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Potential influence of prenatal 2.45 GHz radiofrequency electromagnetic field exposure on Wistar albino rat testis

Viera Almášiová[1], Katarína Holovská[1], Sandra Andrašková[1], Viera Cigánková[1], Zuzana Ševčíková[1], Adam Raček[1], Zuzana Andrejčáková[2], Katarína Beňová[2], Štefan Tóth[3], Eva Tvrdá[4], Ján Molnár[5] and Enikö Račeková[6]

[1]Department of Morphological Disciplines, [2]Department of Biology and Physiology, University of Veterinary Medicine and Pharmacy in Kosice, [3]Department of Histology and Embryology, Faculty of Medicine, Pavol Jozef Safarik University in Kosice, Kosice, [4]Department of Animal Physiology, Slovak University of Agriculture in Nitra, Nitra, [5]Department of Theoretical and Industrial Electrical Engineering, Faculty of Electrical Engineering and Informatics, Technical University of Kosice and [6]Institute of Neurobiology of Biomedical Research Center of Slovak Academy of Sciences, Kosice, the Slovak Republic

Summary. An ever-increasing use of wireless devices over the last decades has forced scientists to clarify their impact on living systems. Since prenatal development is highly sensitive to numerous noxious agents, including radiation, we focused on the assessment of potential adverse effects of microwave radiation (MR) on testicular development. Pregnant Wistar albino rats (3 months old, weighing 282±8 g) were exposed to pulsed MR at a frequency of 2.45 GHz, mean power density of 2.8 mW/cm^2, and a specific absorption rate of 1.82 W/kg for 2 hours/day throughout pregnancy. Male offspring were no longer exposed to MR following birth. Samples of biological material were collected after reaching adulthood (75 days). *In utero* MR exposure caused degenerative changes in the testicular parenchyma of adult rats. The shape of the seminiferous tubules was irregular, germ cells were degenerated and often desquamated. The diameters of the seminiferous tubules and the height of the germinal epithelium were significantly decreased (both at **p<0.01), while the interstitial space was significantly increased (**p<0.01) when compared to the controls. In the group of rats prenatally exposed to MR, the somatic and germ cells were rich in vacuoles and their organelles were often altered. Necrotizing cells were more frequent and empty spaces between Sertoli cells and germ cells were observed. The Leydig cells contained more lipid droplets. An increased Fluoro Jade - C and superoxide dismutase 2 positivity was detected in the rats exposed to MR. Our results confirmed adverse effects of MR on testicular development.

Key words: Rats, Fetal development, Microwaves, Testes

Corresponding Author: Viera Almášiová, Department of Morphological Disciplines, University of Veterinary Medicine and Pharmacy in Kosice, the Slovak Republic. e-mail: viera.almasiova@uvlf.sk

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Introduction

The widespread and daily use of wireless devices such as mobile phones, WiFi routers, portable bluetooth gadgets and many others, has significantly increased man-made radiofrequency electromagnetic radiation (RF - EMR) in the environment. Correspondingly, this has raised public concern and scientific interest in elucidation of the potential adverse health effects of RF - EMR (Yahyazadeh et al., 2018; Yahyazadeh and Altunkaynak, 2020a,b). Despite responding to high-priority research demands, the studies investigating the effects of prenatal exposure to RF - EMR are scarce. Embryonic and fetal tissues are extremely sensitive to the effects of various noxious agents including radiation. Several reports have shown that exposure to even low doses of RF - EMR during the fetal period, would have even more profound and conclusive consequences on health (Dietert and Piepenbrink, 2008; Aldad et al., 2012; Balassa et al., 2013; Hanci et al., 2013).

Mammalian testes are generally highly susceptible organs (Kilcoyne and Mitchell, 2019). Their vulnerability is mainly caused by the complexity and long duration of spermatogenesis, which is driven by numerous delicate regulatory mechanisms. Testicular tissue is rich in water molecules, which are polar and responsive to the effect of microwaves. Their vibrations

may cause gain in energy in the form of heat. This phenomenon may result in an increased tissue temperature or a non-uniform distribution of heat with the occurrence of the so-called hot spots. Specific anatomical and physiological features also contribute to testicular radiosensitivity. Testes are superficially located and encapsulated by a thick fibrous tunica albuginea that limits an effective removal of accumulated heat. Furthermore, spermatogenesis, which is strictly dependent on an optimal temperature, is also highly vulnerable to heat stress. As such, it is not surprising that numerous studies have highlighted adverse effects of microwave radiation generated by various wireless devices on male reproduction (La Vignera et al., 2012; Kesari et al., 2018; Singh et al., 2018; Jaffar et al., 2019; Zha et al., 2019).

Any unfavourable internal or external insult during early life has a potential to affect testicular development with detrimental consequences on the future capacity of male gonads to produce spermatozoa (Kilcoyne and Mitchell, 2019). Since wireless technologies are frequently used by pregnant women, the effects of RF - EMR on the fetal tissues and organs have attracted the interest of scientists as well as of the wider public. Several studies have confirmed unfavourable effects of RF - EMR applied *in utero*, on postnatal development and behaviour (Zhang et al., 2015; Othman et al., 2017), brain or liver development (D'Silva et al., 2017; Sharma et al. 2017) as well as on the integrity of DNA and lipids (Guler et al., 2010). In the available literature we found only one study focused on the effects of RF - EMR on the testiculogenesis that evaluated the impact of 900 MHz mobile phone-like radiation on juvenile rats (Hanci et al., 2013).

As such, the aim of this study was to investigate whether the exposure to 2.45 GHz Wi-Fi - like RF - EMR could impact selected male reproductive parameters of adult rats, exposed to MR solely during their prenatal period of life. The selected frequency coupled with the exposure time may be considered as very similar to the actual dose of non-ionizing radiation to which the organism is routinely exposed in the environment.

Materials and methods

Animals and their exposure to radiation

The animals used in this study were bred at the certified vivarium of the Institute of Neurobiology, Biomedical Research Center, Slovak Academy of Sciences, Slovakia, and originated from Velaz Ltd., the Czech Republic. They were housed in standard cages (two animals in each) with *ad libitum* access to water and food, under a controlled temperature (21±1°C) and 12/12 h day light/darkness cycle. Before the onset of the experiment, female Wistar Albino rats (n=6) approximately 3 months old (average body weight of 282±8 g), were mated with a male of the same strain (n=1) at the age of approximately 3 months and the body weight of 297 g. A vaginal swab was observed under a light microscope for the presence of spermatozoa, and in the case of a positive finding this day was considered the first day of pregnancy. Pregnant females were randomly divided into control (n=3) and experimental (n=3) groups. The study was performed in the far-field exposure with an estimated radiation source output below 4 W. The experimental mates were whole-body exposed to pulsed microwave radiation at the frequency of 2.45 GHz for 2 hours/day throughout the pregnancy. The mean power density was 2.8 mW/cm^2 and the specific absorption rate (SAR) was 1.82 W/kg. SAR was calculated according to Valberg et al. (2007), using the following formula:

Rat body surface area: S=0.0247 m^2; Rate of power radiated from the microwave: p=28 W/m^2; Power absorbed by the rat: P=S × p=28 × 0.0247=0.691 W; Weight of the pregnant rat: m=0.38 kg.

$$SAR=\frac{P}{m}=\frac{0.691}{0.38}=1.82W/kg$$

The exposure chamber (100×80×70 cm) was designed for the exposure of free-moving pregnant rats, and the uniformity of the radiation was controlled with a spectrum analyzer (Fig. 1). The control mates were kept under standard conditions without exposition to MR. After parturition, randomly selected progeny - two males per each mother exposed to MR (n=6) and two males per each control mother (n=6) survived until the age of 75 days. During this period, the animals were no longer exposed to MR. After reaching 75 days of age, the rats were deeply anaesthetized with chloral-hydrate and euthanized by transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer and the relevant biological material was collected for examination. All procedures with animals were approved by the Ethical Committee of the Institute of Neurobiology, Biomedical Research Centre, Slovak Academy of Sciences, the Slovak Republic, and the State Veterinary Authority, the Slovak Republic (registration number: Č.k. Ro 2246/18-221/3).

