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Scientific paper

HISTOLOGICAL AND CYTOLOGICAL EXAMINATION OF RAT REPRODUCTIVE TISSUE AFTER SHORT-TIME INTERMITTENT RADIOFREQUENCY EXPOSURE

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The unfavourable outcomes of mobile phone use on male fertility have still not been fully elaborated. To establish the potentially adverse effects of everyday exposure to radiofrequency radiation (RF) on humans, we performed a controlled animal study that aimed to investigate the influence of RF radiation on rat testis histology as well as the amount, mobility, and structure of epididymal free sperm cell population. Eighteen adult male rats were divided into two groups of nine. One group comprised sham-exposed control animals, while the other group endured total body irradiation for an hour daily during two weeks. A 915 MHz RF field, power density of 2.4 W m^{-2} and strength of 30 V m^{-1} was generated in a Gigahertz Transversal Electromagnetic chamber. The specific absorption rate (SAR) was 0.6 W kg^{-1}. Body mass and temperature were measured before and after each exposure treatment. Immediately after the last exposure, the animals were sacrificed and testes removed and prepared for histological analysis. The free sperm cells were collected from the cauda epididymis and their quantity, quality, and morphology were microscopically determined using a haemocytometer. No statistically significant alteration in any of the endpoints was observed. This study found no evidence of an unfavourable effect of the applied RF radiation on testicular function or structure. Based on these results, we can conclude that short-time intermittent exposure to RF radiation does not represent a significant risk factor for rat reproductive functions.

KEY WORDS: *915 MHz, mobile phone frequency, sperm cell, testes*

Recently, serious attention has been devoted to the possible unfavourable health effects of radiofrequency (RF) fields, which are increasingly being used in telecommunications. The potential hazard of RF microwave radiation on cellular phone users has been of particular interest. The Global System for Mobile Communications (GSM) mostly operates on either the 900 MHz or the 1800 MHz band (1). Apart from the most common health problems in users of mobile phones such as memory impairment, headaches, sleep disturbance, depression, tiredness, neuro-endocrine dysfunction, and brain tumours, miscarriages and semen quality impairments have also been detected (2-4). The unfavourable outcomes of mobile phone use on male fertility have still not been fully established, as studies have revealed a wide spectrum of possible effects, ranging from insignificant ones to inconsistent degrees of testicular damage (5). It should be stressed that findings from controlled laboratory studies are often controversial, unrepeatable or inconclusive. As for the consequences of cellular phone exposure on testes, there are several reports of radiation effects on testicular tissue. Several groups of investigators reported no effect of cell phone use

on rat testicular function (6-9). In their case control study, Hardell et al. (10) did not find any correlation between RF radiation from cell phones and testicular cancer in humans. Contrary to this, Akdag (11) found that sperm counts lowered and sperm morphology and weight changed following chronic microwave exposure. It has also been suggested that spermatozoa and male fertility can be affected if a cell phone is repeatedly kept in talk mode near the reproductive organs (e.g., in one's pocket) (12). Yan et al. (13) provided almost an identical conclusion after finding a significantly higher incidence of sperm cell death and abnormal sperm cell clumping in rats exposed to cellular phone emissions.

Given the conflicting data reported by the relevant literature, our study attempted to investigate the influence of RF radiation derived from conventional cellular phones on the histology of rat testes and the amount, mobility, and structure of epididymal free sperm cells.

MATERIALS AND METHODS

Experimental animals

The study included eighteen adult male Wistar rats (12-week-old, approximately 350 g in weight). The animals were divided in two groups of nine. One group endured total body irradiation in the Gigahertz Transversal Electromagnetic (GTEM) chamber, while the other group consisted of sham-exposed control animals kept under the same conditions but without being irradiated.

The rats were bred at the Institute for Medical Research and Occupational Health, Zagreb, Croatia, in accordance with the Animal Protection Act. The study was approved by the Institute's Ethics Committee. The animals were kept in steady-state micro-environmental conditions [(22±1) °C, 50 % to 70 % humidity] and received standard laboratory food and water *ad libitum* (4RF21 GLP, Mucedola srl, Milano, Italy). The rats were kept in alternating 12-hour light and dark cycles.

