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Immunohistopathologic demonstration of deleterious effects on growing rat testes of radiofrequency waves emitted from conventional Wi-Fi devices

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Abstract *Objective:* To investigate effects on rat testes of radiofrequency radiation emitted from indoor Wi-Fi Internet access devices using 802.11.g wireless standards.
Methods: Ten Wistar albino male rats were divided into experimental and control groups, with five rats per group. Standard wireless gateways communicating at 2.437 GHz were used as radiofrequency wave sources. The experimental group was exposed to radiofrequency energy for 24 h a day for 20 weeks. The rats were sacrificed at the end of the study. Intracardiac blood was sampled for serum 8-hydroxy-2′-deoxyguanosine levels. Testes were removed and examined histologically and immunohistochemically. Testis tissues were analyzed for malondialdehyde levels and prooxidant—antioxidant enzyme activities.
Results: We observed significant increases in serum 8-hydroxy-2′-deoxyguanosine levels and 8-hydroxyguanosine staining in the testes of the experimental group indicating DNA damage due to exposure ($p < 0.05$). We also found decreased levels of catalase and glutathione peroxidase activity in the experimental group, which may have been due to radiofrequency effects on enzyme activity ($p < 0.05$).

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Conclusions: These findings raise questions about the safety of radiofrequency exposure from Wi-Fi Internet access devices for growing organisms of reproductive age, with a potential effect on both fertility and the integrity of germ cells.

Introduction

There has been continuing public anxiety about the potential health consequences of wireless communication tools using radiofrequency (RF), despite authorities on the subject having set safety boundaries to protect the community against RF exposure [1—3]. Therefore, the World Health Organization advised research in this area. Animal studies involving rats have been conducted to analyze the effects of Wi-Fi signals on health parameters and stress markers [4]. The environmental wireless 802.11.g device (also called Wi-Fi device, or wireless internet access device or WIAD) has in general higher frequency ranges and longer exposure times than wireless phones [5]. Thus, the level of health hazards associated with Wi-Fi devices might be different from, and possibly higher than, mobile phones (MB). Additionally, Wi-Fi devices commonly expose the whole body to RF, unlike MB which generally irradiate some parts of the body, e.g. cranium, more than others.

Exposure of animals to RF electromagnetic radiation (EMR) may lead to a variety of changes in tissues. The observed changes vary depending on the wireless exposure characteristics, species studied, and histological methodology used for the detection of effects [6,7]. Human beings have also been shown to be adversely affected by prolonged and repeated exposure to RF EMR [8,9]. One adverse effect is on the male reproductive system. Both the number and fertilizing capacity of mouse and human sperm were shown to be decreased after RF exposure [10,11]. Another important issue about the effects of RF EMR is induction of carcinogenesis [12—14]. The mechanism underlying the induction of infertility and carcinogenesis by RF EMR emitted from wireless devices appears to be DNA damage due to oxidative stress (OS) [15,16]. DNA oxidation could occur when guanine, the most electron-rich DNA base, is subjected to the oxidizing influence of RF waves [17]. The purpose of this study was to demonstrate, by measuring serum 8-hydroxy-2′-deoxyguanosine (8OHdG) and testis tissue 8-hydroxyguanosine immunohistochemistry, whether the commonly used indoor WIADs cause DNA damage due to OS, and could be a contributing factor for infertility and the induction of carcinogenesis in rat testes.

Materials and methods

Subjects and animal care

The local ethics committee for the use of laboratory animals approved the study. Ten 8-week-old inbred healthy male Wistar albino rats, weighing 215—285 g, were used in the experiment. The rats were housed in acrylic-glass cages, 20 × 30 × 40 cm in size. The rats were separated into two groups of five: one experimental group and one control group. They were kept in well-ventilated cages and rooms, not restrained. The experimental group was exposed to WIAD RF of 2437 megahertz (MHz), while the control group was shielded against RF radiation.