Conventional light microscopy - LM

Approximately 0.5 cm^3 large tissue samples from the right-sided testis of each animal were fixed in a modified Davidson's Fixative solution - mDF (Latendresse et al., 2002) for 24 hours, subsequently dehydrated in a graded series of ethanol, and embedded in paraplast. Two paraplast blocks were prepared from each right-sided testis. Five tissue sections (5 μm) from the different regions of each testis (2 paraplast blocks) were obtained. The sections were transferred to xylene, stained with haematoxylin-eosin, examined under a light microscope ZEISS Axio Lab A1 (Zeiss Germany) and photodocumented with an Axio Cam ERc5 camera (Zeiss Germany). Fifty randomly chosen fields (10

fields/slide) were observed for each testis at ×400 magnification.

Morphometry

The same tissue sections previously prepared for LM were examined under a light microscope OLYMPUS CX 43 (Olympus, Tokyo, Japan) and selected testicular parameters were measured using the QuickPHOTO MICRO 3.2 software (Promicra, Prague, Czech Republic). The mean diameter of seminiferous tubules was assessed from the right-sided testis of each rat by evaluation of 60 randomly chosen round seminiferous tubules at a total magnification of ×400. Each tubule was measured across two perpendicular axes and the mean diameter was obtained. The germinal epithelium height was measured in 4 equidistances of each cross section of seminiferous tubules and the mean of these was calculated. 40 randomly chosen round seminiferous tubules were evaluated at stage VIII of seminiferous epithelium cycle, identified according to the typical cellular associations. The volumetric proportion between the tubular and interstitial compartments was evaluated from 30 different fields for each animal. The obtained morphometric data are expressed as mean±SEM.

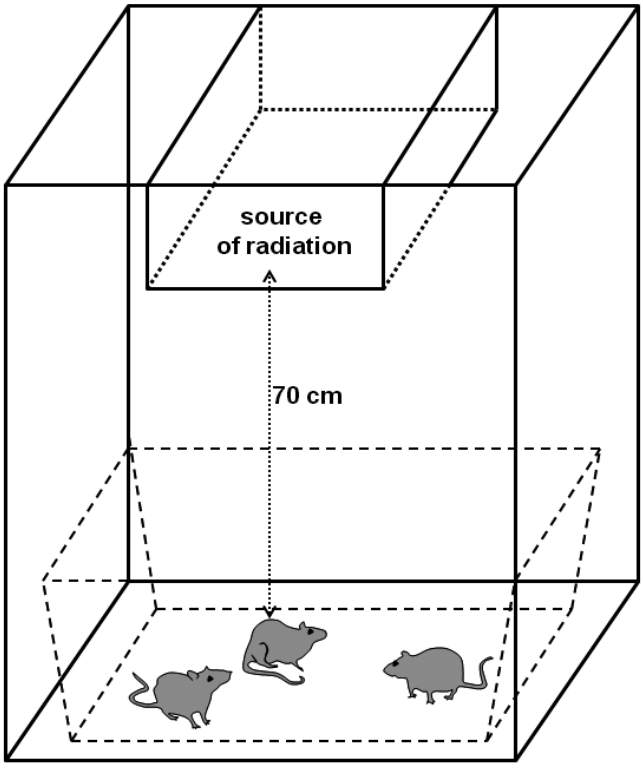

Fig. 1. Experimental scheme: exposure chamber with the source of radiation - magnetron. Experimental animals were placed in the original plastic home cage, allowing them free movement to minimize potential stress. The distance between the magnetron and animals was 70 cm.

Statistical analysis

Statistical analysis was carried out using the GraphPad Prism software (version 8.4.3 for Mac; GraphPad Software, La Jolla California USA, www.graphpad.com). A paired t-test was used to assess the differences between the control and the experimental group. The level of significance was set at ***$p<0.001$; **$p<0.01$; *$p<0.05$.

Transmission electron microscopy - TEM

Samples for TEM (up to 1 mm^3) were obtained from three different parts (superficial, middle, and deep) from the right-sided testis of each animal. The specimens were immediately fixed in buffered 3% glutaraldehyde and 1% osmium tetraoxide, dehydrated in graded acetone series, transferred to propylene oxide, and embedded in Durcupan™ ACM (Sigma-Aldrich Chemie GmbH, Germany). Ultra-thin sections (100 nm) were cut using a LKB-Nova ultramicrotome (LKB, Sweden), and subsequently contrasted with uranyl-acetate and lead-citrate. The sections were examined and photodocumented under a transmission electron microscope Tesla BS 500 (Tesla Brno, Czech Republic).

Qualitative fluoro - Jade C histochemical detection

To visualize the degenerating cells in the testes, we used the Fluoro Jade - C (FJ - C, Histo-Chem Inc., AR, USA) histochemical staining. Approximately 0.5 cm^3 large testicular tissue samples from the left-sided testis of each animal were fixed overnight in 4% paraformaldehyde, subsequently transferred into cryoprotective solution containing 30% sucrose for 24 hours and cut into 30 μm thick sections with the help of a cryostat. The sections were mounted on gelatin-coated slides, air dried at 50°C for 30 min and subjected to FJ - C staining. Subsequently, they were immersed in absolute alcohol for 3 min, 70% alcohol for 1 min and distilled water for 1 min and finally treated with 0.06% potassium permanganate solution for 15 min. Following a rinse in distilled water for 2 min, the sections were incubated in the FJ - C staining solution (1 μg/ml in 0.1% acetic acid) for 30 min, dried at room temperature, cleared with xylene and cover-slipped with the DPX mounting medium. Five histological slides from different testicular regions (2 tissue blocks/each testis) were obtained, and fifty randomly chosen fields (10 fields/each slide) were evaluated.

Qualitative Superoxide dismutase 2 (SOD2) immunodetection

The same paraplast blocks previously prepared for LM (2 blocks/each right-sided testis) were used for this procedure. Four μm thick tissue sections were cut, subsequently deparaffinized and flushed with phosphate buffer (PBS-Tween 20). The endogenous peroxidase

activity was blocked with 3% ethanol solution of H_2O_2, flushed with PBS-Tween 20, citrate buffer and PBS-Tween 20. The blockage of nonspecific background reaction was conducted by 5% milk buffer and the primary monoclonal antibody (ADI-SOD-100-D, Enzo Life Science). Tissue sections were then washed with PBS-Tween 20 and the secondary antibody (Cat. No. K0690, DAKO, USA) was added. After flushing with phosphate buffer, streptavidin peroxidase was used. Subsequently, the proteins were visualized by diaminobenzidin (DAB) and 0.015% ethanol solution of H_2O_2. The sections were finally flushed with distilled water, counter-stained with haematoxylin, examined under the Zeiss Axio Lab A1 light microscope (Zeiss Germany) and photodocumented with the Axio Cam ERc5 camera (Zeiss Germany). The expression of SOD2 positivity was evaluated on five histological slides from the different regions of each right-sided testis. Fifty randomly chosen seminiferous tubules (10 tubules/slide) were evaluated qualitatively for SOD2 positivity as follows: + weak reaction, ++ moderate reaction, +++ intense reaction.

