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The potential adverse effect of 2.45 GHz microwave radiation on the testes of prenatally exposed peripubertal male rats

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Summary. In utero development of organs is easily influenced by many environmental factors. The aim of this study was to elucidate the effect of microwave radiation (MR) at a frequency of 2.45 GHz and a specific absorption rate of 1.73 W/kg on intrauterine development of testis. Pregnant albino rats were exposed to whole-body MR for 2 hours per day throughout the pregnancy. Male offspring (n=12, age 35 days) were not exposed to MR after birth. The study revealed that MR applied in utero induced apparent structural changes in the testes, such as irregular shape of seminiferous tubules, significant decrease in the diameter of seminiferous tubules ($p<0.05$) and in the height of the germinal epithelium ($p<0.01$), disorganisation of germ cells, desquamations of immature germ cells, formation of giant multinucleated cells, and significant ($p<0.01$) expansion of the interstitium. At the level of transmission electron microscopy, there were observed basement membrane irregularities in seminiferous tubules, vacuolation of the cytoplasm and adversely affected organelles in Sertoli cells, germ cells, Leydig cells, peritubular and endothelial cells. The tight junctions between adjacent Sertoli cells were often incomplete, and necrotizing germ cells were more numerous in experimental animals compared to controls. Enhanced necrotizations of germ cells proved by a Fluoro Jade C method, and declined germ cells proliferation confirmed by proliferating cell nuclear antigen analysis, were detected in MR exposed animals. Our results revealed that the prenatal exposure to MR had an adverse effect on the postnatal testicular development in rats.

Key words: Microwave radiation, Rat testes, Postnatal development, Apoptosis, In utero

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Introduction

Radiofrequency fields (100 kHz - 300 GHz) as a part of the ‘‘non-ionizing’’ electromagnetic spectrum represent a potential environmental risk to human health (Hyland, 2001; Markov and Kostarakis, 2007). Nowadays, such risk involves also a wide use of mobile phones and wireless technologies and devices (Wi-Fi) that are the preferred ways of internet communication and connectivity (Makker et al., 2009; Sage and Carpenter, 2009; Kováč et al., 2010). These operate in an unlicensed spectrum range including 2.45 and 5 GHz bands (Zhang et al., 2015) and are used mainly by the younger segment of the population. They contribute to the increasing concern of the public and scientists regarding human health safety. This is evidenced by the number of published studies which have revealed that exposure to radiofrequency electromagnetic radiation (RF-EMR) can alter various organ systems such as cardiovascular system, auditory apparatus, eye, liver, kidney, endocrine and reproductive functions as well as foetal development (Jauchem et al., 2000; Bouji et al., 2012; Gye and Park, 2012; Ebrahim et al., 2016; Hasan and Islam, 2020).

In the context of an increasing number of cases of partial or total infertility of humans in the reproductive age, more attention has been given to reproductive health (Jung and Schill, 2000; Aitken et al., 2004; Mascarenhas et al. 2012; Yahyazadeh et al., 2018). Many

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fertility disorders are diagnosed as idiopathic and 50% are associated with the so called "male factor" (Thonneau et al., 1991; Sharlip et al., 2002; Liu et al., 2004; Kumar and Singh, 2015). The reproductive process in males is very vulnerable due to great cellular diversity and organizational complexity. It depends on a complex set of biological relationships involving different organs, cell types, molecules, and on the precise temporal and spatial coordination of processes. The very period of maturation and acquisition of functionality of gametes is one of the phases most sensitive to various exogenous and endogenous factors (Sly and Carpenter, 2012). Electromagnetic pollution is one of the potential factors that may contribute to increasing male infertility. It is capable of triggering morphological and functional changes in the reproductive system and impairing the male germ line (spermatogenesis and sperm maturation) and thus compromising male reproduction in general (Aitken et al., 2005; Wdowiak et al., 2007; Baste et al., 2008; Agarwal et al., 2008b; Meo et al., 2011; Merhi, 2012). This phenomenon may be related to ever-increasing and excessive usage of the emerging, commercially available wireless technology operating at 2.45 GHz frequency (Saunders and Kowalczuk, 1981; Moon et al., 2006; Saygin et al., 2011; Shokri et al., 2015). Microwave radiation (MR) emitted by various wireless devices may affect biological systems either by thermal molecular action accompanied by elevation of tissue temperature (Oftedal et al., 2000; Jauchem et al., 2000; Foster and Colombi, 2017), or via specific non-thermal effects in which the oxidative stress pathway and formation of reactive oxygen species appear to be the underlying mechanisms of cell damage (Belyaev, 2005; Kibona, 2013; Nazıroğlu et al., 2013; Yakymenko et al., 2016).

Despite an extensive scientific knowledge, the effect of MR on the male reproductive system still raises serious controversy. Some studies report no effect of MR on the male reproductive system (Lebovitz and Johnson, 1983; Dasdag et al., 2003; Ribeiro et al., 2007; Tumkaya et al., 2016) while others noted a wide spectrum of potential harmful effects, ranging from mild to more severe testicular damage (Deepinder et al., 2007; Adams et al., 2014). The most frequently observed phenomena are changes in the histological structure and ultrastructure of the testes (Agarwal, 2007; Khayyat, 2011; Odaci and Ozyilmaz, 2015; Hanci et al., 2018; Hasan et al., 2021) germ cell degeneration, increased germ cell necrotization and apoptotic index, decelerated spermatogenesis, reduced sperm production as well as reduced quality of the semen (Lee et al., 2004; Agarwal et al., 2008a, 2009; Li et al., 2010). Adverse effects on the levels of reproductive endocrine hormones (Bahaodini et al., 2015) were also found. At the cellular level, MR-mediated increased free radical production and impaired calcium ion pathways may lead to inhibition of cell growth, misfolding of proteins and DNA breaks (Aitken et al., 2005; Falzone et al., 2011; Zalata et al., 2015; Akdag et al., 2016; Yahyazadeh et al., 2018).

Prenatal life is a critical stage in term of influence of various noxious environmental factors due to the rapid growth and differentiation of developing tissues (Dietert and Piepenbrink, 2008). MR radiation is undoubtedly one of these agents (Hanci et al., 2013; Othman et al., 2017). It is understandable that pregnant mothers and their unborn offspring attract the interest of researchers. Any unfavourable insult during the early stage of life may affect adversely the testicular development with damaging consequences for their future capacity to produce viable spermatozoa (Kilcoyne and Mitchell, 2019). Although the studies dealing with MR exposure during the pregnancy are very limited, they suggest that the MR may compromise the normal testicular development and reproductive functions in male offspring (Mc Ree et al. 1983; Chen et al., 2009; Tenorio et al., 2011; Hanci et al., 2013).

