

Full Length Research Paper

Short-duration exposure to 2.45 GHz microwave radiation induces DNA damage in Sprague Dawley rat's reproductive systems

Usikalu, M. R.¹, Obembe, O. O.^{2*}, Akinyemi, M. L.¹ and Zhu, J.³

¹Department of Physics, Covenant University, Ota, Ogun State, Nigeria.

²Department of Biological Sciences, Covenant University, Ota, Ogun State, Nigeria.

³Radiobiological Group, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, China.

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The genotoxic effects of 2.45 GHz microwave (MW) radiation on the testis and ovary of Sprague Dawley rats was investigated. The animals were exposed to varying levels of specific absorption rate (SAR) of 0 (control), 0.48, 0.95, 1.43, 1.91, 2.39, 2.90, 3.40, 3.80 and 4.30 Wkg⁻¹, for 10 min. The induction of DNA damages was assessed using DNA direct amplification of length polymorphisms (DALP) and validated with single cell gel electrophoresis (SCGE) comet assay for same cells at SAR 2.39 Wkg⁻¹. Potential damage at the organ level was assessed by histopathological study. The results show significant differences in the Olive moment and % DNA in the blood of the exposed animals when compared with the control ($p < 0.05$). Hyperchromasia was observed in the ovary of the animals exposed to MW radiation. Also, there was reduction in the number of germ cells and cell disorganization in the testis of exposed group with increasing SARs. These results suggest that MW radiation has the potential to affect both male and female fertility adversely.

Key words: 2.45 GHz microwave radiation, histopathology, DNA single strand break, ovary, testis.

INTRODUCTION

Microwave (MW) radiation is a non-ionizing electromagnetic radiation present in the environment and health risks posed by it has been a point of debate for the last two decades. The recent increase in the applications of this radiation has particularly aroused public interest and concerns about possible health hazards (Nakamura et al., 2003; Ruediger, 2009). The low-level radiofrequency radiation at 2.45 GHz in particular, is widely being used in many domestic, industrial and health appliances (Habash et al., 2003). Indeed, there is a body of evidence that suggests potential hazards of exposure to MW radiation in humans (Agarwal et al., 2009; Boscol et al., 2001; Lebedeva et al., 2000; Yadav and Sharma,

2008), animals (Kumar et al., 2011; Nittby et al., 2008; Panagopoulos et al., 2010) and plants (Roux et al., 2008; Tkalec et al., 2009; Vian et al., 2006). Developmental and reproductive impairments resulting from exposure to MW radiation have been copiously reported in small mammals at both the organ and cellular levels (Acar et al., 2009; Aweda et al., 2011, Kesari and Behari, 2010a; Orendáčová et al., 2011; Paulraj and Behari, 2002; Tenorio et al., 2011). Additionally, several reports have given evidence of MW radiation-induced genotoxicity at 2.45 GHz in mice and rats. Recently, Aweda et al. (2010), Kesari et al. (2010) and Chaturvedi et al. (2011) reported mutagenic effects in the brains of mice and rats exposed to 2.45 GHz MW radiation (though at different durations, specific absorption rates and power densities). There are, however, few reports of DNA damage in the reproductive systems at this low-level frequency (Aitken et al., 2005; Sarkar et al., 1994). Most of the previous radiation studies have been conducted over a period of time (5

*Corresponding author.
olawole.obembe@covenantuniversity.edu.ng.
+2348130928965.

E-mail:
Tel:

days to 1 year) with daily exposures, ranging from few minutes to hours, which often leads to increased body temperature, which in turn has been implicated as the main cause of damage in the biological systems (Levitt and Lai, 2010). Nonetheless, non-thermal effects have also been established (Aweda et al., 2010; Campisi et al., 2010; Kesari and Behari, 2010a).

We reported for the first time, DNA damage in the ovary and testis of Sprague Dawley rats following 10 min exposure to 2.45 GHz MW radiation at varying specific absorption rates (SAR) lower than 4 Wkg⁻¹, under isothermal conditions.