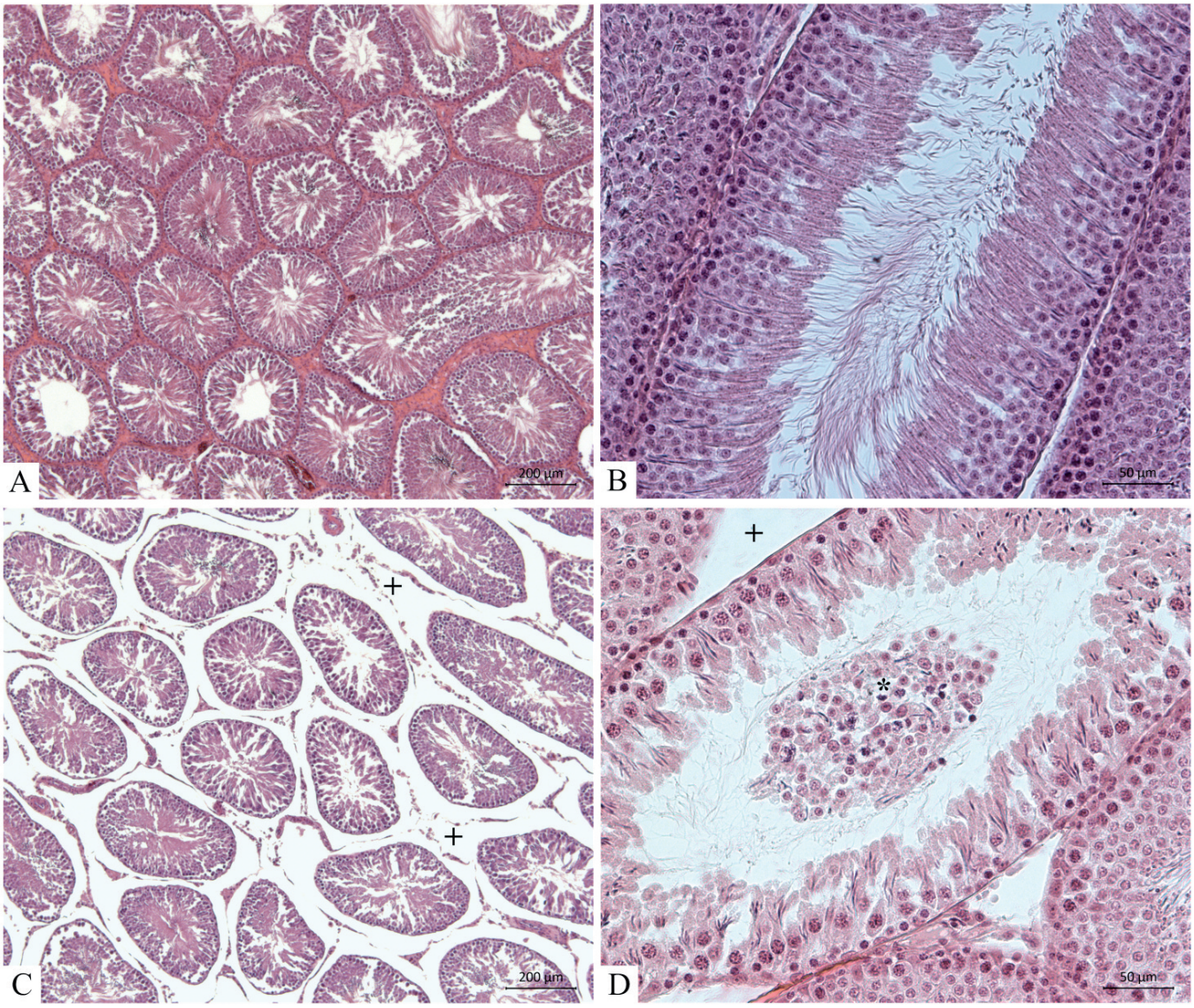

Fig. 2. Representative photomicrographs of the hematoxylin and eosin (H&E) stained tissue sections. The testicular tissue of the adult control rats **(A, B)** exhibited a typical histological appearance. The testicular tissue of adult rats exposed to MR prenatally **(C)** showed slightly irregular seminiferous tubules in addition to tubules with regular shape, and enlarged interstitial space (+). The seminiferous tubule of an adult rat exposed to MR prenatally **(D)** revealed desquamated immature germ cells in the lumen (*) and enlarged interstitium (+). A, C, x 50; B, D, x 200.

Results

Conventional light microscopy - LM

The testes of the control animals had a typical histological structure. The seminiferous tubules were regular in shape, and the germinal epithelium was composed of numerous developing germ cells. The tubular lumen contained typically organized tails of future spermatozoa. The interstitium tightly bounded seminiferous tubules and housed well-formed clusters of Leydig cells and blood vessels. The testicular parenchyma of the experimental animals revealed diffuse, moderate degenerative features. In addition to the approximately regular seminiferous tubules, some irregularly shaped seminiferous tubules (approx. 25%) were observed in the testicular parenchyma. The lumen of the seminiferous tubules contained either only projecting tails of future spermatozoa, or groups of desquamated immature germ cells in some segments. The interstitial space was enlarged and contained groups of Leydig cells of typical shape as well as characteristic blood vessels. The peritubular myoid cells also had a typical structure (Fig. 2A-D).

Morphometry and statistical analysis

A comparison of the data collected from the morphometrical assessment revealed significant differences between the control and experimental group. The diameter of the seminiferous tubules as well as the mean height of the germinal epithelium were significantly decreased (**p<0.01) in the rats prenatally exposed to MR when compared to the control group. The volume percentage of the interstitial compartment was significantly increased (**p<0.01) in the *in utero* exposed rats in comparison with the control group (Fig. 3).

Transmission electron microscopy - TEM

While the ultrastructure of the cells in the testes of the control animals was generally well preserved, the testicular cells in the animals exposed to MR exhibited relatively pronounced ultrastructural changes. The Sertoli cells presented with mostly unchained and typically indented nuclei, however their cytoplasm often contained some electronlucent vacuoles of different sizes. The Sertoli cells also contained an increased number of lysosomes, autophagic and heterophagic vacuoles as well as swollen mitochondria. Some inconspicuous interruptions occurred within the Sertoli - Sertoli tight junctions and irregular empty spaces between the germ and Sertoli cells were observed. All stages of developing germ cells had a vacuolated cytoplasm and affected mitochondria. Necrotizing germ cells with a strongly electron-dense cytoplasm and disintegrated organelles occurred more frequently in the experimental animals in comparison to the controls. Some segments of the basement membrane had an uneven thickness with small irregular electron-lucent hollow spaces. The peritubular myoid cells had mostly a regular shape and typically elongated nuclei, but their cytoplasm often contained electron-lucent vacuoles. The Leydig cells were mostly regular in shape, with