The main objective of this study was to investigate the potential effect of continuous prenatal exposure to a pulsed microwave radiation at a frequency of 2.45 GHz with focus on potential changes in the structure, ultrastructure and selected immunohistochemical parameters of testes in 35-day-old male offspring.

Materials and methods

Animals

Three-month-old, clinically healthy adult female Wistar albino rats (n=6) with mean body weight of 280±10 g were included in the study. They were housed in standard plastic cages (3 animals per cage) on sawdust bedding in an animal facility of Biomedical Research Centre, Slovak Academy of Sciences, Kosice. The animals were kept at standard laboratory conditions with 12/12h light/dark cycle, temperature of 21±1°C and relative humidity 50-55%. They were clinically healthy, with ad libitum access to food (standard rat pellets) and tap water. Primiparous females were mated with a three-month-old male rat (n=1) of the same strain and body weight of 294 g. All animals had originated from Velaz, Ltd., Prague, the Czech Republic. Vaginal smears were examined by light microscopy and the presence of spermatozoa was regarded as the first day of pregnancy. The pregnant rats were subsequently divided into a control and an experimental group (3 rats per group). Each group was placed in a separate cage.

Exposure system

The experimental group of pregnant rats (n=3) was whole-body exposed to a pulsed microwave radiation at a frequency of 2.45 GHz for 2 hours per day throughout their pregnancy. A far-field exposure was used with 70 cm distance of animals from the radiation source (Fig. 1). The homogeneity of the field was measured in a pre-experiment in various MR modes, and the optimal location for a purpose-designed chamber was determined. Power density measurements were

performed with a portable electromagnetic field meter (LUTRON EMF-819, Taiwan). The control animals were kept under normal conditions without exposure to radiation. After the delivery, the mothers from both groups were placed into individual cages and the pups were fed naturally by their own mothers. Randomly chosen male pups (2 males per mother) from the experimental and the control group were no longer exposed to MR and were allowed to survive until the postnatal day 35 (PND 35) when they were anaesthetized by intraperitoneal administration of chloral hydrate (CentralChem, Slovakia). Under deep anaesthesia, the animals were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer and the biological material was collected. Handling and all experimental procedures were performed in accordance with the approval of the Animal Care Ethical Committee of the Institute of Neurobiology of the Biomedical Research Centre, Slovak Academy of Sciences and the State Veterinary and Food Administration of the Slovak Republic (Ro. 2246/18-221/3).

Specific absorption rate calculation

The specific absorption rate (SAR) represents a measure of the rate at which electromagnetic energy is absorbed per unit mass by the body after exposition to the RF-EMR. It is recommended to provide SAR value at frequencies below 10 GHz (ICNIRP, 2000). SAR value was calculated according to Valberg et al. (2007), using the following formula:

Power density: p=28 W/m^2

Rat body surface area: S=0.0232 m^2

Power absorbed by the rat: P=p × S=28 × 0.0232=0.649 W

Weight of the pregnant rat: m=0.375 kg

$$SAR=\frac{P}{m}=\frac{0.649}{0.375}=1.73\ W/Kg$$

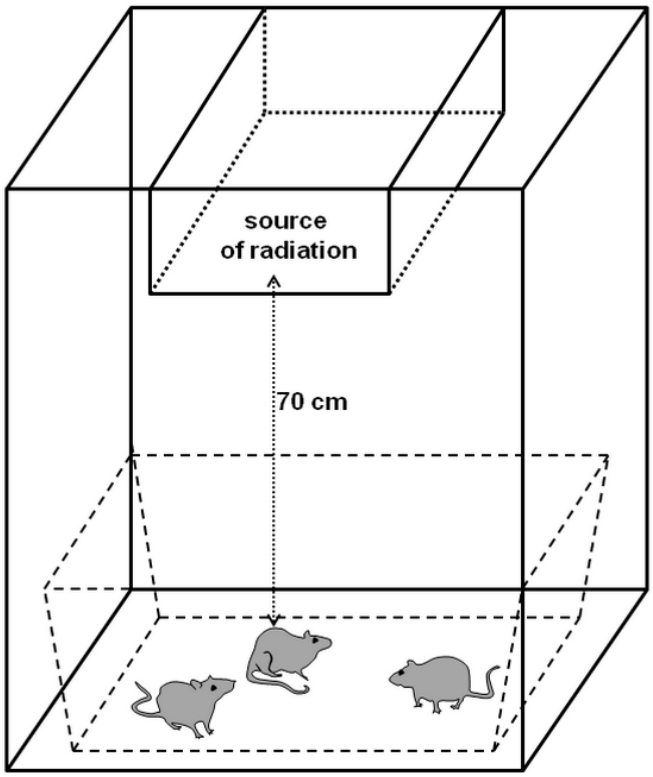

Fig. 1. Exposure chamber - scheme. Experimental rats placed in the original plastic cage with free movement to minimize their potential stress (adapted from Almasiova et al., 2021).

Tissue processing for light microscopy – LM

The testicular tissue (0.5 cm^3) from right-sided testes of each control (n=6) and each experimental (n=6) animal was processed for light microscopic examination. The samples were fixed for 24 hours in modified Davidson's Fixative solution – mDF (Latendresse et al., 2002) and processed for preparation of paraplast blocks (Sigma-Aldrich). Tissue sections approximately 5 µm thick were stained with haematoxylin and eosin (Sigma-Aldrich), examined under an Axio Lab A1 light microscope (Zeiss, Germany) and photodocumented with a digital camera (Axio Cam ERc5, Zeiss Germany). Sixty randomly selected seminiferous tubules from each testis (animal) were evaluated at 200× and 400× magnification.