Experimental design and exposure conditions

In order to generate a uniform RF field, a GTEM cell (Mod. 5402, ETS Lindgren, USA) was used. The GTEM cell is a pyramidal tapered with outer dimensions of 1.4 m x 0.75 m x 0.5 m and a dual-terminated section of a 50 Ω transmission line. The cell is flared to create a test volume within which the object under experiment is placed. At the input, a normal 50 Ω coaxial line is physically transformed to a rectangular cross section with an aspect ratio of 3:2 horizontal to vertical. The central conductor, known as the septum, is a flat, wide conductor which, when driven by a signal generator, produces a sized region of a nominally uniform electric field distribution beneath itself. This region of the nominally uniform field is the test volume for testing (14). The electromagnetic field strength was measured inside the GTEM cell, as was its homogenicity by means of graphical EMF readout (HI 4460, Holaday Instruments) and electric field probe (HI 6005, Holaday Instruments, US).

A 915 MHz signal was generated by the Antritsu 27211A generator (Japan), while GSM basic modulation was obtained by means of a RF 2722 Polaris chip signal modulator (RF Micro Devices, Greensboro, USA). To amplify the signal, an RF 3146 Power Amp Module amplifier (RF Micro Devices, Greensboro, USA) was used.

Throughout the experiment, animals were exposed to a 915 MHz RF field, power density of 2.4 W m^{-2} and strength of 30 V m^{-1}. The temperature within the irradiation set-up was kept at room level. Each animal was placed in its own Plexiglas cage, specially designed to fit into the GTEM cell, the dimensions of which were 25 cm long, 7.5 cm high, and 7.5 cm wide. Three Plexiglas cages with one animal at a time were placed into the GTEM chamber. The animals were total-body irradiated in the GTEM chamber for an hour per day during two weeks. The specific absorption rate (SAR) was calculated to be 0.6 W kg^{-1} (15).

Body weight and temperature were measured before and after each exposure. Body temperature was measured using a thermoscan thermometer (IRT 3020, type 6012, Braun GmbH, Kronberg Germany).

Tissue collection, histology, and sperm morphology assay

Immediately after the last irradiation session, the rats were euthanized under combined Narketan/Xylapan anaesthesia (Narketan®, 80 mg kg^{-1} b.m. + Xylapan®, 12 mg kg^{-1} b.m., *i.p.*, Vétoquinol, Bern, Switzerland).

The testes were removed, cleaned of accessory tissue, weighted, and fixed. The tissue samples were embedded in paraffin and cross-sections were stained with hematoxylin-eosin stain. Histological sections

were evaluated with a light microscope at 1000x magnification.

Spermatogenesis was determined by the semi-quantitative testicular score count in 100 cross-sections in each animal at the same magnification and summed up according to the mean Johnsen (MJS) testicular biopsy score. For the testicular biopsy, the scoring system was standardized ranging from 1 (no seminiferous epithelium) to 10 (full spermatogenesis recorded) (16, 17).

Cauda epididymis was cut into small pieces, transferred into Petri dishes and flushed with a pre-warmed nutrition medium (RPMI 1640, Sigma Chemicals Ohio, USA). The sperm cells were allowed to swim for five minutes at 37 °C. The free sperm cells were collected and their quantity, quality, and morphology were determined using a Neubauer-type haemocytometer under a light microscope.

Immediately after the tissue isolation, sperm motility was evaluated. The ratio of motile sperms was calculated based on the number of motile sperm cells divided by the total number of sperm cells found in a sample. Sperm cells that were not moving at all were considered immotile, while others were considered motile. The results were expressed as total numbers of sperm cells per millilitre of solution.

To distinguish normal from abnormal sperm morphology, two haematoxylin-eosin stained slides were prepared for each rat. Sperm cells that lacked hook-shaped heads (headless), broken, or had their tails bent toward the head were considered abnormal. Two hundred sperm cells per slide were examined and morphologically evaluated under a light microscope at 400-fold magnification.

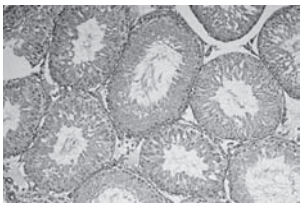

Figure 1 *Photomicrograph of rat testes tissue in sham-control animal group (magnification x1000, hematoxylin-eosin stained)*

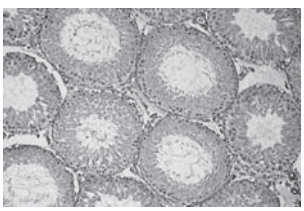

Figure 2 *Photomicrograph of rat testes tissue in RF exposed animal group (magnification x1000, hematoxylin-eosin stained)*

Statistical analysis

Statistical analyses were completed using STATISTICA software version 9.0 (StatSoft, USA). The data were evaluated using non-parametric Mann-Whitney *U*-test. The level of statistical significance was set at $p<0.05$. The Mann–Whitney *U*-test was used because it is a non-parametric test recommended for small sample sizes and because it assumes that cell samples do not follow any specific parameterised distributions, as well as that they have the same shape and are independent of each other.