Wireless Internet access device — whole body exposure

Two commercially available wireless ADSL 2 + gateways (USR9108 MAXg, US), operating at 802.11.g standards, were used in this study. The output power was 95 mW, average, as equivalent isotropically radiated output power. The maximum specific absorption rate (SAR) of the wireless gateways in the conformity assessment test was 0.091 W/kg.

A network storage link was attached to each wireless device, by which a 250 GB capacity Universal Serial Bus hard disk was connected. Gateways were wirelessly bridged to each other. Rats' cages were placed in between the gateways so that the distance between the cages and the gateways was 25 cm, as suggested by the regulatory information in the producer's user guide according to the FCC (Federal Communications Commission) Radiation Exposure Statement [18]. Data transmission was performed after each hard disk was assigned as a network drive. Exposure was continued for 24 h a day for 20 weeks (Fig. 1).

At the end of the 20 weeks all rats were sacrificed by decapitation under anesthesia and intracardiac blood samples were obtained. Sera were separated by

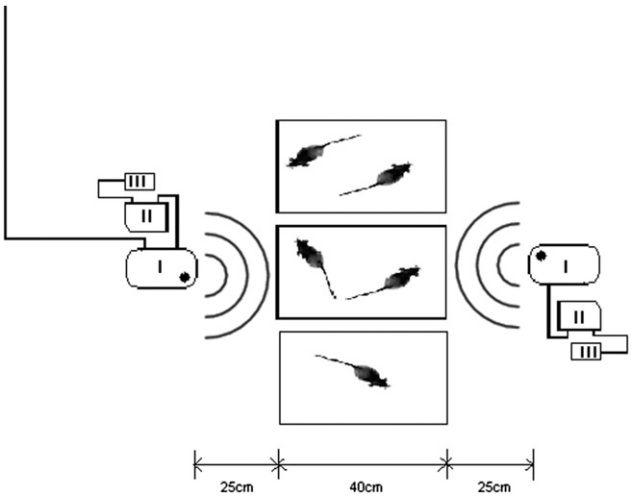

Figure 1 Experimental design of the study: (I) hard disk, (II) network storage link, (III) wireless gateway.

centrifugation (at 1250 g for 15 min at 25 °C) and stored at −80 °C. Testes were removed, one of the testes snap-frozen in liquid nitrogen (−196 °C) and stored at −80 °C until biochemical analyses, and other testis tissues fixed in a solution of 10% formaldehyde for histopathologic examination.

Determination of 8-hydroxy-2′-deoxyguanosine serum levels

8OHdG was measured with the highly sensitive 8OHdG competitive sandwich enzyme immunoassay kit (Catalog No. 839320, Cayman Chemical Co., MI, USA). The test principle is based on competition between 8OHdG and 8OHdG acetylcholinesterase conjugate via 8OHdG monoclonal antibodies. Plates were read by a Bio-Rad Benchmark Plus microplate reader at 420 nm (Bio-Rad Laboratories Inc., CA, USA). Results were expressed in nanograms per milliliter.

Histologic examination of testes

After fixation, the testes were embedded in paraffin, sectioned at 4 μm, deparaffinized, and stained with hematoxylin & eosin. The testicular tissues were evaluated histopathologically by using Johnsen's testicular biopsy score (TBS) count [19] (Table 1).

8-Hydroxyguanosine immunohistochemistry of testes

Tissue sections from every paraffin block were immunostained with antibodies for 8-hydroxyguanosine, which were mouse-raised monoclonal antibodies (ab62623; 1 mcg/mL) obtained from *AbCAM*. Nuclear or perinuclear cytoplasmic staining was considered as 'positive'. Intensity was graded as 0 (no staining), 1 (weak staining), 2 (strong staining) or 3 (very strong staining). On each slide, the intensity of staining in 10 seminiferous tubules was counted and averaged. The modified immunohistochemical histologic score (HSCORE) was used for semiquantitative estimation of 8OHdG antibody staining of the testicular tissue. This is a score used for the evaluation of intensity and percentage of cells that stain at each intensity [20]. The HSCORE is formularized mathematically as follows:

$$\text{HSCORE} = \sum(\text{Pi} \times I/100)$$

where Pi is the percentage of stained cells, ranging from 1% to 100%, and I is the intensity of staining, ranging from 0 to 3. Staining intensities were calculated as shown in Table 2.