Morphometry

The above mentioned tissue sections prepared for light microscopy were also used for quantitative assessment of selected testicular parameters. Data were obtained via light microscope Olympus CX 43 (Japan) and measured using the Quick-Photo Micro 3.2 image analyser software (Promicra, Czech Republic). Histomorphometric parameters were assessed at a magnification of 200×. Diameter of the seminiferous tubules and seminiferous epithelial height were evaluated in sixty randomly chosen round seminiferous tubules per testis (animal). Proportion between the tubular and interstitial area was evaluated from sixty fields per testis (animal) and expressed in volume percentage using the following formula:

$$\text{Interstitial area (\%)}=\frac{\text{total field area } (\mu m^2)-\text{tubular area } (\mu m^2)}{\text{total field area } (\mu m^2)}\times 100$$

Statistical analysis

The collected data were computerized and statistically analysed using GraphPad Prism version software 8.4.3 (GraphPad Software, San Diego, CA). The data were expressed as means±standard deviation

(SD). The significance of the differences between the control and the exposed group of animals was analysed by paired t-test. The results were considered statistically significant at $p<0.05$ and highly significant at $p<0.001$.

Transmission electron microscopy - TEM

Small samples (1 mm^3) of right-sided testis from each male pup were fixed in 3% glutaraldehyde in 0.1M cacodylate buffer (pH 7.3) immediately after collection. The specimens were post-fixed in 1% osmium tetraoxide. After dehydration, the samples were transferred to propylene oxide and embedded in Durcupan™ ACM capsules (Sigma-Aldrich, Germany). Semithin sections (1 μm thick) were prepared on glass slides and stained with toluidine blue to select the representative areas of seminiferous tubules. Subsequently, ultrathin sections (90-110 nm) were prepared by an ultramicrotome (LKB, Sweden) and double stained with 1% uranyl acetate and 0.3% lead citrate for TEM examination (Tesla BS 500, Brno, Czech Republic). The sections were photodocumented and the prints were assessed.

Fluoro - Jade C histochemical analysis

A Fluoro Jade C (FJ-C) histochemistry staining method (FJ-C; Histo-Chem Inc., AR, USA) was used to label the degenerating testicular cells. Samples of testicular tissue (0.5 cm^3) from the left-sided testis of each male rat were fixed in 4% paraformaldehyde overnight. The samples were then transferred to a cryoprotective solution containing 30% sucrose in 0.1M phosphate buffer. After 24 h the tissue was frozen and sectioned by a cryostat. Five sections per testis (animal) of thickness 30 μm were obtained and mounted on 2% gelatine coated slides. After drying (50°C for 30 min) the slides were first immersed in absolute alcohol for 3 min, then in 70% alcohol for 2 min. After rinsing in distilled water, the sections were incubated in 0.06% potassium permanganate solution for 15 min. Following a water rinse, the slides were transferred to FJ-C staining solution (1μg/ml) dissolved in 0.1% acetic acid. After 30 min of staining, the slides were rinsed with distilled water and dried overnight. After being cleared in xylene, the slides were cover-slipped with DPX mounting medium (FlukaChemie AG, Switzerland). The tissue was evaluated under a fluorescence microscope with reflected fluorescence system U-RFL-T (Olympus BX51). The images were captured at 200× magnification with a digital camera (Olympus DP71) and forty randomly selected fields (8 fields per slide) from each animal were evaluated. The FJ-C positive cells were selected based on fluorescent signal strength, and their total number was counted manually with supporting Image Tool software (Image J1.46r software, USA). The data were expressed as the mean number of positive cells per field.

Proliferating cell nuclear antigen (PCNA) immunohistochemical analysis

Proliferative activity of testicular cells was determined via one of the key proliferation markers - proliferating cell nuclear antigen (PCNA). Testicular tissue sections (6 μm) obtained from paraplast blocks previously prepared for conventional light microscopy were mounted on silanized slides, deparaffinised with xylene and rehydrated in ethanol series. This was followed by heat-mediated antigen retrieval in 10 mM sodium citrate buffer (pH 6.0). To block the endogenous peroxidase activity, the tissue sections were quenched with 3% hydrogen peroxide in methanol for at least 10 minutes. Dilution tests were performed with primary antibody to identify dilutions as appropriate. Subsequently, the sections were incubated overnight at 4°C with primary antibody for the protein of interest in a humidified chamber. The proliferating cells were labelled by marker of proliferation - PCNA antibody (anti- PCNA, Mouse Monoclonal Antibody, BioLegend, SanDiego, CA, US; Ms-106-P1; 1:100). After washing the slides with phosphate buffered saline (PBS), the related secondary antibody was applied to the slides for 1 hour at room temperature. Immunoreactivity to the protein was visualised by the reaction with diaminobenzidine as chromogen (DAB), and counterstained with Mayer's haematoxylin (GHS132, Sigma-Aldrich). Omitting the primary antibody was considered as a negative control. Five histological slides per testis (animal) were obtained and forty selected seminiferous tubules in stage VII of seminiferous epithelial cycle (8 tubules per slide) were examined in a blinded study by two independent histologists at 400× magnification. Representative microphotographs were taken using an Olympus CX 43 light microscope with an Olympus SP350 camera (Olympus, Tokyo, Japan). The cells with strong nuclear positivity (brown colour) were manually counted using Quick-Photo Micro 3.2 image analyzer software (Promicra, Czech Republic). The obtained data were calculated as the mean number of positive cells per seminiferous tubule section area. The data were expressed as the mean±standard deviation (SD).

Results

Light microscopy

Light microscopic examination of the testicular parenchyma in the control group showed a well-preserved histological structure typical for the peripubertal period. Regular seminiferous tubules were closely packed with a compact interstitial tissue in between. The basement membranes, encircled by a layer of flat myoid cells were intact and had a regular contour. The normal histological arrangement of the seminiferous epithelium was observed from the basal compartment to the lumen. There were typical Sertoli cells with their

large, pale and basally located elongated or pyramidal nuclei and prominent nucleoli wedged between the compact layers of germ cells. Most of the tubules lumens were empty, with currently absent spermatozoa. The interstitium housed groups of typical polyhedral Leydig cells with rounded vesicular nuclei and prominent nucleoli, and also well designed blood vessels (Fig. 2A,B).