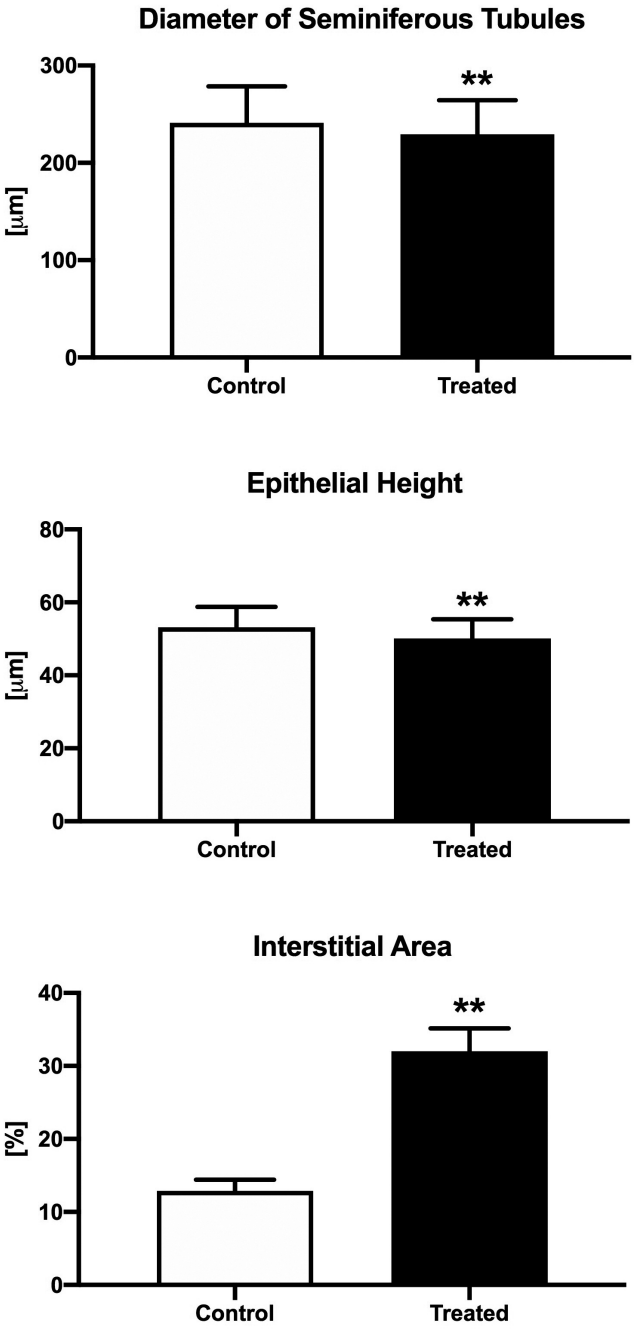

Fig. 3. Morphometric evaluation of selected testicular parameters in the control and experimental rats exposed to MR prenatally. Significant differences (**p<0.01) were observed between the control and treated animals (mean±SEM).

unchained nucleus, but their cytoplasm contained swollen mitochondria, a reduced smooth endoplasmic reticulum and an increased number of electron-lucent vacuoles and lipid droplets of different sizes. The endothelial and pericapillary cells were typical, except for the occurrence of electron-lucent vacuoles in their cytoplasm (Fig. 4A-D).

Qualitative Fluoro - Jade C histochemical detection

Degeneration of different forms of developing germ cells in seminiferous epithelium with a clear FJ - C positivity were observed in both control rats and rats exposed to MR. Specifically, the seminiferous tubules in the control animals contained only a relatively low number of FJ - C positive degenerating spermatogonia and spermatocytes in addition to FJ - C positive residual bodies (Fig. 5A). The interstitium did not contain any positive components (Fig. 5A,B). The testicular parenchyma of the experimental group showed a more pronounced FJ - C positivity, and in addition to residual bodies, numerous spermatogonia and spermatocytes, as well as rounded and elongating spermatids were FJ - C positive. Within the interstitial constituents, sporadic Leydig cells were also labelled with the FJ - C marker in the rats prenatally exposed to MR (Fig. 5C,D). The residual bodies were found to be FJ - C positive to a comparable extent between the control and experimental animals (Fig. 5A-D).

Qualitative superoxide dismutase 2 (SOD2) immunodetection

With respect to the SOD2 reaction in the testicular parenchyma there were clear variations between the control and experimental groups. While the seminiferous epithelium in both groups showed a comparably weak SOD2 reaction in the case of somatic Sertoli and peritubular cells, the reaction of Leydig cells revealed an intense positivity in both groups of animals. Regarding

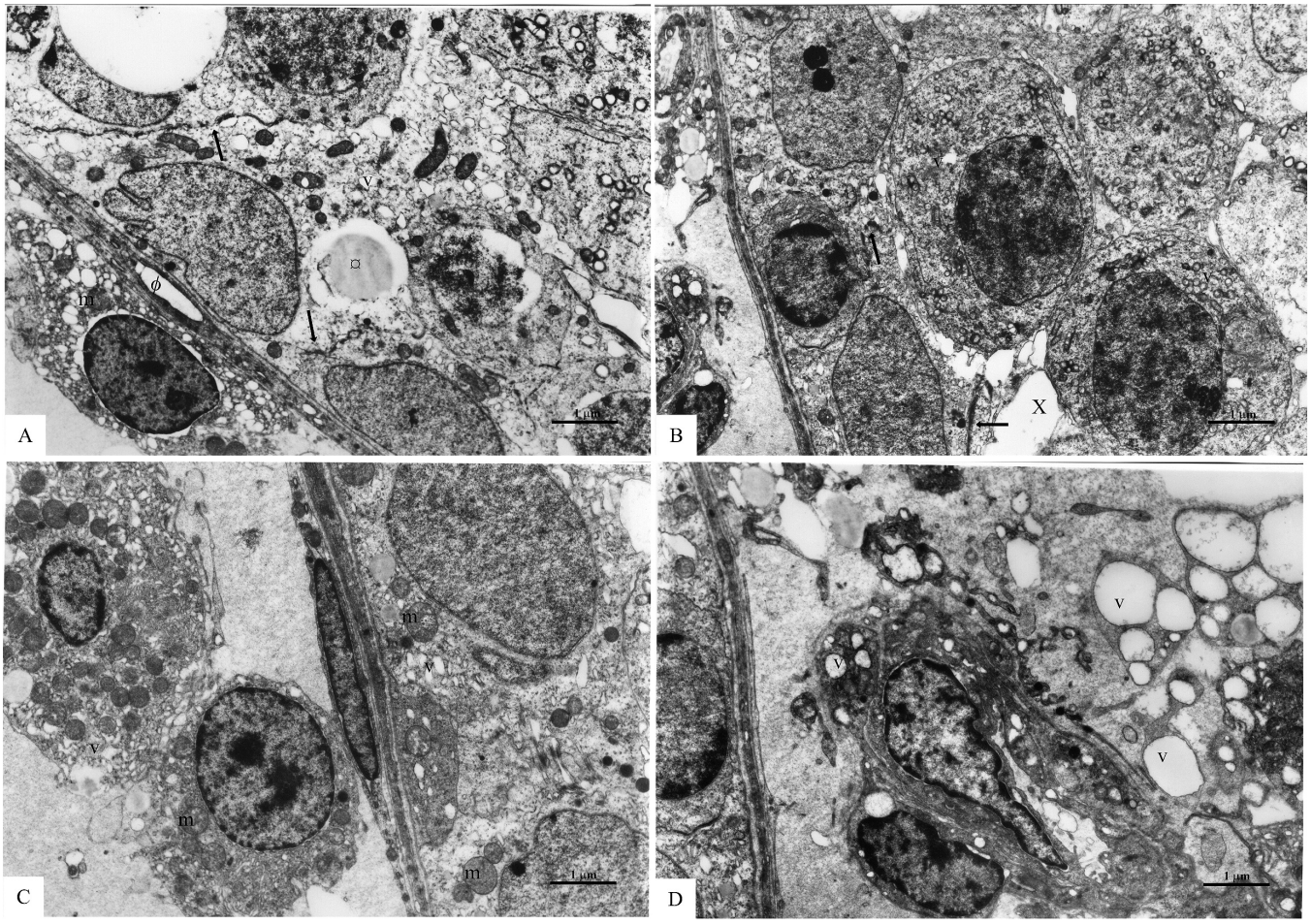

Fig. 4. Representative electron micrographs of the details of seminiferous epithelium and adjacent interstitial space of adult rats exposed to MR prenatally taken **(A-D)** showed specific ultrastructural changes (v - vacuoles in cytoplasm, m - affected mitochondria, ↓ - affected tight junction, ¤ - phagocytosed material, ø - empty space within the basement membrane, × - empty space between the adjacent cells). x 2,800.

the germ cells, the SOD2 reactivity differed depending on their developmental stage as well as on the group of animals. In the control group, spermatogonia and preleptotene and leptotene/zygotene spermatocytes showed a weak SOD2 positivity, which was comparable with the positivity of the Sertoli cells. Early small pachytene spermatocytes and round spermatids showed a moderate SOD2 reaction. Late large pachytene spermatocytes exhibited a high SOD2 reaction. High SOD2 positivity was also detected in the elongating spermatids and residual bodies. In the group of animals prenatally exposed to MR, every developmental stage of the germ cells except for spermatids appeared as highly SOD2 positive. On the contrary early rounded and late elongating spermatids revealed a weakened SOD2 reaction. The residual bodies also demonstrated attenuated SOD2 positivity in the MR - exposed group (Fig. 6A-D, Table 1).

