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Genome toxicity upshot of low 2.45 GHz microwave radiation exposures on Sprague Dawley rats

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Abstract

This paper investigates the genotoxic effects of 2.45 GHz microwave (MW) radiation exposure at low specific absorption rates (SAR) . 200 Sprague Dawley rats were exposed to SAR values between 0.48 and 4.30 W.kg^{-1} and the DNA of different tissues extracted, precipitated and quantified. Induced deoxyribonucleic acid (DNA) damages were assessed using the methods of DNA Direct Amplification of Length Polymorphisms (DALP) and the Single Cell Gel Electrophoresis (SCGE). Densitometric gel analysis demonstrated distinctly altered band patterns within the range of 40 and 120 bp in exposed samples and in the tail DNA of the same animals before exposure compared with control. Results were re-affirmed with SCGE (comet assay) for the same cells. Different tissues had different sensitivities to exposures with the brains having the highest. DNA damages were sex-independent. There was statistically significant difference in the Olive moment and % DNA in the tail of the exposed tissues compared with control ($p < 0.05$). Observed effects were attributed to magnetic field interactions and production of reactive oxygen species. We conclude that low SAR 2.45 GHz MW radiation exposures can induce DNA single strand breaks and the direct genome analysis of DNA of various tissues demonstrated potential for genotoxicity.

Keywords: Microwave radiation, single strand break, genotoxicity.

INTRODUCTION

Recently, microwaves (MW) have gained significant importance in the numerous industrial, military, medical and domestic uses. 2.45 GHz MW are currently widely used in domestic ovens as kitchen appliances. The recent increase in MW radiation uses has generated growing public interest and concerns about the potential health hazards associated with exposures. Research into possible biological effects of MW exposures has focused on biological endpoints such as DNA damage (Velizarov et al., 1999; Yariktas et al., 2005) changes in cell proliferation and free radical release (Paredi et al., 2001; Aweda et al., 2003; Hinrikus et al., 2005; Moustafa et al., 2001). Also, the mechanisms of how MW fields exert possible non-thermal effect on various cellular processes have not yet been well elucidated. Cytotoxic effects of MW exposure have been observed in some studies (Lai and Singh, 1996; Pilla, 1979; Sargentini and Smith, 1985) Authors have reported on induction of DNA damage in rat brain cells after exposure to 2.45 GHz, and MW enhancement of genotoxic effects of mitomycin C using micronucleus test and comet assay *in vitro* (Zhang et al., 2002).

In contrast, (Velizarov et al., 1999) found no significant increase in DNA damage in leukocytes exposed to MW signals for 3-4 h, but exposure for longer time than 24 h at SAR of 5.0 and 10.0 Wkg^{-1} resulted in significant increase of the frequency of micronucleated lymphocytes. Significant increase in the number of micronuclei following exposure to phase-modulated 1,800 MHz MW fields for 15 min to approximately 5 Wkg^{-1} SAR was observed in human lymphocytes (d'Ambrosio et al., 2002). On the other hand, there are several studies reporting no cytotoxic effects (Simko, 2004; Dubreuil et al., 2003; Cassel et al., 2004; Cosquer et al., 2005).

The direct non-thermal effects of MW exposure and

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their interactions with the mammalian tissues have been a matter of discussion for a long time. However recent research on alterations in neurological function due to low-level exposures has indicated the need to con-sider possible mechanisms for the biological effects of MWradiations. d'Andrea et al. (2003) reported significant changes in different psychopatho-physiological functions in exposed subjects even when the power is quite low (W to mW range).