On the contrary, the testicular structure in the experimental animals was obviously altered and variable degrees of alteration to seminiferous tubules were detected. Some segments of seminiferous tubules showed marked irregular outlines, and seminiferous epithelium often showed an impaired arrangement. Adversely affected epithelial segments revealed disorganization of germ cells, the occurrence of wide empty spaces between cellular components as well as a significant sectoral loss of germ cells. The desquamated immature germ cells were often accumulated in wide lumens of some seminiferous tubules. Giant multinucleated cells were rarely observed in the adluminal compartment of seminiferous epithelium in some seminiferous tubules. In addition to tubular impairment, a large expansion of the interstitial space

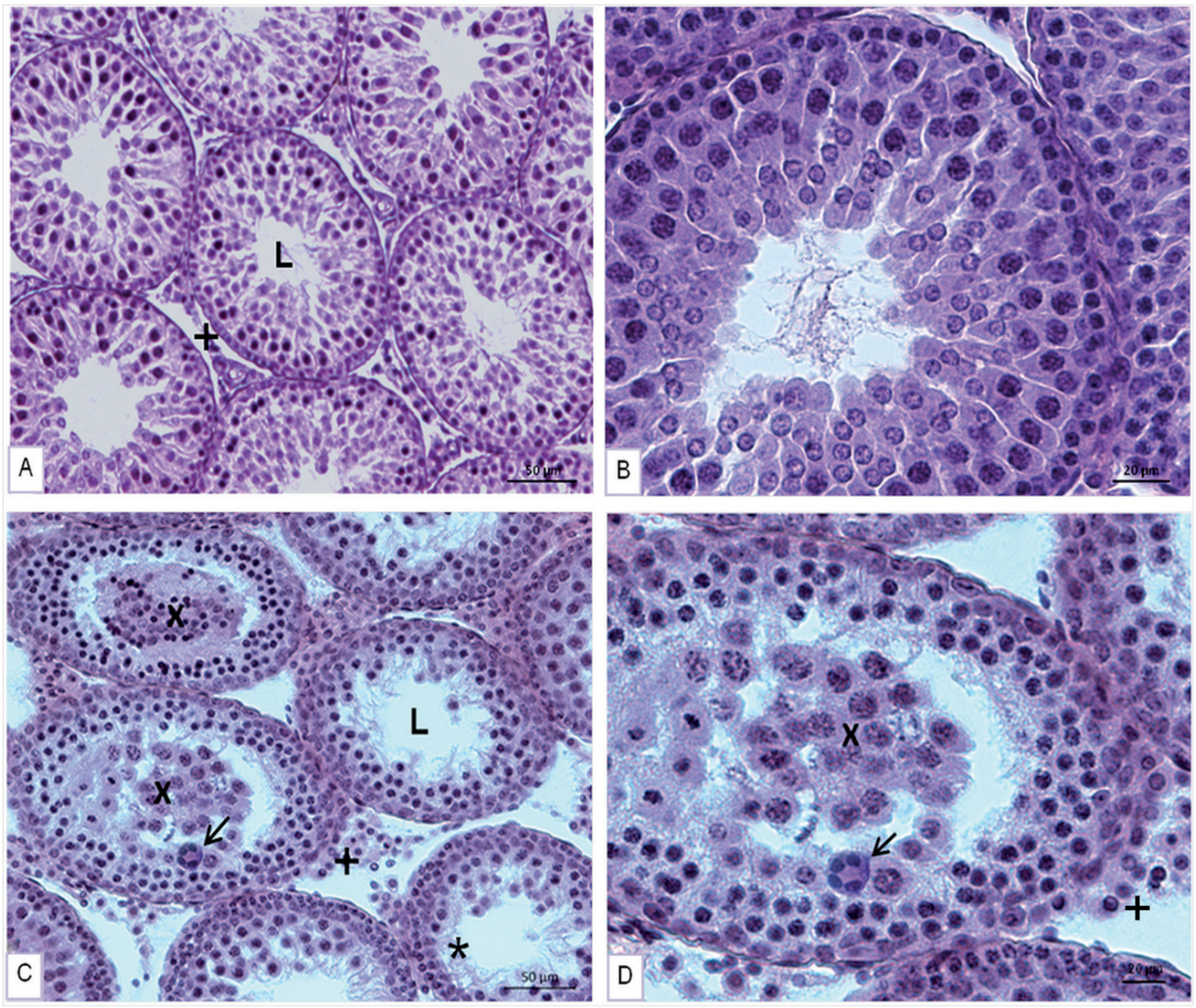

Fig. 2. Photomicrographs of testicular tissue of 35-day-old rats stained with haematoxylin and eosin (H&E). Well organized seminiferous tubules are separated by narrow interstitium (+) and show normal histological structure in the group of control rats **(A, B)**. Compromised testicular structure of the seminiferous tubules reveals focal areas of spermatogenic cell loss (*) and groups of sloughed germ cells (x) in the lumens (L) of seminiferous tubules and extended interstitial space (+) in the rats prenatally exposed to MR **(C)**. Higher magnification image shows the presence of a giant multinucleated cell in the seminiferous tubule (arrow) in MR group **(D)**. A, C, × 200; B, D, × 400.

was evident in the group of experimental animals. The structure of the interstitial Leydig cells at the light microscopy level appeared unchained. The blood vessels showed normal histological structure with no dilatations (Fig. 2C,D).

Morphometry

The morphometric analysis of some parameters of H&E stained testis sections showed a significant decrease in the diameter of seminiferous tubules ($p<0.05$) as well as reduced epithelial height ($p<0.01$) in the group of rats exposed to MR in utero. On the other hand, a significantly increased ($p<0.01$) interstitial space was observed in the testes from the MR group of rats compared to the control group, as shown in Table 1 and Fig. 3.