MATERIALS AND METHODS

Animals' exposure to MW radiation

A total of 60 (30 males and 30 females) Sprague Dawley rats of 16 weeks old, weighing between 160 and 190 g were used for this study. An experimental grouping of two males and two females per group was adopted. All rats were housed in standard plastic cage under 12 h light and 12 h darkness and were all provided with rat chow and water *ad libitum* from Pfizer Pharmaceutical Company, Nigeria. The laboratory animal house guidelines on animal handling and euthanasia, as approved by the ethical committee of College of Medicine, University of Lagos, were duly observed. All the rats except the control rats were given whole body irradiation by exposure to various SAR delivered from a controlled radiation chamber for 10 min. The MW generator model ER6660E from Toshiba UK Ltd at a power density of 6 mWcm⁻², available in the Department of Radiation Biology and Radiotherapy, College of Medicine, University of Lagos was used for irradiation. The detector of MW used was the non-interacting thermistor, which has a resistance of 4.7 KΩ at 25°C. The thermometer was calibrated in a 12 x 6 x 4 cm size water phantom with the aid of a digital multimeter as readout and mercury-in-glass thermometer as reference. The thermistor response values as indicated by the digital multimeter were recorded against the corresponding readings from the thermometer in °C. SAR was measured by inserting the thermistor probe into each animal's rectum during exposure following an earlier described method (Guy, 1987). The irradiation chamber surfaces were lagged with water to minimize the reflective properties, which may increase the heating rate (Bren, 1996). The generator was operated at room conditions of 25 ± 2°C and 56 ± 4% relative humidity. Exposures were whole body irradiation with the animal at 12 cm from the MW antenna of dimensions 12 x 5 cm. The animals were divided into ten groups of three males and three females per group as follows: Control group A that were not exposed to radiation and blood of some of the animals were obtained before exposure in order to compare them with same animal after exposure, and Groups B to J were exposed to 0.48, 0.95, 1.43, 1.91, 2.39, 2.9, 3.4, 3.8 and 4.3 Wkg⁻¹ SAR radiation, respectively. The SAR were obtained using $SAR = \frac{C\Delta T}{\Delta t}$ where

ΔT is small temperature change, Δt is the small change in duration of exposure and C is the specific heat capacity of the tissue with the value of C taken as 3334 Jkg⁻¹K⁻¹ given by Durney et al. (1980) and Mc Ree and Davies (1987).

Chemicals

Low melting point agarose, normal melting point agarose, ethidium

bromide and DNase free proteinase K were procured from Sigma Chemical Co., USA. The rest of the chemicals were purchased from Takara Biotechnology Co., Dalian.

Removal of reproductive organs and DNA extraction

Each animal was anesthetized by placing them in a glass jar containing cotton dipped in anesthetic ether (Kesari et al., 2010; Paulraj and Behari, 2006); ovary and testis were dissected out for comet assay (2.39 Wkg⁻¹) and DNA extraction using the procedure of Egler et al. (2005) as follows: isolation of DNA from tissues was performed using the method of Egler et al. (2005). The ovary and testis were teased and the pellets were suspended in 25 µl of lysis buffer [40 mM Tris pH 8.0, 80 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 2% sodium dodecyl sulfate (SDS), proteinase K 5 mgml⁻¹]. Polymerase chain reaction (PCR) water was added to a final volume of 100 µl and incubated overnight in a water bath (Uniscope SM801A by Surgifriend Medicals) at 37°C. 300 µL of distilled water was added to the mixture and mixed by vortexing. An equal volume of phenol-chloroform isoamyl-alcohol (25:24:1) was added, vortexed and centrifuged at 13000 rpm for 10 min, after which the aqueous phase was collected. This step was repeated once, then an equal volume of chloroform was added, vortexed and centrifuged, after which the aqueous phase was collected. This step was also repeated. The aqueous phase was then transferred to another tube and 45 µl of 3.0 M sodium acetate (pH 5.0) was added and twice the volume of cold absolute ethanol was added. The contents of the tube were mixed by rapid but gentle inversion of the tube. The tubes were incubated overnight at 20°C; the DNA was recovered by centrifuging at 13000 rpm at room temperature. 1 ml of 70% ethanol was used to wash the DNA. The DNA was recovered by centrifuging at 13000 rpm and the supernatant was carefully removed with a pipette and the tubes were inverted and left to dry. The DNA was reconstituted in 20 µl TE buffer solution.