Table 1. Summary of the intensity of SOD 2 reaction determined by visual inspection in different developmental stages of germ cells in seminiferous epithelium of control rats and rats exposed to MR prenatally (+ weak reaction, ++ moderate reaction, +++ intense reaction).

Group	Spermatogonia	Preleptotene spermatocytes	Leptotene/zygotene spermatocytes	Early pachytene spermatocytes	Late pachytene spermatocytes	Round spermatids	Elongating spermatids	Residual bodies
Control	+	+	+	++	+++	++	+++	+++
Prenatally exposed to MR	+++	+++	+++	+++	+++	+	+	++

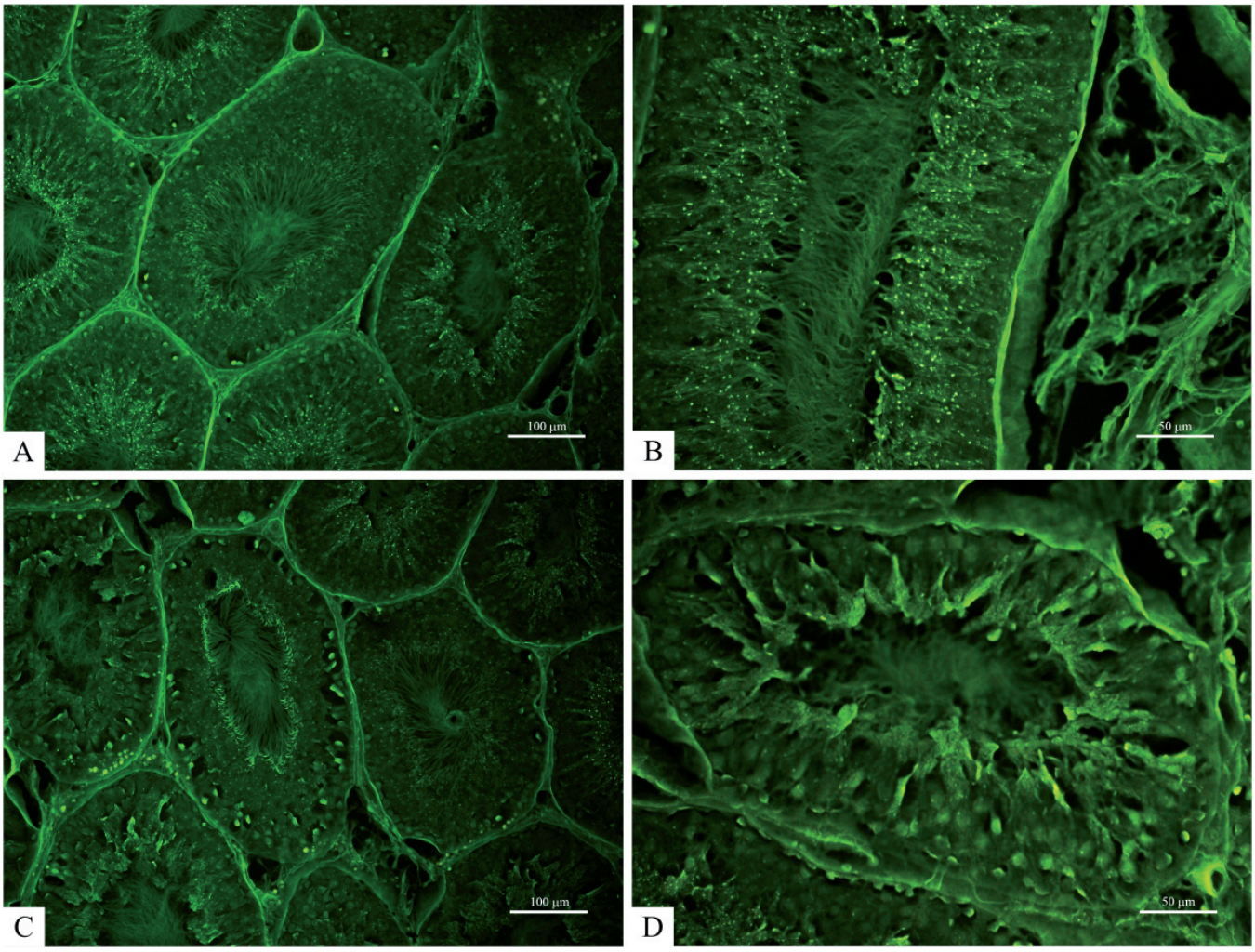

Fig. 5. Representative photomicrographs of the Fluoro - Jade C positive cells (bright green spots). The testicular tissue of adult control rats **(A, B)** and adult rats exposed to MR prenatally **(C, D)**. Note the increased number of Fluro - Jade C positive cells in the seminiferous epithelium and interstitium of adult rats exposed to MR prenatally. A, C, x 100; B, D, x 200.

Discussion

It is well known that spermatogenesis is a process exceptionally sensitive to RF - EMR (Santini et al., 2018; Jaffar et al., 2019; Zha et al., 2019). Although the exact mechanism of action of RF - EMR on the testicular tissue is not yet completely understood, the production of free radicals and oxidative damage to the tissue, are the most likely contributors (Atasoy et al., 2013; Özorak et al., 2013; Oksay et al., 2014; Yahyazadeh and Altunkaynak, 2019; Yahyazadeh et al., 2020). Only a few reports focused on the consequences of exposure to RF - EMR on the prenatal development of different organ systems (Ait-Aissa et al., 2012; Aldad et al., 2012; Zhang et al., 2015; Alchalabi et al., 2016; Othman et al., 2017) and the intrauterine development of the male reproductive system (Hanci et al., 2013).

Since non-ionizing radiation has become a ubiquitous component of our environment (Kováč et al., 2010, 2017) and at the same time, the entire prenatal development is generally a highly susceptible period, we considered it important to investigate the impact of Wi-Fi-like microwave radiation on the testicular development in rats. This study was performed using a