Transmission electron microscopy

Testicular ultrastructure of the control rats was typical and reflected the condition representative of the given age category. Seminiferous tubules had regular basement membranes ensheathed with characteristic elongated myoid cells. Sertoli cells cytoplasm revealed well-formed organelles and was rich in mitochondria and lipid droplets. The nucleus of Sertoli cells was typically indented, euchromatic, with prominent nucleolus. Type A spermatogonia possessed oval, finely granular nucleus with peripherally located stringy nucleolus. Intermediate spermatogonia had oval to round nucleus with one or two compact nucleoli and spotted heterochromatin rimming the inner nuclear membrane. The oval shaped spermatogonia of type B were more frequently observed. They owned rounded nucleus with periodically clumped heterochromatin along the inner nuclear membrane and a small, compact, centrally located nucleolus. All types of spermatogonia had characteristic cytoplasm with well organized organelles such as mitochondria, strands of granular endoplasmic reticulum, free ribosomes, and an inconspicuous Golgi complex. The primary spermatocytes appeared as large rounded cells above the spermatogonia, with large rounded nucleus containing typical synaptonemal complexes which frequently extended from the nuclear membrane. Their cytoplasm was abundant and contained well defined organelles such as free ribosomes, mitochondria and a large Golgi complex surrounded by vesicles. The secondary spermatocytes were less frequent and their nucleus was smaller than that in the primary spermatocytes. The early round spermatids were connected by intercellular bridges and created typical cellular associations. They had a large ovoid euchromatic nucleus and their cytoplasm was rich in mitochondria. The Golgi complex and formation of the acrosome was observed. The elongated late spermatids occurred only in small numbers. Their nucleus was more condensed and prolonged. The acrosome became prominent in the supranuclear portion. Occasional apoptotic germ cells exhibiting membrane blebbings and fragmented nucleus were observed. Leydig cells had rounded nucleus, prominent nucleolus, and peripherally clumped heterochromatin. Their cytoplasm contained numerous mitochondria, cisternae of smooth endoplasmic reticulum and a number of lipid droplets.

Contrary to the control animals, obvious ultrastructural alterations in both the seminiferous epithelium and interstitial components were present in the group of rats prenatally exposed to MR (Fig. 3). The basement membrane of the seminiferous tubule was thickened and irregular in some segments, and often possessed empty-like electronlucent spaces. Peritubular myoid cells had typical elongated shape and had typical nuclei, but their cytoplasm contained small electronlucent vacuoles. Sertoli cells had large, deeply indented nuclei with a homogeneous nucleoplasm and a prominent nucleolus. The cytoplasm of Sertoli cells contained altered organelles such as swollen mitochondria, enlarged cisternae of the agranular endoplasmic reticulum and more numerous lysosomes compared to the control group. In the cytoplasm of Sertoli cells there were many electronlucent vacuoles of various size as well as abundant lipid droplets. Slightly affected tight junctions were often seen between adjacent Sertoli cells. Some sections of the seminiferous epithelium contained small, irregular widenings between the germ cells and Sertoli cells. The germ cells were

Table 1. Morphometric analysis of selected testicular parameters in the control and experimental rats prenatally exposed to microwave radiation.

	Control group	Group exposed to MR
Seminiferous tubule diameter (μm)	219.7±23.8	190.3±15.4*
Seminiferous epithelium height (μm)	68.2±4.6	48.3±8.1**
Interstitial space (volume %)	21.7±2.2	34.2±3.8**

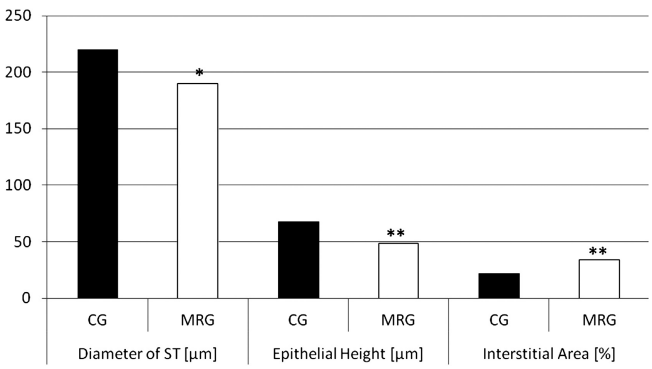

Fig. 3. Selected morphometrical testicular parameters in the control group (CG) and in utero microwave exposed group of rats (MRG). The data are expressed as means±SD; *p<0.05, **p<0.01 - significant difference compared to the control group.

regular in shape but their cytoplasm possessed numerous electronlucent vacuoles and moderately affected organelles. Nuclei of the germ cells were mostly unchanged. The necrotizing germ cells were more frequent in the group of rats in utero exposed to MR in comparison to the control group. These cells were typically shrivelled, with the pycnotic nuclei and strongly electron-dense and disintegrated cytoplasm. The interstitial space contained typical polyhedral Leydig cells with unchanged nucleus, but the cytoplasm contained numerous electronlucent vacuoles in addition to moderately swollen and less numerous mitochondria. The reduced agranular endoplasmic reticulum was distributed throughout the cytoplasm along with great abundance of lipid droplets of various sizes (Fig. 4A-C).

Assessment of cell degeneration by Fluoro - Jade C detection

FJ-C positive cells manifested by green fluorescence were observed within the seminiferous tubules of both control and experimental groups of rats. In the seminiferous tubules of the control rats there were only sporadic FJ-C positive germ cells (Fig. 5A). In the experimental group, there was observed a significant increase ($p<0.01$) in the number of degenerating FJ-C positive cells in comparison with the control (Fig. 5C). The degenerating cells, mainly spermatogonia and spermatocytes, were heterogeneously distributed within the seminiferous epithelium. Degenerations of spermatids were much less common (Fig. 5B). The interstitium did not reveal any FJ-C positivity.

Quantitative PCNA immunodetection

We examined the density of proliferating testicular cells in the seminiferous tubules (stage VII of seminiferous epithelium cycle) by means of the key proliferation marker PCNA. Clear PCNA positivity was found in the nuclei of spermatogonia and primary spermatocytes in the control group (Fig. 6A). On the contrary, the number of proliferating PCNA-labelled cells was slightly decreased in the experimental group of rats (Fig. 6B). Our results showed that the mean number of PCNA positive cells in the experimental group decreased, however only non-significantly ($p>0.05$), in comparison with the control group (Fig. 7).