Quantification of DNA sample

The DNA samples were quantified by adding 114 µl double distilled water (ddH₂O) to 6 µl of sample to give 20 dilution of the solution, and was mixed properly. 50 µl of the mixture was added to quartz curvette of Eppendorf Biophotometer (AG 2331 Hamburg, Germany) available at the Institute of Modern Physics Laboratory, Lanzhou, China and digital output was recorded.

DNA amplification and electrophoresis

The DNA amplification and electrophoresis were carried out according to Desmarais et al. (1998). Each reaction was carried out with combination of one of the selective primers spanning the 16-kb rat genome sense, 5'-GTTTTCCAGTCACGACGC-3', and antisense, 5'-TTTCACACAGGAAACAGCTATGAC-3' (Takara Biotechnology Co., Dalian). The PCR were carried out in a final volume of 15 µl starting from 1 µl DNA sample, 1 µl of each primer, 7.5 µl of Premix Ex-taq (Takara Biotechnology Co., Dalian) using biometra thermocycler (12014, Germany) (Ali and Epplen, 1991). The PCR was carried out with the following parameters: 94°C 10 min; 94°C 1.5 min; 50°C 1 min; 72°C 2 min; for 30 cycles. 5 µl formamide loading dye was added to the PCR products; this is essential to achieve sufficient resolution between very close bands in the multi-locus pattern. Electrophoresis was performed on 10% gels, prepared with 6 ml acrylamide/bisacrylamide (29:1); 2 ml 10 x Tris-borate EDTA (TBE) pH 8.0; 11.7 ml ddH₂O; 200 µl ammonium persulfate (APS) and 10 µl tetramethylethylenediamine (TEMED) in an Hoefer VE (Vertical Electrophoresis System) connected to Electrophoresis power supply (Amersham Pharmacia Biotech,

Table 1. DNA single strand break following exposure to 2.45 GHz microwave radiation at 2.39 W/kg⁻¹.

Parameter	% DNA tail	Olive moment
Ovary		
Control	0.87 ± 0.13	0.64 ± 0.11
2.39 W/kg	5.63 ± 0.57*	3.20 ± 0.15*
Testis		
Control	0.15 ± 0.01	0.50 ± 0.01
2.39 W/kg	3.35 ± 0.55*	2.46 ± 0.13*

*Indicates significant difference ($p < 0.05$) as compared to the control.

Sweden) and run at 120 V for 2 h. Staining was carried out by transferring the gel into AgNO₃ solution in the dark and agitated for 7 min and washed twice in distilled water for 1 min, stained using very sensitive staining solution (3.5 g NaOH, 0.0475 g Na₂B₄O₇·10H₂O and 1 ml formaldehyde in 250 ml distilled water) until the bands were visible, then washed thrice in distilled water for 1 min each. The gel photographs were taken using both digital camera and multimage light cabinet connected to computer system using Chemilmager software (Alpha Innotech Corporation, USA). Densitometry analysis of the tracks was carried out with Image J gel analyzer software (NIH).

DNA single-strand breaks estimation

Comet assay also referred to as single cell gel electrophoresis (SCGE) was used to determine DNA damage in terms of DNA single strand break (SSB) in ovary and testis of the animals after exposure to 2.39 W/kg⁻¹ MW radiations. Whole tissue was washed two to three times with phosphate buffered saline (PBS) (1.37 mM NaCl, 4.3 mM Na₂HPO₄, 2.7mM KCl, 1.4 mM KH₂PO₄, pH 7.4) to remove the red blood cells (Paulraj and Behari, 2006). The cells were minced into small pieces by adding 1 ml PBS in a medimachine (Becton Dickinson, Italy) and a single cell suspension was collected using a 200 µl pipette.

