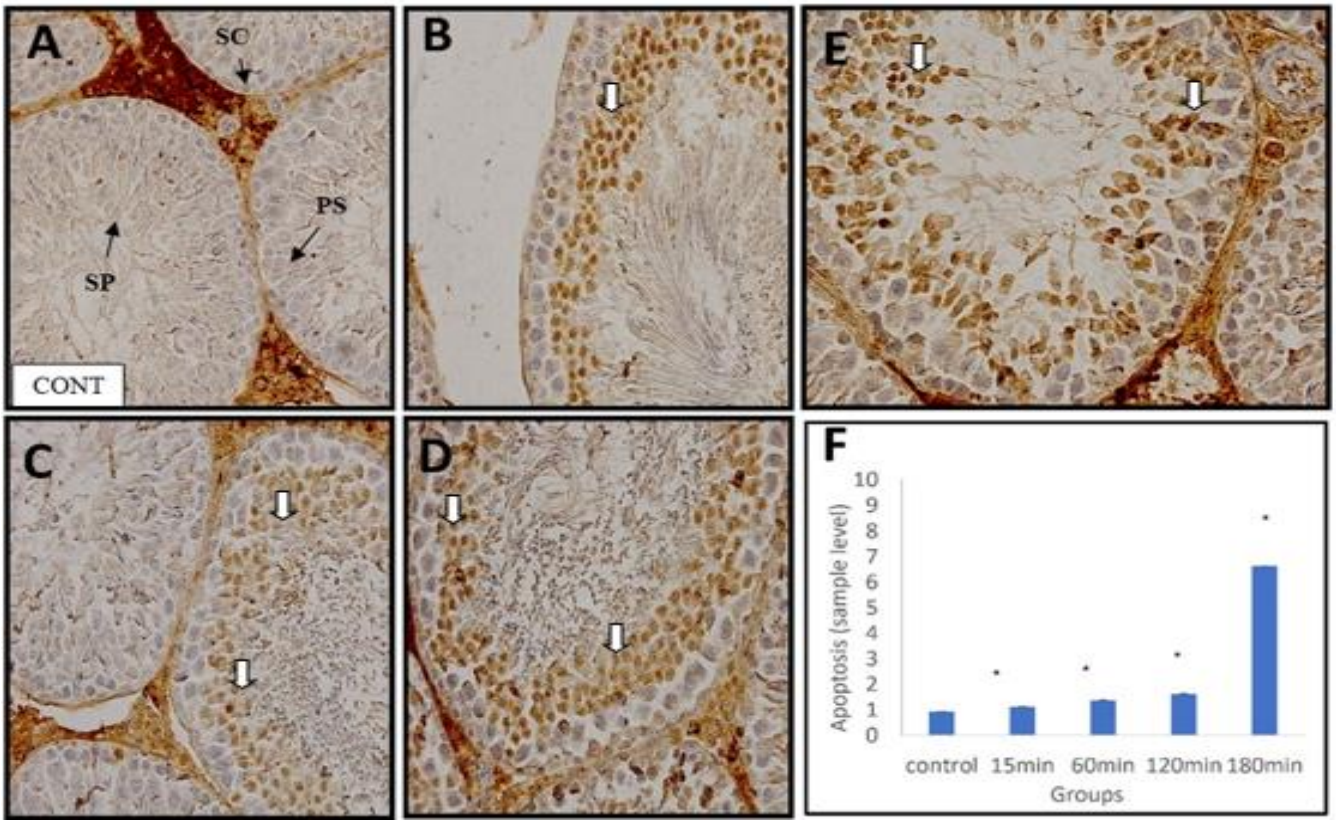

Figure 2. Immunohistochemical staining of Caspase-3. The testicular tissue was exposed to HFEMW in different groups. A) The control group, B) Group 1, exposure for 15 minutes, C) Group 2, exposure for 60 minutes, D) Group 3, exposure for 120 minutes, E) Group 4, exposure for 180 minutes. Also increase in apoptosis was observed during the increasing exposure time of the groups under the influence of high frequency electromagnetic waves (Brown color). F) Quantitative results of the Caspase-3 immunohistochemistry staining. * Indicates a Significant difference ($P < 0.001$) compared to the control group. The arrows represent SG: spermatogonia, PS: primary spermatocytes, SP: spermatids, CONT= Control, White arrows: Apoptotic cells, Magnification: ×400.

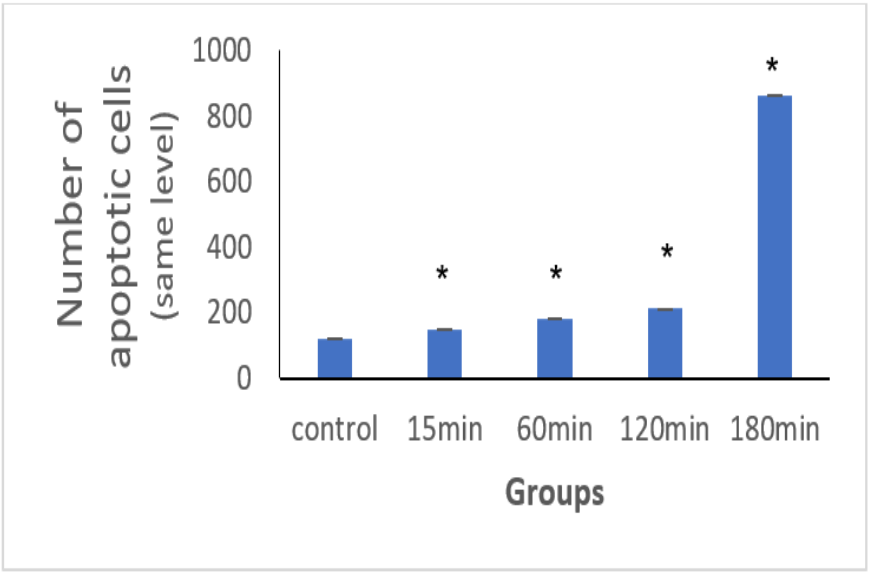

Figure 3. The average count of apoptotic cells in rats exposed to high-frequency radiation ($P \leq 0.001$).