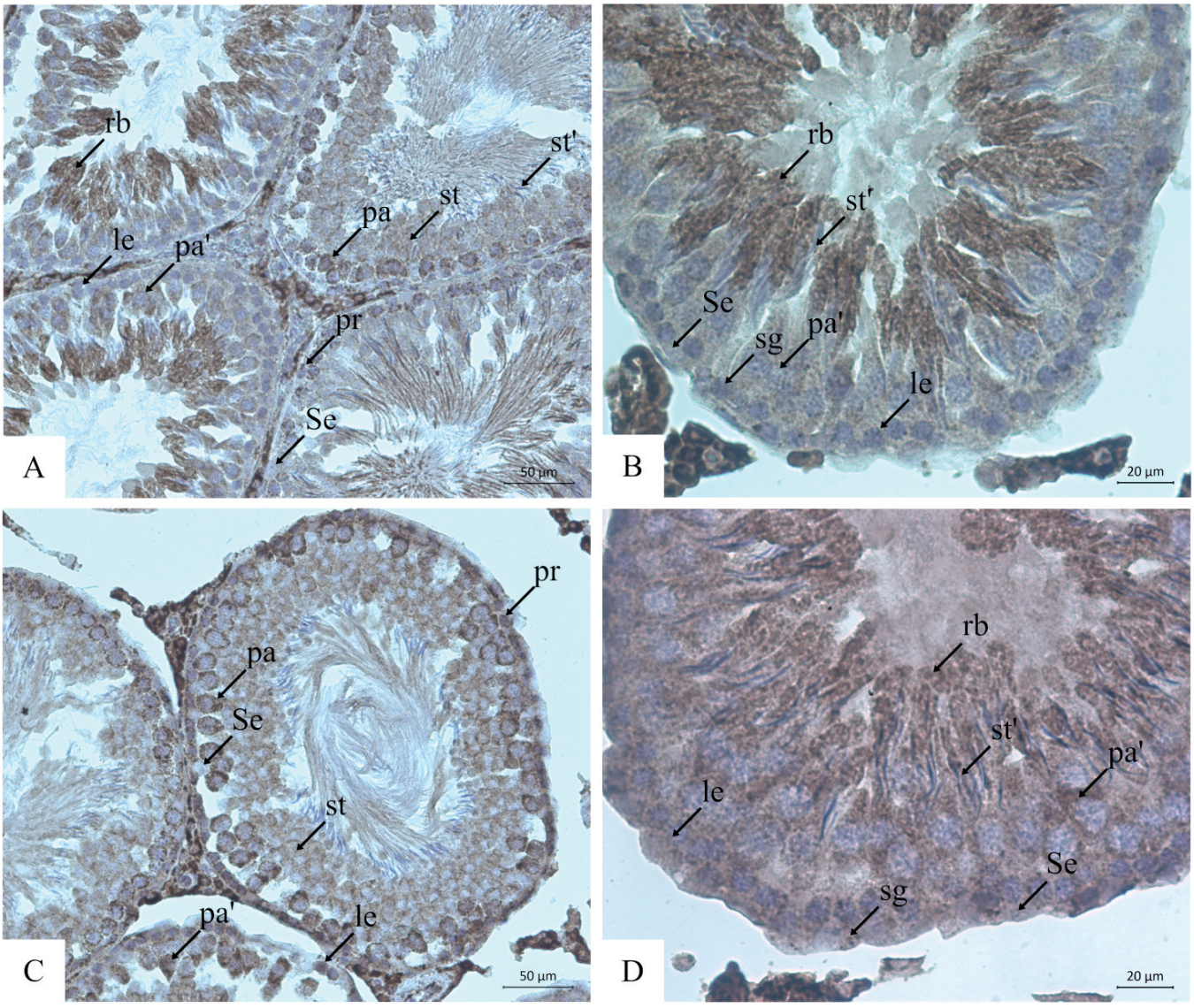

Fig. 6. Representative photomicrographs of the immunohistochemical localization of SOD2 (brown granular material) in the testicular tissue of adult control rats **(A, B)** and adult rats exposed to MR prenatally **(C, D)**. Note the increased SOD2 reactivity in spermatogonia (sg), preleptotene spermatocytes (pr), leptotene/zygotene spermatocytes (le), early and late pachytene spematocytes (pa, pa'), a decreased SOD2 reactivity in the rounded and elongating spermatids (st, st'), and a decreased SOD2 reactivity of residual bodies (rb) in the seminiferous epithelium in adult rats exposed to MR prenatally. The sections were counter-stained with haematoxylin. A, C, x 200; B, D, x 400.

far-field exposure that corresponds to various transmitters of indoor or outdoor antennas. Pregnant mates were exposed to 2.45 GHz RF - EMR throughout the pregnancy. The selected mean power density of 2.8 mW/cm^2 and SAR of 1.82 W/kg together with the applied time of the exposure (2h/day) and the distance of animals from the source of radiation (70 cm) may be considered close to the real long-term effects of low RF - EMR doses in the environment. However, due to an inconsistent methodology in the experiments involving non-ionizing radiation, further studies will be required in this area.

Our histopathological and histomorphometric observations demonstrated diffuse, moderate, degenerative testicular changes in the rats exposed to MR during their intrauterine development. These manifested themselves as specific irregularities in the shape of the seminiferous tubules as well as the occurrence of desquamated immature germ cells located in the lumen of the seminiferous tubules. The seminiferous tubules had a significantly reduced diameter as well as the height of the seminiferous epithelium (**$p<0.01$), while the interstitial volume was significantly increased (**$p<0.01$). This is in close agreement with changes observed in young 21-days old rats exposed to MR *in utero* (Hanci et al., 2013) or sexually mature rats exposed to MR postnatally (Saygin et al., 2011; Al-Damegh, 2012; Shahin et al., 2014; Odaci and Özyilmaz, 2015; Shokri et al., 2015). Furthermore, similar observations were reported in prepubertal animals exposed to MR solely after birth (Saygin et al., 2016; Jonwal et al., 2018; Simaiova et al., 2019). According to the above authors, the most frequently observed histopathological changes included irregular seminiferous tubules with a decreased diameter and a reduced epithelial height.

According to the available literature, the testicular ultrastructure in rats exposed to MR *in utero* has not been studied yet. Our transmission electron microscopy evaluation revealed degenerative changes particularly in the Sertoli cells, Leydig cells, endothelial and pericapillary cells as well as all stages of developing germ cells. This is in close agreement with our observations conducted with light microscopy, histomorphometric analyses, Fluoro - Jade C and SOD2 assays. Similar changes at the subcellular level such as alterations to a normal conformation of the blood-testis barrier (Wang et al., 2008; Gao et al., 2016) or ultrastructural injuries to the Sertoli and germ cells (Celik et al. 2012; Gao et al., 2016) were described in adult animals exposed to MR postnatally.

Our results indicating a more extensive testicular degeneration in rats prenatally exposed to MR, detected by light and electron microscopy, were also supported by the Fluoro Jade - C histochemical method. This is a very sensitive technique primarily intended for the localization of neurons undergoing degeneration and a subsequent necrotization (Schmued et al., 2005). Recently, it was shown that under some conditions the FJ - C method could also label degenerating non-neuronal cells (Ikeda et al., 2017; Ikenari et al., 2020). Since the loss of the germ cells by apoptosis normally occurs during spermatogenesis (Shaha et al., 2010), it is very important to distinguish a normal rate of apoptosis from the pathological one. We did not observe any signs of an increased degeneration in the control group of animals, as only a few spermatogonia and spermatocytes were FJ - C positive. In contrast, the seminiferous epithelium of the experimental rats presented with a high number of FJ - C labelled cells. Our findings are consistent with the results of Hanci et al. (2013) who noted a significant increase in the number of TUNEL positive germ cells in juvenile rats exposed to microwaves *in utero*, as well as with those of other authors who reported a more pronounced RF - EMR induced cellular necrotization in adult animals exposed to the radiation postnatally (Saygin et al., 2011; Hanci et al., 2013; Shokri et al., 2015; Jaffar et al., 2019).

Since oxidative stress appears to be the most relevant mechanism of RF-EMR induced testicular impairment (Sage and Carpenter, 2009; Atasoy et al., 2013; Meena et al., 2014; Odaci and Özyilmaz, 2015; Ding et al., 2018; Jonwal et al., 2018; Santini et al., 2018), and a notable feature of the testicular antioxidant system is a high SOD activity (Sikka et al., 1995; Gu and Hecht 1996), we focused on the evaluation of a range of testicular oxidative changes via a qualitative assessment of the intensity of SOD2 in individual developmental stages of the testicular cells. High levels of SOD2 expression under physiological conditions were detected, especially in late pachytene spermatocytes (Sakai et al., 2010) and early post-meiotic germ cells (Gu and Hecht, 1996). Based on the comparison of the SOD2 reaction within individual testicular cells between the control and MR - exposed rats, we may generally conclude that RF-EMR enhanced the SOD2 production within the germ cells undergoing cell divisions during spermatocytogenesis (spermatogonia and spermatocytes). On the other hand, a lower cellular SOD2 antioxidant capacity was found in the post-meiotic germ cells (rounded to elongating spermatids). These changes in the SOD status may be attributed to morphological abnormalities such as irregular seminiferous tubules, enlargement of the interstitial space and a decreased diameter of the seminiferous tubules accompanied with a reduced height of the seminiferous epithelium, as observed in the present study. This also coincides with our electron microscopy investigation and FJ - C analysis, where the spermatocytes exhibited a more intense degeneration due to RF - EMR. A similar response was noted in our previous studies conducted on adult (Almasiova et al., 2017) or infant (Simaiova et al., 2019) rats exposed to MR postnatally, where the spermatogonia as well as spermatocytes were highly SOD positive. Most studies that evaluated the total testicular antioxidant status (including SOD-s) in homogenized tissues of adult animals following the exposure to RF - EMR, agree

upon a significant reduction in reactive oxygen species (ROS) quenching mechanisms (Atasoy et al., 2013; Shahin et al., 2014; Odaci and Özyilmaz, 2015; Jonwal et al., 2018). Oxidative damage to the testicular tissue was evident in adult rats exposed to MR postnatally (Kesari and Behari, 2010; Atasoy et al., 2013; Shahin et al., 2014; Saygin et al., 2016; Jonwal et al., 2018) or juvenile rats exposed to MR prenatally (Hanci et al., 2013). The radio-protective effect of selected antioxidants on the testicular structures has also been clearly demonstrated by some authors (Sikka et al., 1995; Yahyazadeh and Altunkaynak, 2019; Yahyazadeh et al., 2020).