The comet assay was performed as described by Singh et al., (1995), normal melting point agarose (Amersco, NMA) and low melting point agarose (Amersco, LMA) were suspended in PBS at 37°C. Then, 100 µl of 1% NMA was added to comet slides and the slides were allowed to solidify. 22.5 µl of cell suspension was mixed with 67.5 µl, 1% agarose (3:1) added on the solidified gel and covered with cover slip for 5 min in the refrigerator. After removing the cover slips, the slides were submerged in lysing solution [2.5 M NaCl, 100 mM EDTA-Na₂, 10 mM Tris-HCl, pH 10; 1% Triton X-100 and 10% dimethyl sulphoxide (DMSO), pH 10] for 2 h in the dark. The slides were then placed in unwinding buffer (1 mM EDTA and 300 mM NaOH, pH 13) for 25 min. In the same buffer, electrophoresis was carried out at 4°C for 25 min at 25 V using Amersham Pharmacia Biotech power supply and current was adjusted to 302 mA by modulating the buffer level. After electrophoresis, the slides were neutralized by washing three times with neutralization buffer (400 mM Tris-HCl, pH 7.4) for 5 min each. Slides were immersed in 70% ethanol for 10 min to precipitate the DNA and dehydrate the gels. Slides were left in vertical position to dry and then stained with 50 µl of 10 µgml⁻¹ ethidium bromide (EB). The slides were examined using a Roper Scientific (RS) image analysis system (Alpha Innotech Corporation, USA) fitted with an Olympus BX51 fluorescence microscope equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm.

Comet scoring

A total of 100 cells were randomly selected, each of which was used to prepare three slides (three replicates per cell) and scored. DNA damage was evaluated from the pictures obtained from the slides by RS image analysis system by calculating the Olive moment and % DNA tail as input to the comet score software 1.5 (TriTek Cor., Virginia) (Hiroyuki et al., 2010). In order to compare each sample of the tissue with its control, two-tailed paired *t*-test was used and $P < 0.05$ value was regarded as statistically significant.

Histopathological studies on the ovary and testis

The abdomen of each animal was carefully opened up and the ovary and testis were removed. The sectioning of the tissue was done in the Department of Morbid Anatomy, College of Medicine, University of Lagos. The tissue samples were fixed in 10% neutral buffered formalin; these were dehydrated with ethanol, and embedded in paraffin according to the procedure of Masuda et al. (2006). Tissue sections of 5 to 7 µm thickness were cut and stained with hematoxylin and eosin (H&E) for microscopic examination.

Sperm count

The caput from the epididymis was immersed in 5 ml normal saline in a measuring cylinder and its volume measured. It was matched into suspension, from which the count was done. Sperm count was done under microscope using improved Neubauer hemocytometer. Count in 5 large Thomas square was taken and adjustment was made for the volume of the normal saline added. The count was therefore calculated as:

$$\text{Count ml}^{-1} = \text{Number of sperm cell in 5 large Thomas square} \times 32000 \times \text{dilution}$$

RESULTS

DNA single-strand break

There was significant increase in the tail of DNA and olive moment of the exposed animals both in testis and ovary as compared to the control animals ($p < 0.05$) as shown in the comet score display in Table 1. Densitometric