Discussion

There is a considerable body of evidence from numerous high-quality studies indicating health hazards from radiofrequency electromagnetic radiation (RF-

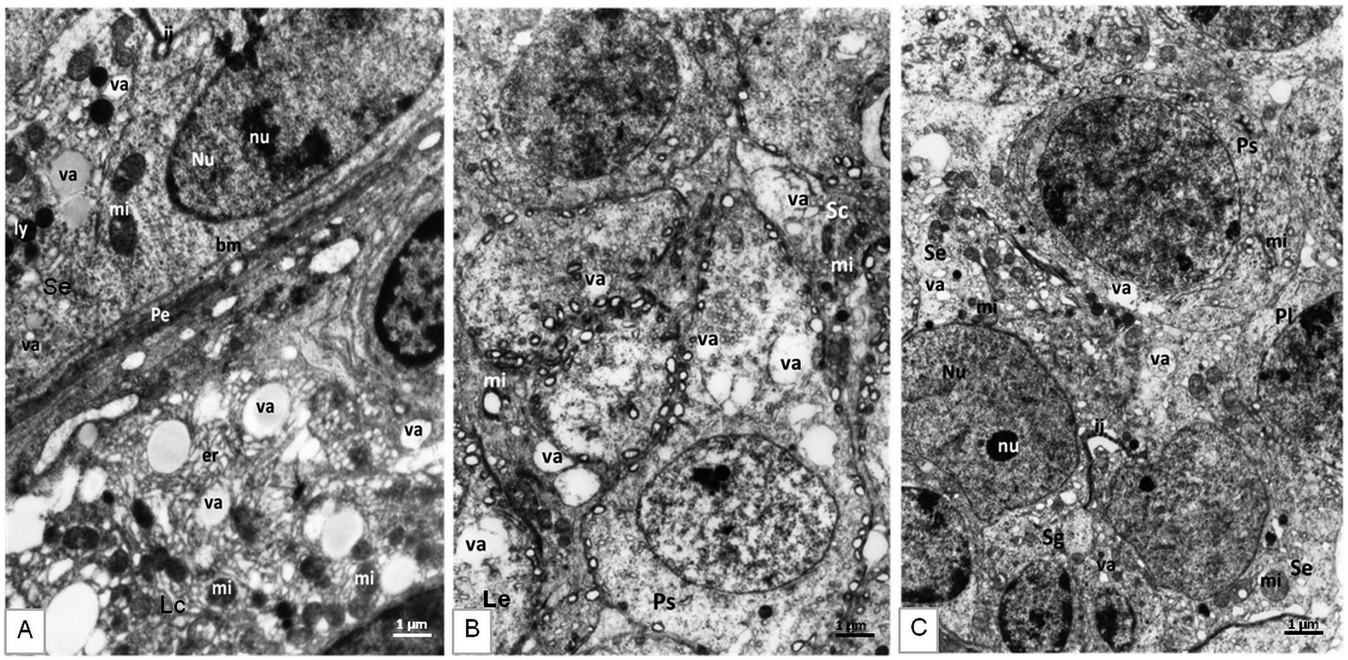

Fig. 4. Electron micrographs of sexually immature rat testis prenatally exposed to MR. The panel **A** shows the interstitium and basal compartment of the seminiferous epithelium with slightly irregular basement membrane (bm) and peritubular cells (Pe). The Leydig cells (Lc) contain vacuolated cytoplasm (va), swollen mitochondria (mi) and enlarged and irregular cisternae of agranular endoplasmic reticulum (er). The Sertoli cells (Se) show obvious cytoplasmic vacuolation (va) and contain numerous lysosomes (ly) and affected intercellular junctions (ij). The panels **B** and **C** demonstrate components of the seminiferous epithelium such as Sertoli cell (Se) with typical nucleus (Nu) and nucleolus (nu), spermatogonia (Sg), leptotene spermatocyte (Le), and a large pachytene spermatocyte (Ps). All forms of germ cell as well as somatic Sertoli cells reveal vacuolated cytoplasm (va), and swollen mitochondria (mi). Slightly interrupted intercellular junctions are present between adjacent Sertoli cells (ij). x 2,800.

EMR) emitted by many popular wireless technologies (Belpomme et al., 2018; Simkó and Mattsson, 2019). Many environmental factors including RF-EMR may seriously adversely affect various developing tissues and organs that are especially sensitive and vulnerable due to their intensive proliferation and differentiation activities (Sly and Carpenter, 2012).

The testis is a complex organ with multiple cell types all of which are coordinated to produce viable spermatozoa. A number of recent reports have suggested a possible link between widespread use of wireless devices and male fertility disorders (Derias et al., 2006; Jaffar et al., 2019; Yahyazadeh et al., 2020). Only a few reports focused on the RF-EMR and its potential adverse effect on the prenatal development of male reproductive organs (Hanci et al., 2013, Almasiova et al., 2021).

Therefore, we decided to use pregnant rats for whole-body exposure to RF-EMR for 2 hours per day throughout the pregnancy. The selected frequency of 2.45 GHz and SAR 1.73 W/kg corresponds to the long-term exposure to low doses of RF-EMR commonly emitted by the conventional Wi-Fi devices that do not exceed safety limits set by legislation (Agarwal et al., 2011; ICNIRP, 2020). We evaluated various testicular parameters in the sexually immature, 35-day-old male offspring, prenatally exposed to RF-EMR

Our histopathological evaluation at the light microscopy level showed that prenatal exposure to RF-EMR induced moderate diffuse alterations in the testicular parenchyma, manifested as enlarged interstitial spaces, specific irregularities in the shape of the seminiferous tubules and injury to germinal epithelium together with the occurrence of giant multinucleated cells and sloughed immature germ cells in the tubular lumen. Quantitative histomorphometric analysis revealed a decrease in the diameter of seminiferous tubules ($p<0.05$), significantly reduced height of the germinal epithelium ($p<0.01$) as well as a significantly enlarged interstitial space ($p<0.01$). Our results are in close agreement with the observations of other authors