The combination of two factors - anatomical characteristics of the testes and the process of spermatogenesis, which is extremely sensitive to an elevated temperature, forces us to consider a potential thermal effect of RF - EMR on the testicular tissue. In our previous study (Almasiova et al., 2017) using a thermal camera, the rise in testicular temperature was confirmed in sexually mature rats exposed to MR postnatally. Frequency and power density of MR were identical to those used in the present study. However, due to the impossibility of performing thermometry in the tissues of a developing fetuses, we cannot unambiguously confirm or reject involvement of the thermal effect in the current experiment. Based on the obtained results, we assume that there is most likely an interplay between the thermal and non-thermal mechanism of action, that may be involved in the testicular injury.

Because the rats investigated in the present study were exposed to MR exclusively during their embryonal and fetal periods of life, we may assume that the causes of testicular changes observed in sexually mature rats may be related to the developmental alterations in the Sertoli cells, Leydig cells and potentially also stem germ cells. Undoubtedly, a potential negative impact of RF - EMR on the development of other organ systems in the fetal body that are involved in the regulation of the male reproductive system can not be excluded from the proposed etiology.

We can conclude that the pulsed microwave long-term exposure radiation at a frequency of 2.45 GHz, mean power density of 2.8 mW/cm^2 and SAR of 1.82 W/kg applied in the prenatal period of life, caused developmental alterations with a direct unfavorable impact on the testicular structure and spermatogenesis in adult rats. Oxidative damage and an affected blood-testis barrier were considered as the possible mechanisms of the tissue injury, which manifested as germ cell degeneration and desquamations. Transmission electron microscopy also revealed alterations to the somatic testicular cells, which suggested that the origin of impaired spermatogenesis can result from an improper conformation of the Sertoli cells as the typical post-mitotic elements, or a combination of Sertoli, Leydig and stem germ cell dysgenesis. Because a relatively permanent exposure to RF - EMR has become very common in modern life, there is no way to eliminate it completely. However, a simulation of a realistic human exposure remains still challenging, and extrapolation of experimental results to humans requires further studies.

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References

Ait-Aissa S., Billaudel B., de Gannes F.P., Ruffié G., Duleu S., Hurtier A., Haro E., Taxile, Athané M.A., Geffard M., Wu T., Wiart J., Bodet D., Veyret B. and Lagroye I. (2012). *In utero* and early-life exposure of rats to a Wi-Fi signal: screening of immune markers in sera and gestational outcome. Bioelectromagn. 33, 410-420.

Alchalabi A.S.H., Aklilu E., Aziz A.R., Malek F., Ronald S.H. and Khan M.A. (2016). Different periods of intrauterine exposure to electromagnetic field: Influence on female rats' fertility, prenatal and postnatal development. Asian Pacif. J. Repr. 5, 14-23.

Aldad T.S., Gan G., Gao X.B. and Taylor H.S. (2012). Fetal radiofrequency radiation exposure from 800-1900 MHz-rated cellular telephones affect neurodevelopment and behavior in mice. Sci. Rep. 2, 312-318.

Al-Damegh M.A. (2012). Rat testicular impairment induced by electromagnetic radiation from a conventional cellular telephone and the protective effects of the antioxidants vitamins C and E. Clinics 67, 785-792.

Almasiova V., Holovska K., Simaiova V., Benova K., Racek A., Racekova E., Martoncikova M., Mihalik J., Horvathova F., Tarabova L., Slanina T. and Cigankova V. (2017). The thermal effect of 2.45 GHz microwave radiation on rat testes. Acta Vet. Brno 86, 413-419.

Atasoy H.I., Gunal M.Y., Atasoy P., Elgun S. and Bugdayci G. (2013). Immunohistopathologic demonstration of deleterious effects on growing rat testes of radiofrequency waves emmited from conventional Wi-Fi devices. J. Pediatr. Urol. 9, 223-229.

Balassa T., Varro P., Elek S., Drozdovszky O., Szemerszky R., Vilagy I. and Bardos G. (2013). Changes in synaptic efficacy in rat brain slices following extremely low-frequency magnetic field exposure at embryonic and early postnatal age. Int. J. Dev. Neurosci. 31, 724-730.

Celik S., Aridogan A., Izol V., Erdogan S., Polat S. and Doran S. (2012). An evaluation of the effects of long-term cell phone use on the testes via light and electron microscope analysis. Urology 79, 346-350.

Dietert R.R. and Piepenbrink M.S. (2008). The managed immune system: protecting the womb to delay the tomb. Hum. Exp. Toxicol. 27, 129-134.

Ding S.S., Sun P., Tian H., Huo Y.W., Wang L.R., Han Y., Zhang Z., Liu X. and Xing J.P. (2018). Association between daily exposure to electromagnetic radiation from 4G smartphone and 2.45-GHz wi-fi and oxidative damage to semen of males attending a genetics clinic: a primary study. Int. J. Clin. Exp. Med.11, 2821-2830.

D'Silva M.H., Swer R.T., Anbalagan J. and Rajesh B. (2017). Effect of radiofrequency radiation emitted from 2G and 3G cell phone on developing liver of chick embryo - A comparative study. J Clin. Diagn. Res. 11, 5-9.

Gao X.H., Hu H.R., Ma X.L., Chen J. and Zhang G.H. (2016). Cellphone

electromagnetic radiation damages the testicular ultrastructure of male rats. Zhong. Nan Ke Xue. 22, 491-495.

Gu W. and Hecht N.B. (1996). Developmental expression of glutathione peroxidase, catalase, and manganese superoxide dismutase mRNAs during spermatogenesis in the mouse. J Androl. 17, 256-262.

Guler G., Tomruk A., Ozgur E. and Seyhan N. (2010). The effect of radiofrequency radiation on DNA and lipid damage in non-pregnant and pregnant rabbits and their newborns. Gen. Physiol. Biophys. 29, 59-66.

Hanci H., Odaci E., Kaya H., Aliyazicioglu Y., Turan I., Demir S. and Colakoglu S. (2013). The effect of prenatal exposure to 900-MHz electromagnetic field on the 21-old-day rat testicle. Reprod. Toxicol. 42, 203-209.

Ikeda M., Wakasaki R., Schenning K.J., Swide T., Lee J.H., Miller M.B., Choi H.S., Anderson S. and Hutchens M.P. (2017). Determination of renal function and injury using near-infrared fluorimetry in experimental cardiorenal syndrome. Am. J. Physiol. Renal. Physiol. 312, 629-639.

Ikenari T., Kurata H., Satoh T., Hata Y. and Mori T. (2020). Evaluation of Fluoro-Jade C staining: Specificity and Application to damaged immature neuronal cells in the normal and injured mouse brain. Neurosci. 425, 146-156.

Jaffar F.H.F., Osman K., Ismail N.H., Chin K.Y. and Ibrahim S.F. (2019). Adverse effects of Wi-Fi radiation on male reproductive system: A systematic review. Tohoku J. Exp. Med. 248, 169-179.

Jonwal Ch., Sisodia R., Saxena V.K. and Kesari K.K. (2018). Effect of 2.45 GHz microwave radiation on the fertility pattern im male mice. Gen. Physiol. Biophys. 37, 453-460.

Kesari K.K. and Behari J. (2010). Effects of microwave at 2.45 GHz radiations on reproductive system of male rats. Toxicol. Environ. Chem. 92, 1135-1147.