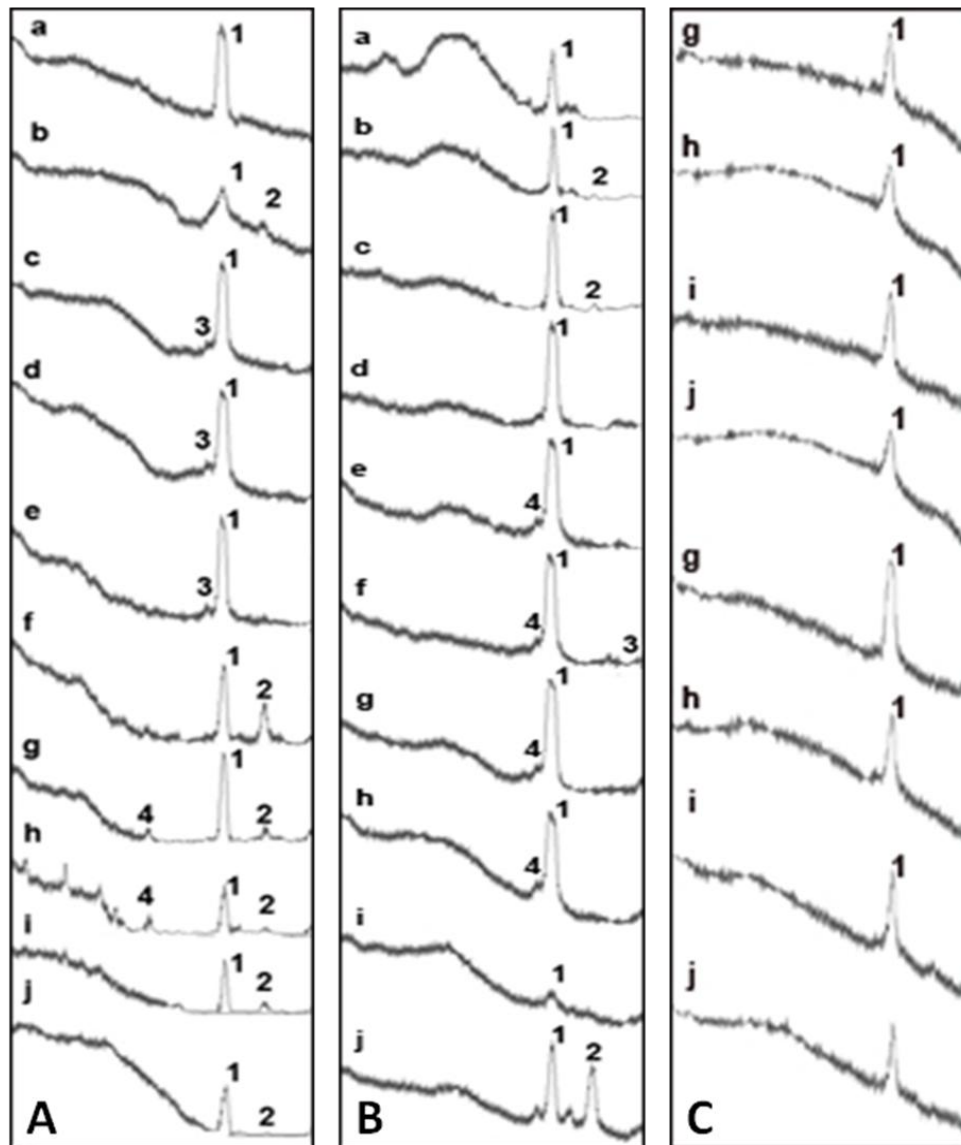


Figure 1. Densitometric track analyses of ovary DNA (A), testis DNA (B) and male and female blood DNA before exposure having peak 1 only (C). Lane (a) is the DNA of the control; (b to j) are DNA from exposed animals. Peak 1 is present in both control and exposed animals while other peaks appeared in all exposed animals.

analysis of the control DNA for testis and the ovary as well as for the blood of the animals before exposure to MW radiation consistently have sharp peak marked 1 at the same molecular weight (Figure 1C) while in the exposed animals, there were always additional peaks either before or after this particular peak; marked "2", "3" and "4" (Figure 1A and B).