RESULTS

Results, presented as mean value and standard deviation of nine rats per group, including animal body and testis weight, testis histology score count, body temperature before and after 915 MHz RF radiation, in addition to the epididymal sperm cell count, sperm motility ratio together with their absolute value, and morphology in the exposed and shame-exposed rats are given in Table 1.

In comparison with sham controls, non-significant decrease in body weight was noted in RF exposed animals, rectal body temperature after exposure differed at a biologically irrelevant level (0.3 °C). Although the slight decrease in testis weight in irradiated animals was noted, it was not considered biologically relevant.

Spermatogenesis was determined by testicular biopsy score count and the results of the histopathological study of testicular tissue showed a normally developed histological image in the irradiated group of animals in comparison to the sham-exposed controls. The exposed group exhibited complete spermatogenesis, numerous spermatozoa, a germinal epithelium of regular height, and a tubular lumen of normal diameter. (Figures 1 and 2).

Statistical analyses revealed that there were no significant differences in the obtained quantitative or qualitative parameters between the irradiated and sham-exposed rats. Sperm count, motility, and shape were not affected to a higher extent.

This paper evaluated testicular and epididymal weight, and epididymal sperm count. Maturation phase spermatid retention at stage IX-X, interstitial

Table 1 *Descriptive parameters of body and testes mass, testes histology score count (MJS), body temperature, epididymal sperm count, sperm motility ratio together with their absolute value and morphology in the 915 MHz exposed and control rats*

Animal group	Irradiation	Control rats (*n*=9) x±SD	Exposed rats (*n*=9) x±SD	Significance (*p*)
Body mass / g	First day	370.3±24.9	372.1±31.9	>0.05
	Last day	376.9±24.5	375.8±32.2	>0.05
Body temperature / °C	First day	37.9±0.5	37.7±0.6	>0.05
	Last day	38.0±0.6	38.0±0.6	>0.05
Testes mass / g		2.1±0.1	2.2±0.2	>0.05
Testes histology score count (MJS)		9.5±0.4	9.4±0.4	>0.05
Sperm count / $x10^6$ mL^{-1}	Total	245.0±46.4	238.0±31.0	>0.05
	Motile	185.6±37.0	159.2±21.5	>0.05
	Normally shaped	219.0±8.4	211.6±7.5	>0.05
	Irregularly shaped	26.0±8.4	26.4±7.5	>0.05
Sperm motility ratio / %		75.7	66.9	>0.05

infiltration, cellular vacuolation and multinucleate giant cells were among the qualitative testicular histopathological endpoints analysed. Each rat had ten consecutive round seminiferous tubules at stage VII-VIII evaluated for the mean seminiferous tubular diameter measurement, the crude histological count of round spermatids, pachytene spermatocytes and Sertoli's cells with evident nucleoli, and true histological count (Abercrombie's correction factor) of round spermatids and pachytene spermatocytes.

DISCUSSION

The findings of the present study suggest that total-body short-time intermittent irradiation of male rats with a 915 MHz RF field for an hour per day during two weeks did not result in significant disturbances of testicular function or structure. As the exposure conditions investigated here are within the range of the RF to which humans are exposed on a daily basis due to frequent cellular phone use, this study suggests that this RF does not present a significant risk to male reproductive function.

As for data on humans, several reports point to unfavourable effects of RF exposure on male fertility (18, 19), while others contradict them (20). One study on humans (21) suggested that the duration of phone possession and daily transmission time correlated negatively with the proportion of rapid progressive motile sperm and positively with the proportion of slow progressive motile sperm. Furthermore, acute adverse effects on human sperm motility in the progressive, slow progressive, and no-motility categories of sperm movement after RF radiation emitted by an activated 900 MHz cellular phone have also been observed (22). In an analysis of mobile phone frequency radiation on human semen, an increase in the percentage of sperm cells of abnormal morphology was associated with the duration of exposure (23). The decrease in the percentage of sperm cells in vital progressing motility in the semen was correlated with the frequency of using mobile phones (23). Semen quality in men using cell phones for two, four, and more than four hours a day has been found to seriously diminish, since sperm count, motility, viability, and morphology decreased (18). The decrease in sperm parameters depended on the duration of daily exposure and was independent of initial semen quality (5). De Iuliis et al. (24) found not only a decline in the motility and vitality of human spermatozoa exposed to RF radiation, but also DNA fragmentation.