Table 1 Evaluation of testicular biopsy score (TBS) count [19].

Score	Description
1	No cells in tubular section
2	No germ cells, but Sertoli cells present
3	Spermatogonia are the only germ cells present
4	Only a few spermatocytes (<5), but no spermatids or spermatozoa present
5	No spermatozoa or spermatids, but many spermatocytes present
6	No spermatozoa and only a few spermatids present (<5–10)
7	No spermatozoa, but many spermatids present
8	Only a few spermatozoa present (<5–10)
9	Much spermatogenesis, but germinal epithelium disorganized with marked sloughing or obliteration of lumen
10	Complete spermatogenesis with many spermatozoa

Biochemical analysis of prooxidant–antioxidant parameters of testis tissues

The tissues were removed from the freezer (−80 °C) and disaggregated thoroughly with physiological saline (1 g in 5 mL) in a homogeniser (Heidolph Diax 900, Germany). After centrifugation at 4000 g for 20 min (Harrier 18/80 MSB080.CR2.K, UK), the supernatants were removed to be used in the analyses.

Malondialdehyde (MDA) level was measured by the thiobarbituric acid reactive substances method. MDA reacts with thiobarbituric acid to form a colored complex that has maximum absorbance at 532 nm. Xanthine oxidase (XO) activity was determined by measuring uric acid formation from xanthine at 293 nm. Superoxide dismutase (SOD) activity was measured by a method based on the nitroblue tetrazolium reduction rate. Catalase (CAT) activity was determined by measuring decrease in absorbance of hydrogen peroxide (H_2O_2) at 240 nm. Glutathione peroxidase (GPX) activity was measured by following changes in NADPH absorbance at 340 nm. In the activity calculations, extinction coefficients of H_2O_2, NADPH and uric acid were used for CAT, GPX and XO, respectively. Absorbances were read by a Unicam Heλios α (UV–VIS) spectrophotometer (Spectronic Unicam, Cambridge, UK).

Statistical analysis

Five animals per group were required for the study with a power of 87% and error type I of 5% to detect a one unit difference between the groups in nonparametric unpaired tests. For central tendency and dispersion of data we used median and quartile deviation (QD), respectively. The Mann–Whitney *U*-test was used for group comparisons. Multivariate analysis of variance was used to search for any significant relationship with dependent variables of 8OHdG and parameters of biochemical OS in groups. Pillai's trace significance test was used for significance of the overall relationship between dependent variables and RF groups. A

Table 2 Evaluation of HSCORE count [20].

Score	Description
0	Percentage of unstained cells × 0
1	Percentage of weakly stained cells × 1
2	Percentage of moderately stained cells × 2
3	Percentage of intensely stained cells × 3

p value less than 0.05 was considered as significant. PASW version 17 (Chicago, IL, USA) was used for the statistical procedures.

Results

There was no difference between the weights of the control and experimental groups before and after the experiment. Median values of TBS, as evaluated using Johnsen's scale, were lower in the experimental than the control group (9 ± 0 vs 10 ± 0). The difference in the TBS between the control and experimental groups was statistically significant ($p < 0.01$). 8OHdG levels in the experimental group were significantly higher than in the control group. Routine histological examinations of the experimental and control group were not different. 8-Hydroxyguanosine immune reactivity, which shows oxidative damage to DNA, was positive in both groups. The HSCOREs of the experimental group were significantly higher than those of the control group ($p < 0.01$) (Table 3). GPX and CAT activities of the experimental group were statistically lower than those of the control group ($p < 0.05$ and $p < 0.01$, respectively). SOD and XO activities and MDA levels of the experimental group were lower than in the control group, but the difference was not statistically significant ($p > 0.05$) (Table 4). The histological and immunohistochemical changes in the experimental and control groups are shown in Fig. 2. Multivariate analysis of variance showed a significant relationship between RF group and the OS parameters of 8OHdG, MDA, GPX, CAT, XO and SOD (F value 19.66; $p = 0.017$).