Histopathological analysis

No histological or macroscopic alterations were observed in the ovary and testis of the control rats, as there was

normal arrangement of the cells (Figures 2A and 3A). Figure 2B present the result for testis exposed to SAR 0.48 Wkg^{-1} showing reduction in the number of germ cells as compared to control. The testis in the group exposed to SAR 0.95 W/kg showed reduction in the number of germ cells as compared to the control (Figure 2C). Figure 2D display result of the testis exposed to SAR 1.43 Wkg^{-1} showing mild reduction in the number of germ cells as compared to the control. The result obtained in testis of the group exposed to SAR 1.91 Wkg^{-1} is similar to that exposed to 2.39 Wkg^{-1} ; they both showed cells disorganization and reduction in the number of germ cells as compared to the control (Figure 2E and F). Figure 3B

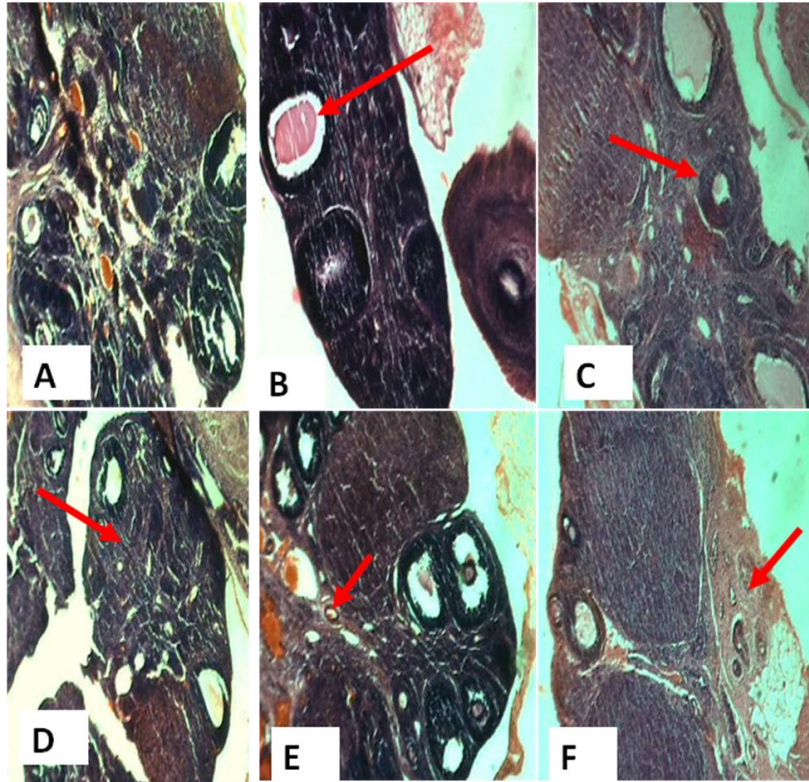


Figure 2. Micrographs of follicle distribution and arrangement in the ovaries for the unexposed control group (A), group exposed to SAR 0.48 Wkg^{-1} (B), SAR 0.95 Wkg^{-1} (C), SAR 1.43 Wkg^{-1} (D), SAR 1.91 Wkg^{-1} (E) and SAR 2.39 Wkg^{-1} (F) (magnification 40x).

shows the result obtained for the ovary exposed to SAR 0.48 Wkg^{-1} having thick sectioning coarse chromatin and oedema. Figure 3C shows ovary exposed to SAR 0.95 Wkg^{-1} containing few follicles of varying sizes. The ovary in the group exposed to SAR 1.43 Wkg^{-1} showed increased hyperchromasia coarse nuclear chromatin and cellular oedema (Figure 3D). The ovary in the group exposed to SAR 1.91 Wkg^{-1} showed vascular congestion hyperchromatic granulosa cell and cellular oedema (Figure 3E). Figure 3F shows the result of the ovary exposed to SAR 2.39 Wkg^{-1} showing cellular oedema and hyperchromatism in some of the follicles.

Sperm count

It was also striking to observe a significant reduction in the number of sperm cells in animals exposed to SAR 0.48 Wkg^{-1} and above, as compared to the control (Figure 4).

DISCUSSION

The effect of 2.45 GHz microwave radiation on the

behavioural modification of rats was previously reported by Aweda et al. (2010). Our observation that the rats were unable to recover totally from both anxiolytic and exploratory effects after two weeks of exposure to 2.39 Wkg^{-1} radiation informed our choice of this SAR for the comet assay in the present study. We hypothesized that short exposure to 2.45 GHz microwave radiation might have genotoxic effect and impact reproductive capacity on the rats. In this study, whole body irradiation was administered to the rats by exposing them to various SAR for 10 min.