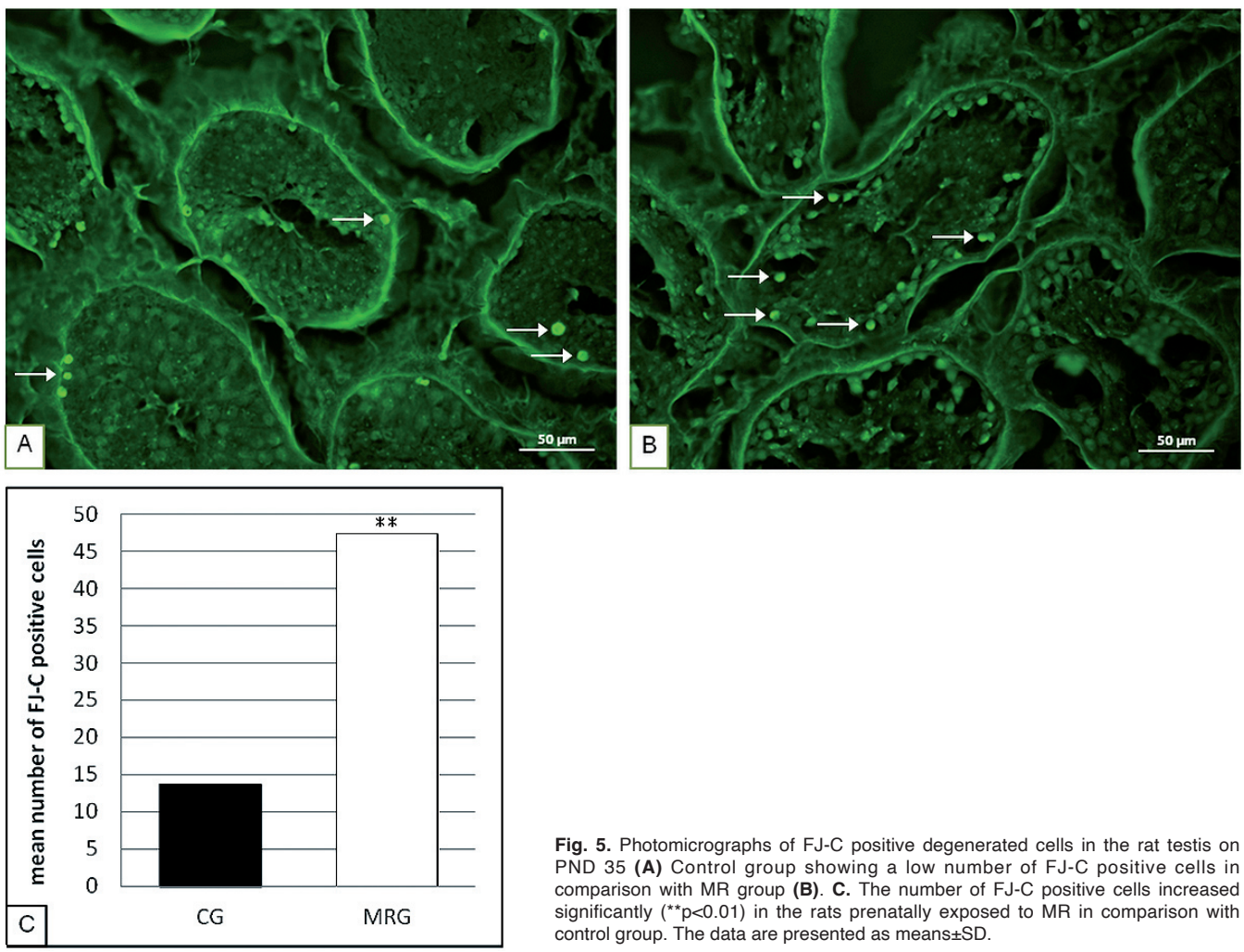

Fig. 5. Photomicrographs of FJ-C positive degenerated cells in the rat testis on PND 35 **(A)** Control group showing a low number of FJ-C positive cells in comparison with MR group **(B)**. **C.** The number of FJ-C positive cells increased significantly (**$p<0.01$) in the rats prenatally exposed to MR in comparison with control group. The data are presented as means±SD.

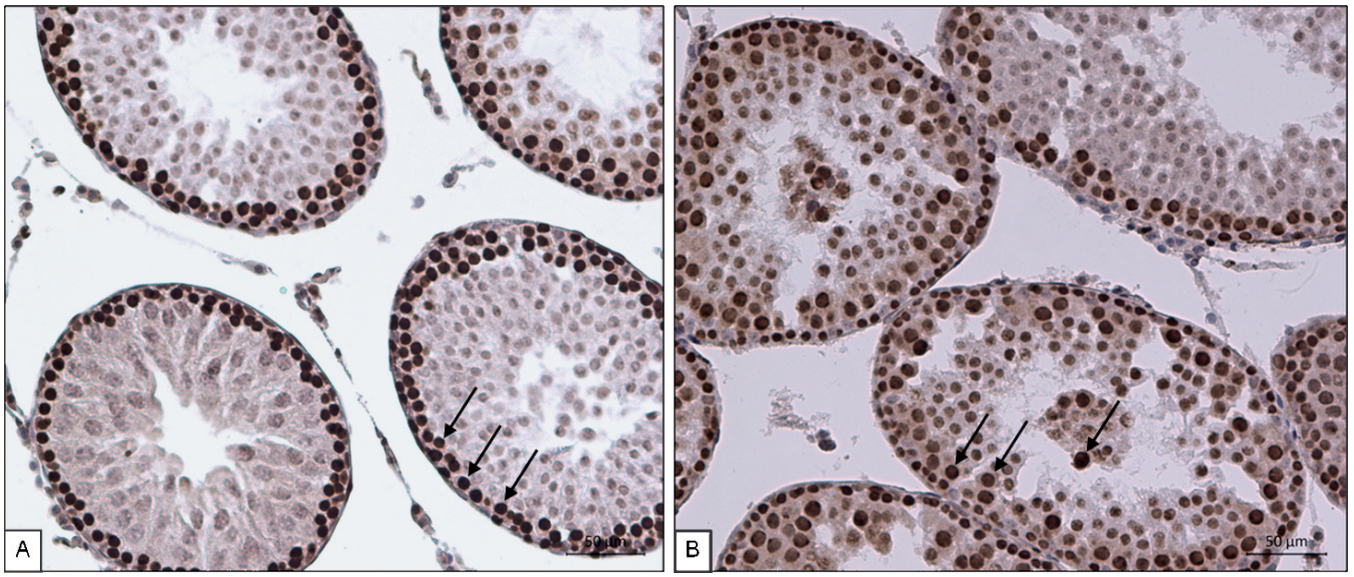

Fig. 6. PCNA immunohistochemical staining of testicular tissue. Spermatogonia and spermatocytes in the seminiferous epithelium of both the control **(A)** and experimental **(B)** rats showed a strong nuclear expression of PCNA (arrow). The number of PCNA positive cells was slightly decreased in the group of experimental rats. x 200.