Discussion

Various studies have investigated the effects on the human body of RF EMR from mobile phones, with inconclusive results, but there have been no studies as yet on the health effects of WIADs, which are associated with higher frequency radio waves and longer exposure times than MBs. Currently, the most popular wireless technology employs the 802.11.g protocol, which is used in many wireless electronic devices, including baby video monitors, Wi-Fi MBs and television devices. A wireless 802.11.g RF device has unique properties, e.g. with regard to frequency range, output power and exposure time, which may have an untoward influence on health. It uses the 2400-MHz band, which is higher than the frequencies used by GSM (Global System for Mobile Communications) or 3G (third generation) phones. The output power of Wi-Fi devices ranges from 20 mW to 4 W, and is similar to or greater than that of MBs, which range from 1 mW to 2 W [21,22]. WIADs are generally 'on' while transceiving data from the net and they cause longer human exposure than MBs. Furthermore, they commonly expose the whole body to RF EMR, unlike MBs, which generally cause a more uneven and local irradiation of some parts of the body, e.g. the head and pelvic organs.

The reason why young growing rats were used in this study is that their growing organs may be more prone to the effects of RF EMR, like children and adolescents who spend a lot of time using wireless computers at home and school. We studied the effects on testes since there have been several studies analyzing the effects of wireless phones on male fertility [23,24], and the reproductive system is more sensitive than other organs to RF devices such as laptop computers. Whole body irradiation with WIADs was employed in this experiment, and thus the methodology used in this study differs significantly from that of the previous investigators, and is unique because it explores the effects of the commonly used conventional WIADs on the health of living organisms.

Previous animal studies have not found any significant effect of RF EMR on testicular histology, sperm count and morphology. Cairnie and Harding [25] studied the effects of RF in the mouse testis and found no significant differences in sperm count and no increase in the percentage of morphologically abnormal sperm in microwave-exposed animals. They used a 2.45-GHz continuous wave for shorter periods of up to 30 days (16 h/day). Ono et al. [26] could not find any mutagenic effect of 2.45-GHz intermittent whole body RF exposure in the testes of mouse embryos. The methodological differences compared to those studies may account for the different outcomes we observed in our study. Since we suspected that the continuous exposure to Wi-Fi devices may have more pronounced effects compared with the shorter and intermittent exposure to MBs, we used a longer and continuous exposure as the test environment in this study. One may suspect that this does not reflect real life conditions, where the duration and intensity of exposure may be more variable. However, the growing technological involvement of the modern human being (with wireless modems, wireless

Table 3 Clinical and laboratory characteristics of rats exposed to WIAD RF radiation and control group.

	Control group	Experimental group	p
No. of rats	5	5	
Weight (g)	230 (30)	250 (9)	0.456
8OHdG (ng/mL)	1.15 (0.67)	3.00 (0.44)	0.021[a]
HSCORE	30 (5)	250 (30)	0.008[a]
TBS	10 (0)	9 (0)	0.003[a]

Values are medians, with QD in parentheses.
[a] Statistically significant difference.

Table 4 MDA levels and prooxidant–antioxidant enzyme activities in testicular tissues of experimental and control groups.

	Control group ($n = 5$)	Experimental group ($n = 5$)	p
MDA (nmol/mg)	0.23 (0.025)	0.21 (0.005)[a]	0.166
XO (mIU/mg)	0.0083 (0.0010)	0.0064 (0.0005)	0.209
SOD (U/mg)	0.84 (0.02)	0.76 (0.01)	0.074
GPX (IU/mg)	0.0040 (0.0003)	0.0024 (0.0009)	0.036[a]
CAT (IU/mg)	5.57 (0.115)	2.65 (0.180)	0.009[a]

Values are medians with QD in parentheses.
[a] Statistically significant difference.