Our results reveal that short exposure to low level 2.45 GHz microwave radiation alters the DNA genome band pattern, causes DNA single strand break and modifies the cells in the testis and ovary of the exposed animals. The use of the DALP method for the assessment of the band profiles is very sensitive for detecting any loss or gain of band due to sequence rearrangement or sporadic mutation. In this work, the control and the exposed animals have somewhat similar profile except for additional fragments of different molecular weight, which are observed in some exposed animals. Since this particular fragment is not present in the control or even in the same animals before exposure to MW radiation but appears after microwave exposure, it is suggested that

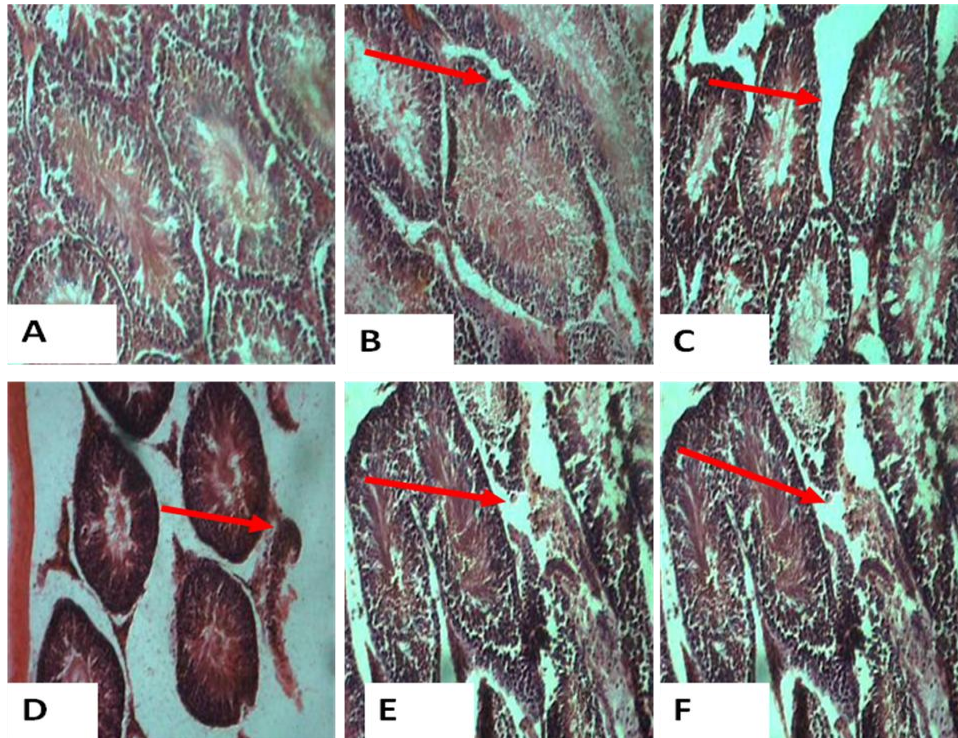


Figure 3. Micrographs of germ cells distribution and arrangement in the testes for the control group (A), group exposed to SAR 0.48 Wkg⁻¹ (B), SAR 0.95 Wkg⁻¹ (C), SAR 1.43 Wkg⁻¹ (D), SAR 1.91 Wkg⁻¹ (E) and SAR 2.39 Wkg⁻¹ (F) (magnification 40x).