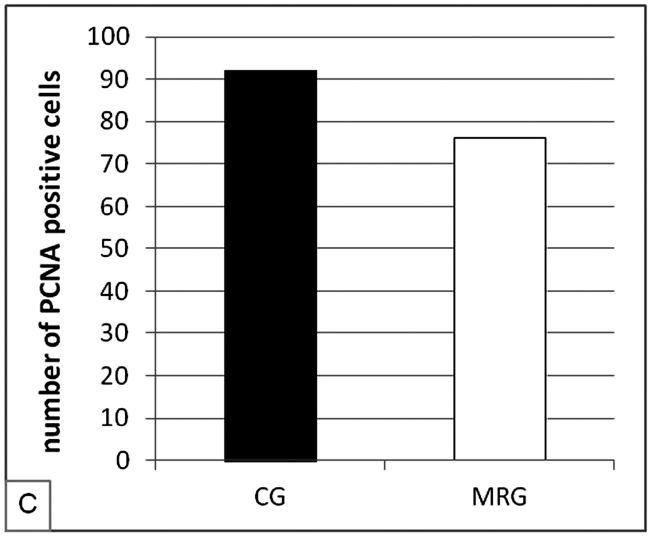

Fig. 7. The mean number of PCNA positive cells in the control (CG) and experimental (MRG) group of rats decreased insignificantly in the group of rats prenatally exposed to MR compared to the control group. The data are expressed as means±SD.

who noted similar structural changes in the testes of prenatally exposed prepubertal rats (Hanci et al., 2013; Odaci et al., 2016) or juvenile rats exposed solely after birth (Tenorio et al., 2011; Ozlem et.al., 2012; Bahaodini et al., 2015; Jonwal et al., 2018; Simaiova et al. 2019). Deceleration of spermatogenesis accompanied with various morphological changes in the testicular parenchyma of adult rats and mice exposed to MR after birth were also observed by a large number of authors (Aydin et al., 2007; Yahyazadeh and Altunkaynak, 2019; Yahyazadeh et al., 2020; Hasan et al., 2021). Our previous investigations that focused on the effect of prenatal exposure to RF-EMR on the male reproductive system of sexually mature individuals clearly demonstrated various adverse effects of RF-EMR (Almasiova et al., 2021).

Our transmission electron microscopy (TEM) evaluation proved various degenerative features in the seminiferous epithelium and interstitium constituents in the testes of prenatally exposed rats. There were observed alterations in tight junctions between the adjacent Sertoli cells accompanied with a formation of wide empty spaces within germinal epithelium and irregularities in the segmental basement membrane. Leydig cells, Sertoli cells as well as various developing germ cells revealed cytoplasmic vacuolation and adversely affected organelles. These TEM observations are in agreement with the structural alterations observed in the current study at the light microscopy level. We also noted an elevated number of necrotizing germ cells which were identified by pycnotic nuclei, and the disintegrated, strongly electrondense cytoplasm in the group of in utero exposed animals. Similar RF-EMR related ultrastructural changes, such as vacuolation of cytoplasm of Sertoli cells and blood-testis barrier disruption, were noted in our previous study focused on in utero exposed adult rats (Almasiova et al., 2021) as well as in studies dealing with prepubertal or adult rats exposed to RF-EMR postnatally (Khillare and Behari, 1998; Wang et al., 2008; Celik et al., 2012; Almasiova et

al., 2014; Gao et al., 2016; Simaiova et al., 2019).

An extensive loss of germ cells during the early testicular development is a frequently observed physiological phenomenon, and up to 75% of developing spermatogenic elements undergo apoptosis in various mammals, including rats (Maheshwari et al., 2012). Increased apoptosis during the first wave of spermatogenesis plays an important regulatory role in establishing spermatogenesis in the mature testis (Jahnukainen et al., 2004; Shaha et al., 2010). Apoptosis further occurs during the complete process of spermatogenesis and is essential for the elimination of excess of germ cells (Shaha et al., 2010). Enhancement of the degenerative processes in testicular tissue, which is often associated with subsequent necrotization of developing germ cells, which we recorded in a group of prenatally exposed rats at the level of light and electron microscopy, was also quantitatively evaluated by the histochemical method FJ-C. In the group of exposed animals, we found significant ($p<0.01$) increase in FJ-C positivity of immature germ cells undergoing degeneration and necrotization compared to the control group of animals. This is in close agreement with our previous study conducted on adult rats which were prenatally exposed to RF-EMR (Almasiova et al., 2021) as well as with the results of authors who evaluated RF-EMR and its impact on testicular germ cells apoptosis in prenatally exposed juvenile rats (Hanci et al., 2013), in utero exposed adult rats (Odaci et al., 2016) or adult rats exposed postnatally (Shokri et al., 2015; Jaffar et al., 2019).

Under physiological conditions, substantial spermatogenesis in most mammals is highly dependent on the balance between controlling mechanisms providing germ cells proliferation and apoptosis. There are numerous cell divisions of spermatogonia and two divisions of spermatocytes that produce numerous germ cell populations for normal maintenance of successful spermatogenesis. This high proliferation activity makes testicular tissue considerably susceptible to various external harmful factors. We evaluated the density of proliferating germ cells through the key cell cycle regulatory marker - PCNA, and slight suppression in proliferative activity ($p>0.05$, non-significant) of spermatogonia and spermatocytes was detected in prenatally exposed rats. This allows us to state that in addition to the increased necrotization of spermatogenic cells, also slight decrement in their proliferation activity may markedly contribute to impaired spermatozoa production. An adverse effect of RF-EMR on spermatogenesis through the suppressed proliferation and differentiation was similarly noted by several authors (Moon et al., 2006; El-Kott and Bin-Meferij, 2015).

The exact cellular pathways mediating the adverse effect of RF-EMR on testicular development and future spermatogenesis are still under investigation and further studies are still required. Many authors suggested that the most likely mechanism of RF-EMR associated testicular tissue injury is oxidative damage (Valko et al., 2006; Höytö et al., 2008; Desai et al., 2009; Tök et al., 2014; Yakymenko et al. 2016). This is in close agreement with our observation of the impaired testicular structure, ultrastructure, and regression of proliferative capacity of spermatogenic elements, accompanied by enhanced degeneration. Given the chosen methodology and the calculated SAR value, we have assumed that the primary source of testicular tissue damage was the non-thermal effect of RF-EMR. However, due to the impossibility of monitoring the hot spot phenomenon in the developing testes of rat foetuses, we cannot exclude the thermal effect.

In conclusion, our data attest to the harmful impact of 2.45 GHz microwave radiation on development of testes during the prenatal period which subsequently compromises the spermatogenic cycle and male reproductive function in the postnatal period of life. To preserve the steady reproductive health, it would be necessary to avoid or minimize exposure to RF-MR, especially in the most vulnerable period of intrauterine development of the individual.

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