Studies on RF exposure are also frequently conducted on rats. One such study on rats exposed to cellular phone radiation for six hours a day over 18 weeks, showed a significantly higher incidence of sperm cell death and abnormal sperm cell clumping (13). Irreversible destructive changes, both in the seminiferous tubules and testicular tissue, along with high sensitivity of rat testes and epididymis to applied radiation (3 GHz frequency, 0.25 mW cm^{-2}), that lasted 2 h a day for 4 months, have been demonstrated (25).

Otherwise, no evidence of an adverse effect of cell phone exposure on rats' testicular function or structure, to be precise; on testis mass, epididymis, and seminal vesicles; daily sperm production per testis and per gram of testis, sperm morphology and the number of epididymal sperm of pulse-modulated microwave radiation (1.3 GHz, 6.3 mW g^{-1}) were determined in a study on rats exposed to cell phone frequency for 20 min per day, 7 days a week for a month (6). Also, Ribiero et al., (9) found that low-intensity pulsed RF does not impair testicular function in adult rats, when they are exposed to RF emitted from a conventional GSM cellular telephone (1.835 MHz to 1.850 MHz) for one hour per day over eleven weeks. Moreover, the results of yet another study (7) showed that a 2 h per day, 7 day a week, exposure of rats to 900 MHz radiation over ten months did not activate testes caspase-3, a well-known feature of typical apoptosis. Ogawa et al. (26) and Sommer et al. (27) studied four generations of exposed rats and found no negative effect. Lee et al. (28) also did not find any effects on rat spermatogenesis after simultaneously combined Code Division Multiple Access (CDMA) and Wideband Code Division Multiple Access (WCDMA) exposure. It is evident that findings on adverse power of mobile phones on male fertility are still controversial. Our paper therefore aimed to evaluate the influence of RF radiation on rat testis histology and the amount, mobility and structure of epididymal free sperm population. Under the given experimental conditions, no significant evidence of an unfavourable effect of applied radiation on rat testicular function or structure was established. Based on these results we can conclude that short-time intermittent exposure to RF radiation does not represent a significant risk factor to male rat reproductive function. However, the potential effects of long-term exposure, which is evident in some cell phone users, should be further investigated using a broader range of frequencies and exposure scenarios.

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Sažetak

HISTOLOŠKA I CITOLOŠKA ISTRAŽIVANJA TKIVA REPRODUKTIVNOG SUSTAVA ŠTAKORA NAKON KRATKOTRAJNE ISPREKIDANE IZLOŽENOSTI RADIOFREKVENCIJSKOM ZRAČENJU

Svrha ovog istraživanja bila je ispitati utjecaj radiofrekvencijskog zračenja (RF) na histologiju testisa štakora i slobodnu populaciju epididimalnih spermija. Osamnaest odraslih štakora (12 tjedana starosti, približne tjelesne mase 350 g) podijeljeno je u dvije skupine po devet životinja. Unutar gigahercne transverzalne elektromagnetske komore (GTEM) stvoreno je RF polje frekvencije 915 MHz, gustoće snage 2,4 W m^{-2} i snage polja 30 V m^{-1}. Jedna skupina životinja (n=9) bila je dva tjedna zračena u GTEM komori jedan sat na dan. Specifična brzina apsorpcije (SAR) iznosila je 0,6 W kg^{-1}. Tjelesna masa i temperatura bile su mjerene prije i nakon svakog ozračivanja. Odmah nakon posljednjeg izlaganja životinje su žrtvovane, testisi su uklonjeni, očišćeni od okolnog tkiva i fiksirani. Uzorci tkiva bili su uklopljeni u parafin, presjeci obojeni hematoksilin-eozinskom bojom. Histološki su preparati analizirani pomoću svjetlosnog mikroskopa. Cauda epididimis isprana je toplim hranjivim medijem. Prikupljene su slobodne stanice, spermiji, te im je hemocitometrom određena količina, kakvoća i morfologija. U usporedbi s kontrolnom skupinom, ni jedan praćeni parametar nije statistički značajno odstupao u ozračenoj skupini životinja. Rezultati su pokazali da primijenjeno RF zračenje frekvencije 915 MHZ nije utjecalo na funkciju i strukturu testisa u štakora.

KLJUČNE RIJEČI: *915 MHz, frekvencija mobilnih telefona, spermiji, štakori, testisi*

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