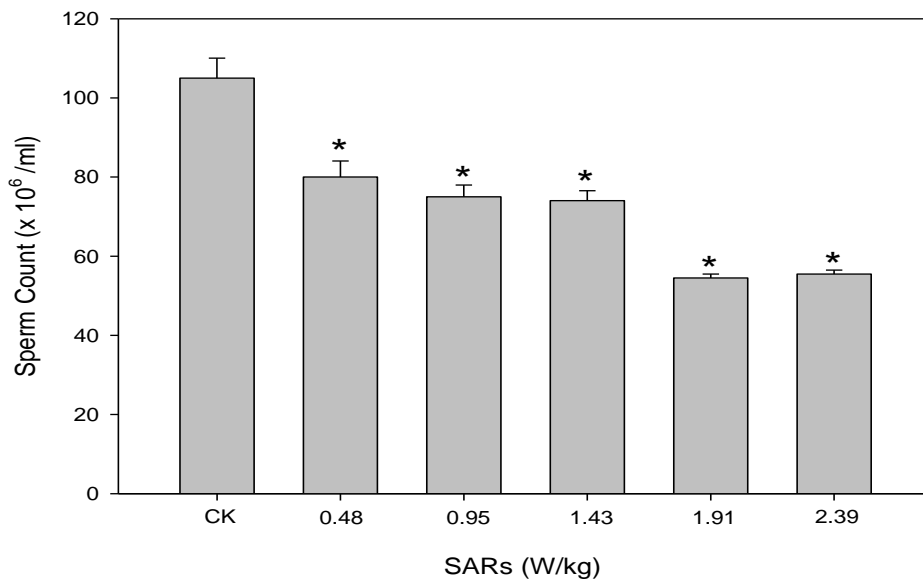


Figure 4. Sperm counts after exposure to SAR 0.48 Wkg⁻¹ and above.

the copy number of these repeat sequences is not sufficient to form a distinct band in the unexposed animals. Microwave exposure may have led to stress-induced amplification of these tandem sequences,

generating more copies of the sequences in this particular region. At present, it has not been established that exposure to mutagens can increase mutation rate in particular regions where the extra bands appear even

though it was suggested that stress is capable of inducing amplification and extra replication of DNA segment in the non-coding repeat sequences (Ramel, 1989). Therefore, the alteration recorded in the DNA could be linked to the non-specific stress generated by microwave field and not in any way related to indirect thermal effects, as there was proper control of temperature throughout the experiment (Sarkar et al., 1994). This result agrees with that of Pilla (1979) that reported the capability of interaction of electromagnetic fields with biological systems with no accompanying cell heating. According to the studies of Sagripanti and Swicord (1986), MW radiation is capable of inducing both single and double strand breaks in the DNA molecule and this damage can be correlated to mutagenic and cancerogenic changes (Sargentini and Smith, 1985; Aitken et al., 2005; Paulraj and Behari, 2006; Campisi et al., 2010; Kesari et al., 2010). Our result, which shows a significant difference in DNA single strand breaks in the exposed tissues, is in agreement with the above-mentioned studies. DNA damage is closely related to human health risk, to the extent that unrepaired or inaccurately repaired DNA damage can lead to cell death as well as to genomic instability, mutations and ultimately to cancer, aging and other diseases (Halliwell, 2002). Theoretically, MW/RF is not able to induce genotoxic effects by direct interaction with DNA, because their intrinsic (quantum) energy is too low to dislodge an electron from a molecule but these may arise as a result of increase in the free radicals production and life span (Grissom, 1995; Lupke et al., 2004).

The reduction in the male germ cells and decrease in sperm counts of the rats exposed to microwave in this study corroborates previous studies that reported decrease in sperm counts (Aweda et al., 2011; Kesari and Behari, 2010a), decreased sperm motility, increased abnormal sperm cells (Aweda et al., 2011), increased apoptosis and significant effect on the level of antioxidant enzymes (Kesari and Behari, 2010b).

Conclusion

We reported for the first time, alteration in DNA bands pattern, single strand break and reduction in the number of male germ cells of Sprague Dawley rats as a result of exposure to 2.45 GHz microwave for just 10 min without change in body temperature. Our findings reveal that exposure to microwave radiation of 0.48 Wkg^{-1} and above produces genotoxic effects on testis and ovary as observed from the genomic result, comet assay and the histopathology results. Thus, there is high possibility of compromising the fertility in the exposed rats. Although, rats are known to be more metabolically active than humans, however, these results give an indication of possible long term effects that may be expected on the reproductive organs in humans when exposed at similar microwave radiation for a considerable period of time.

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