Discussion

As a result of the coronavirus pandemic, the rise in online learning, and the growing use of electronic devices, human exposure to HFEMW has increased. This study aimed to examine whether these HFEMW have a negative impact on testicular tissue. The study discovered that prolonged exposure (2-3 hours daily) to HFEMW can lead to changes in testicular apoptosis, and eventually, severe damage to testicular tissue.

A study revealed that the duration of exposure to consistent electromagnetic waves significantly impacts cellular and tissue-level outcomes. Similarly, Kang's 1997 report highlighted that three factors—intensity, frequency, and exposure duration—play a crucial role in cellular functioning. That investigation focused on how varying exposure durations (ranging from 4 to 24 h) affected the brain and kidney tissues in mice (13,14), which is consistent with the findings of the present study.

Prior research has indicated that electromagnetic waves may influence the testicular tissue differently. Although numerous mobile phone manufacturers have argued that these devices do not negatively impact human health (13, 15), this study aimed to improve the accuracy of such assessments by immunohistochemically evaluating the damage caused by electromagnetic waves to testicular tissue.

In natural settings, electromagnetic waves affect all living organisms, including humans and animals (16). Histological analysis of testicular tissues in various experimental groups demonstrated irregularities, the loss of seminiferous tubule walls, and reduced sperm counts. Exposure to HFEMW led to increased tissue damage. Previous research suggests that electromagnetic waves can affect spermatogenic cells, causing degeneration, germinal layer disruption, and incomplete spermatogenesis; the findings of this study support these previous conclusions (16-18).

Notably, that the seminiferous tubules were atrophied in this study. It is plausible that prior studies contributed to the alterations in spermatogenesis. Apoptosis is a crucial physiological process that selectively removes cells, maintaining cell numbers by disposing of excess and damaged cells in testicular tissue. This process counteracts cellular proliferation.

The quantitative analysis of Caspase-3-stained slides revealed a substantial increase in apoptotic indices, which coincided with a considerable decrease in the number of germ cell layers in the experimental groups. This increase in Caspase-3 levels offers a plausible explanation for the testicular apoptosis observed in the experimental animal groups. These findings align with the increased apoptosis in spermatogenesis and Caspase-3 activity reported by Kesari and Behari (2010) (2.45 GHz) (19). Using flow cytometry, their study demonstrated a significant reduction in sperm percentage and an increase in apoptotic cells following exposure to mobile phones for 2 h daily over 35 days (19).

In another study, Razavi et al. (2015) reported a significant increase in brain tissue apoptosis across all experimental groups following exposure to HFEMW (20). Although the duration and intensity of electromagnetic field exposure differed among the experimental groups, the results of the present study are consistent with those of Kesari and Behari (2010) and Razavi et al. (2015) (19,20).

In a recent investigation, the analysis of apoptotic cells across all groups revealed a substantial increase in apoptosis in cells exposed to HFEMW. These findings align with the research conducted by Kassar, Li, Yan, and Adassi, despite the differing frequencies employed in their studies (2.45 GHz for Kassar, 60 Hz for Li, radiofrequency electromagnetic waves for Yan, and 900 MHz for Adassi) (19-24).

Conversely, in Desdouits' immunohistochemical study involving rats exposed to 900 MHz radio waves (2 h daily, 7 days weekly, for 10 months), no apoptosis was detected in the testes (25, 26). While this contrasts with the findings of the current study, various factors, including animal species, radiation exposure devices, and wave exposure duration, may lead to the diverse outcomes observed in animal experiments (27, 28).

Conclusions

This study explored the potential influence of HFEMW, similar to those produced by mobile phones, on testicular histology and immunohistochemistry in male rats. The study revealed that exposure to these waves could lead to negative consequences on testicular tissue even after periods as short as of 15 min, with more severe effects following a 3-hour exposure. Given these findings, it is hypothesized that the use of modern mobile phones operating at high frequencies might cause severe changes in the normal status of animals, including reproductive parameters, increased apoptosis, decreased sexual characteristics, and reduced fertility.

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Authors' Contributions

Fariba Ghasemian-Nejad Jahromi: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software. **Ahmadreza Raji:** Conceptualization, Data curation, Project administration, Formal analysis, Investigation, Methodology, Resources, Supervision. **Mohsen Maleki:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources. **Pezhman Mirshokraei:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources. **Morteza Kafaee Razavi:** Conceptualization, Data curation, Software, Formal analysis, Investigation, Methodology, Resources.

Data Availability

All datasets generated or analyzed during this study are fully provided within the article.

Ethical Approval

The Ethics Committee for the Care and Use of Laboratory Animals at Ferdowsi University of Mashhad (IR.UM.REC.1400.338) approved the present study.

Conflict of Interest

The authors declare that they have no conflict of interest.

Consent for Publication

Not applicable.

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