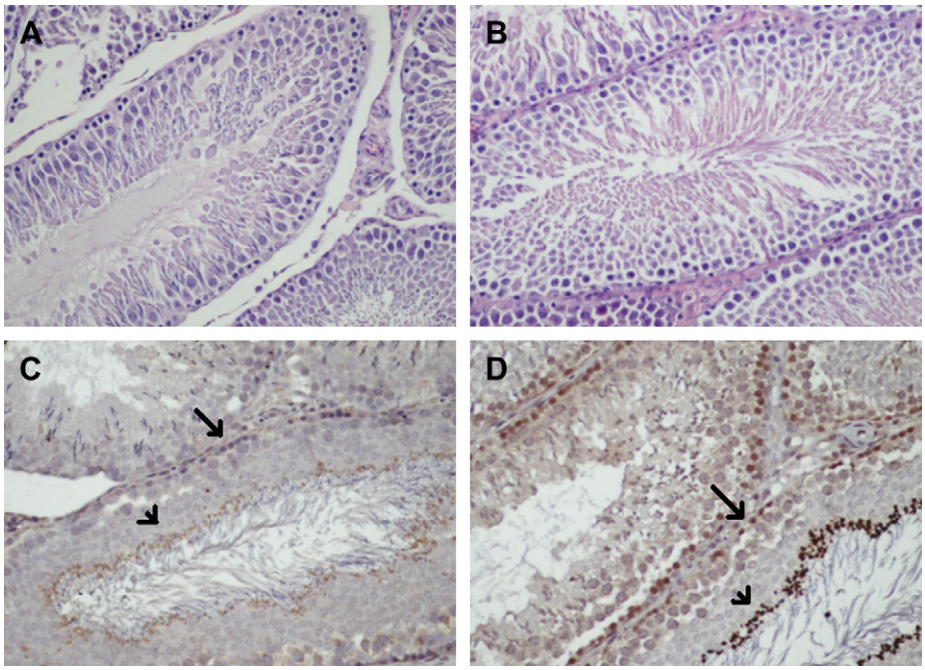

Figure 2 Hematoxylin & eosin (A, B) and immunohistochemical (C, D) staining of testicular tissues of control and Wi-Fi RF-exposed rats. (A,B) No difference was detected in routine histological examinations of the control and Wi-Fi groups (original magnification × 200). (C) In the control group, 8-hydroxyguanosine nuclear positivity is nearly undetectable (original magnification × 200). (D) Diffuse and intense bead-like nuclear immunopositivity (short and long arrows) is seen in the spermatocytic cells of the Wi-Fi group (original magnification × 200).

routers, wireless home monitoring systems, wireless baby monitoring systems, wireless data bridges in between buildings, multiple Wi-Fi mobile phones that interact with wireless routers, use of wireless facilities for various entertainment purposes, etc.) probably exposes many children and adults to RF energy levels comparable to those used in this study. We also used a highly sensitive method to demonstrate any histopathologic changes that might have occurred.

In this study, 8-hydroxyguanosine immune reactivity was observed in both the control and study group. We think that the immune reactivity of the control group may have resulted from endogenous DNA damage caused by background electromagnetism emitted by the Earth or electronic devices or the metabolic rate of animals [27]. We avoided using reverberating or shielding chambers since we did not want to eliminate the effects caused by the background electromagnetism of the Earth on both groups. If we had eliminated this interference for the control group, the significant difference we found in our laboratory findings would not have reflected the differential real effect of RF from our wireless gateways, and would have yielded a falsely higher statistical difference between the two groups.

De Iuliis et al. [9] also showed a correlation between increasing SAR and decreased sperm motility and vitality, and increased OS and 8OHdG markers, stimulating DNA base adduct formation and ultimately DNA fragmentation. Although their results are in very good accordance with ours, they studied purified human spermatozoa ex vivo and used one night's exposure time with lower RF frequency and higher SAR values. To the best of our knowledge, our study is the first in the literature to show oxidative DNA damage in the reproductive tissues of living organisms caused by conventional Wi-Fi devices.