Kesari K.K., Agarwal A. and Henkel R. (2018). Radiations and male fertility. Reprod. Biol. Endocrinol. 16, 118.

Kilcoyne K.R. and Mitchell R.T. (2019). Effect of environmental and pharmaceutical exposures on fetal testis development and function: a systematic review of human experimental data. Hum. Reprod. Update 25, 397-421.

Kováč D., Vince T., Molnár J. and Kováčová I. (2010). Modern internet-based production technology. In: New Trends in Technologies: devices, computer, communication and industrial systems. Er M.J. (ed). Sciyo. Rijeka. pp 145-164.

Kováč D., Kováčová I., Vince T., Molnár J., Perdu'ak J., Bereš M. and Dziak J. (2017). An automated measuring laboratory (VMLab) in education. Int. J. Engin. Educ. 32, 2250-2259.

Latendresse J.R., Warbrittion A.R., Jonassen H. and Creasy D.M. (2002). Fixation of testes and eyes using a modified Davison's fluid: Comparison with Bouin's and conventional Davison's fluid. Toxicol. Pathol. 30, 524-533.

La Vignera S., A Condorelli R., Vicari E., D'Agata R. and E Calogero A. (2012). Effects of the exposure to mobile phones on male reproduction: A review of literature. J. Androl. 33, 350-356.

Meena R., Kumari K., Kumar J., Rajamani P., Verma H.N. and Kesari K.K. (2014). Therapeutic approaches of melatonin in microwave radiations-induced OS-mediated toxicity on male fertility pattern of Wistar rats. Electromagn. Biol. Med. 33, 81-91.

Odaci E. and Özyilmaz C. (2015). Exposure to a 900 MHz electromagnetic field for 1 hour a day over 30 days does change the histopathology and biochemistry of the rat testis. Int. J. Radiat. Biol. 91, 547-554.

Oksay T., Naziroglu M., Dogan S., Güzel A., Gümral N. and Kosar P.A. (2014). Protective effects of melatonin against oxidative injury in rat testis induced by wireless (2.45 GHz) devices. Andrologia 46, 65-72.

Othman H., Ammari M., Rtibi K., Bensaid N., Sakly M. and Abdelmelek H. (2017). Postnatal development and behavior effects of in-utero exposure of rats to radiofrequency waves emitted from conventional WiFi devices. Environ. Toxicol. Pharmacol. 52, 239-247.

Özorak A., Naziroğlu M., Çelik Ö., Yükse I.M., Özçelik D., Özkaya M.O., Çetin H., Kahya M.C. and Kose S.A. (2013). Wi-Fi (2.45 GHz)-and mobile phone (900 and 1800 MHz)-induced risks on oxidative stress and elements in kidney and testis of rats during pregnancy and the development of offspring. Biol. Trace Elem. Res. 156, 221-229.

Sage C. and Carpenter D.O. (2009). Public health implications of wireless Technologies. Pathophysiology 16, 233-246.

Sakai Y., Aminaka M, Takata A., Kudou Y., Yamauchi H., Aizawa Y. and Sakagami H. (2010). Oxidative stress in mature rat testis and its developmental changes. Develop. Growth Differ. 52, 657-663.

Santini S.J., Cordone V., Falone S., Mijit M., Tatone C., Amicarelli F. and Di Emidio G. (2018). Role of mitochondria in the oxidative stress induced by electromagnetic fields: Focus on reproductive systems. Oxid. Med. Cell Longev. 2018, 5076271.

Saygin M., Asci H., Ozmen O., Cankara F.N., Dincoglu D. and Ilhan I. (2016). Impact of 2.45 GHz microwave radiation on the testicular inflamatory pathway biomarkers in young rats. The role of garlic acid. Environ. Toxicol. 31, 1771-1784.

Saygin M., Caliskan S., Karahan N., Koyu A., Gumral N. and Uguz A. (2011). Testicular apoptosis and histopathological changes induced by a 2.45 GHz electromagnetic field. Toxicol. Ind. Health 27, 455-463.

Schmued L.C., Stowers CH. C., Scallet A.C. and Xu L. (2005). Fluoro-Jade C results in ultra high resolution and contrast labeling of degenerating neurons. Brain Res. 1035, 24-31.

Shaha Ch., Tripathi R. and Mishra D.P. (2010). Male germ cell apoptosis: regulation and biology. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 365, 1501-1515.

Shahin S., Mishra V., Singh S.P. and Chaturvedi C.M. (2014). 2.45 GHz microwave irradiation adversely affects reproductive function in male mouse, *Mus musculus* by inducing oxidative and nitrosative stress. Free Radic. Res. 48, 511-525.

Sharma A., Kesari K.K., Saxena V.K. and Sisodia R. (2017). The influence of prenatal 10 GHz microwave radiation exposure on a developing mice brain. Gen. Physiol. Biophys. 36, 41-51.

Shokri S., Soltani A., Kazemi M., Sardari D. and Mofrad F.B. (2015). Effects of Wi-Fi (2.45 GHz) exposure on apopotosis, sperm parameters and testicular histomorphology in rats: a time course study. Cell J. 17, 322-331.

Sikka S.C., Rajasekaran M. and Hellstrom W.J.G. (1995) Role of oxidative stress and antioxidants in male infertility. J. Androl. 16, 464-468.

Simaiova V., Almasiova V., Holovska K., Kiskova T., Horvathova F., Sevcikova Z., Toth S., Racek A., Racekova E., Benova K., Dvorak P. and Cigankova V. (2019). The effect of 2.45 GHz non-ionizing radiation on the structure and ultrastructure of the testis in juvenile rats. Histol. Histopathol. 34, 391-403.

Singh R., Nath R., Mathur A.K. and Sharma R.S. (2018). Effect of radiofrequency radiation on reproductive health. Ind. J. Med. Res. 148, 92-99.

Valberg P.A., van Deventer T.E. and Repacholi M.H. (2007). Base

stations and wireless networks - Radiofrequency (RF) exposures and health consequences. Environ. Health Persp. 115, 416-423.

Wang X.W., Shi CH., Zhao T., Zhang J., Zeng L.H. and Guo G.Z. (2008). Effect of electromagnetic pulse exposure on permeability of blood-testicle barrier in mice. Biomed. Environ. Sci. 21, 218-221.

Yahyazadeh A. and Altunkaynak B.Z. (2019). Protective effects of luteolin on rat testis following exposure to 900 MHz electromagnetic field. Biotech Histochem. 23, 1-10.

Yahyazadeh A. and Altunkaynak B.Z. (2020a). Effect of luteolin on biochemical, immunohistochemical, and morphometrical changes in rat spinal cord following exposure to a 900 MHz electromagnetic field. Biomed. Environ Sci. 33, 593-602.

Yahyazadeh A. and Altunkaynak B.Z. (2020b). Neuroprotective efficacy of luteolin on a 900-MHz electromagnetic field-induced cerebellar alteration in adult male rat. Brain Res. 1744, 146919.

Yahyazadeh A., Deniz Ö.G., Kaplan A.A., Altun G., Yurt K.K. and Davis D. (2018). The genomic effects of cell phone exposure on the reproductive system. Environ. Res. 167, 684-693.

Yahyazadeh A., Altunkaynak B.Z. and Kaplan S. (2020). Biochemical, immunohistochemical and morphometrical investigation of the effect of thymoquinone on the rat testis following exposure to a 900-mhz electromagnetic field. Acta. Histochem. 122, 151467.

Zha X.D., Wang W.W., Xu S. and Shang X.J. (2019). Impacts of electromagnetic radiation from cellphones and Wi-Fi on spermatogenesis. Zhong. Nan. Ke Xue. 25, 451-455.

Zhang Y., Li Z., Gao Y. and Zhang CH. (2015). Effects of fetal microwave radiation exposure on offspring behaviour in mice. J. Radiat. Res. 56, 261-268.

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