OS is a well-established cause of male infertility and carcinogenesis [28,29]. Reactive oxygen species (ROS) from spermatozoa and invading leukocytes cause infertility by affecting sperm motility [30]. Physiologically, ROS are kept at low levels by intracellular antioxidant mechanisms of spermatozoa. Glutathione (GSH), a major thiol in mammals, is an important antioxidant, and plays a major role in the antioxidant defense mechanism against prooxidant offenders. Tissue GSH reflects the capability of tissues to scavenge the radicals, preserve the cellular reduction—oxidation balance, and defend the cells. The depletion of GSH in RF EMR-exposed animals could be responsible for the low percentages of motile sperm by affecting the spindle microtubule formation. Though we did not measure the GSH level of testis tissues, the decreased activity of GPX in the experimental group reflects a disturbance in the antioxidant defense system, and one possible mechanism for this may be the reduction in the availability of its substrate, GSH. The decreased activity of CAT, which is also an important part of the cellular antioxidant defense mechanism, may result in increased levels of prooxidants such as superoxide radicals and hydrogen peroxide. These findings support the theory that the decreased activities of antioxidant enzymes leading to OS in testis tissues might be responsible for the increased DNA oxidation as demonstrated by increased serum levels of 8OHdG and staining of spermatocytic cells by nuclear and perinuclear 8-hydroxyguanosine antibodies. The decreased SOD and XO activities, though insignificant, could imply a generalized

RF-induced decrease in enzyme activities. Similar findings were reported for human ejaculate exposed to RF EMR from MBs [31]. As MDA levels did not differ between our RF groups, we suggest that RF EMR from WIADs induces OS leading to DNA oxidation but not lipid peroxidation which disturbs testicular function and structure in rats exposed to these devices. The decrease in enzyme activities in general together with the increased 8OHdG serum levels and immunohistochemical staining observed are of great interest, since both would appear to result from WIAD RF.

The association of parameters used in our study with fertility is not certain. Even so, recent studies have demonstrated the relationship between oxidative DNA damage and infertility [32,33]. Others have found decreased CAT and GPX testicular activities to be responsible for fertility problems in animal models and humans [34–36]. Very similar to these studies, we have immunohistologically demonstrated DNA damage and found decreased CAT and GPX activities in the testicular tissues of rats, which may cause infertility. It should be noted that the DNA damage that occurred in the rat testes may be heritable. In order to demonstrate how this DNA damage affects the fertility and organ histology of offspring from mated rats, further functional and structural studies about fertility should be performed.

Due to their height, small children are more likely to be affected by wireless devices positioned close to the floor. Also, children using wireless computers or staying between two wireless devices are exposed to higher levels of radiation than the dose used in our study. The model we have used may not be the perfect one for demonstrating the deleterious effect of Wi-Fi devices, which would be a limitation. However, achieving a model 100% compatible with humans is nearly impossible in experimental animal studies.

Studies on the effects of MBs have used RF sources in direct or very close contact to the organs studied, while in real life this is hardly the case [37]. Unlike in previous MB studies where animals were restrained during the exposure or shielded against background Earth or exogenous electromagnetism, we allowed the animals to move freely, did not shield the control group from background radiation, and did not radiate the experimental group in direct contact with the RF antennae, in order to set up an experimental design more akin to real world conditions.

Our study is in agreement with previous studies that showed the role of MBs in male infertility and carcinogenesis [23,38]. Although the energy levels used in this study were derived from the parameters suggested by the manufacturer, such as distance, power output and duration, they were not safe for rat testicles. The question of whether tissues or organs other than the testicles are affected by these energy levels was not within the scope of this study and requires further research to be resolved. Obviously, extrapolating our findings to humans may not be justified, since the human body may have a more effective shielding capacity against RF energy compared to rats. Further studies are required to evaluate the long-term effects of the commonly used current (*g* type) and newer (*n* type) versions of WIADs on male infertility and germ cell mutagenesis in other species and human beings.

Conclusion

The results of the present study demonstrate that continuous long-term WIAD exposure oxidatively affects the testes in growing rats. Further human studies are needed to answer the question of whether RF waves emitted from Wi-Fi devices affect fertility.

Conflict of interest

None.

Funding

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Author's themselves.

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