It has also been reported that chronic exposure to leakage of MW radiation of low energy and non-thermal is harmful and can alter different physiological variables (Bachmann et al., 2005; Hook et al., 2004; Aweda et al., 2004; Lai and Singh, 1995). Published data indicating changes in the permeability of the blood brain barrier due to cell damage by extremely low levels of the radiation suggest possible effects on the physiology of brain (Velizarov et al., 1999; Pilla, 1979). In order to confirm previous findings (Guy, 1987; Moustafa et al., 2001; Lai and Singh, 1996), we investigated the genotoxic effects on vital mammalian cells following 10 min exposure to 2.45 GHz at various low SAR values. The frequency used for this study represents that to which unsuspecting members of the public and people working with MW emitting sources have the possibility of being exposed, both in the environment and from household devices. The results from the study will hopefully serve as vital information in the determination of safe exposure level and establish or otherwise the need for precaution-nary measures in the numerous MW radiation-based technology applications.

MATERIALS AND METHODS

Animals and MW radiation treatment

60 male and female Sprague Dawley rats of about 16 weeks old, weighing between 100 - 120 g were used for the study. All the rats were housed in standard plastic cages under 12 h light and 12 h darkness and were provided with rat chow and water *ad libitum* from Pfizer Pharmaceutical Company, Nigeria. The College of Medicine, University of Lagos Laboratory Animal House guidelines on animal handling and euthanasia were duly observed. All the rats except the control were giving whole body irradiation by exposure to various SAR values delivered from a controlled irradiation chamber.

The MW generator model ER6660E from Toshiba UK Ltd. at a power density of 6 mW cm^{-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 with a resistance of 4.7 k at 25°C. The thermistor was calibrated in a 12 × 6 × 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 values were measured by inserting the thermistor probe into the rectum during exposure following an earlier described method (Aweda et al., 2003). 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 total body, with the rat at 12 cm from the MW antenna of dimensions 12 cm × 5 cm.

The rats were divided into 10 groups of 6 each as follows: The rats in the control group (a) were not exposed to radiation. Detailed physical conditions of each rat were noted before, for the purpose of comparison with same rat after exposure. Groups (b) to (j) were exposed to 0.48, 0.95, 1.43, 1.91, 2.39, 2.90, 3.40, 3.80 and 4.30 Wkg^{-1} SAR respectively. After the exposure to various SAR for 8 days the animals were sacrificed by cervical dislocation and various tissues (blood leucocytes, brain, lung, spleen) were extracted for comet assay and the isolation of DNA done. DNA direct genome analysis was performed on different cells and the single cell gel electrophoresis (SCGE) was at various SAR values.

DNA extraction and washing of the blood leucocytes cell

2.5 L of 0.1% saponin was added to 0.5 ml of whole blood. The mixture was vortexed using the autovortex mixer SA2 (Stuart Scientific, UK) and left for 2 min at room temperature to allow for lysis. The contents were centrifuged at 9450 g for 5 min, this was repeated 2-3 times until the samples were fully clean. The other tissues were teased and the pellets suspended in 25 L in lysis buffer (40 mM Tris pH 8.0, 80 mM ethylene diamine tetra acetic acid (EDTA) pH 8.0, 2% Sodium Dodecyl Sulfate (SDS), Proteinase K 5 mg/ml). Polymerase chain reaction (PCR) water was added to a final volume of 100 l and incubated overnight in a water bath at 37°C.

Phenol extraction and precipitation of DNA

300 L 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 9450 g for 10 min, after which the lower layer was removed, leaving the interface and the aqueous layer. This was repeated, and then equal volume of chloroform was added, vortexed and the lower layer removed. This step was also repeated. The aqueous layer was transferred to another tube, 45 L of 3.0 M sodium acetate (pH 5.0) was added and twice the volume of cold absolute ethanol added. The contents of the tube were mixed by rapid inversion of the tube. DNA may or may not become visible at this stage as fine strand. The tubes were left overnight at 20°C. The DNA was recovered by centrifuging at 9450 g at room temperature and 1 ml of 70% ethanol was used for washing. The supernatant was carefully removed by gently inverting the tube and left to dry. The DNA was reconstituted in 20 L Tris EDTA solution.

Quantification of DNA sample

6 L of sample was added to 114 L doubly deionized water (ddH_2O) to give 20 dilution of the solution; this was mixed properly. 50 L of the mixture was added to quartz curvette of Eppendorf Biophotometer (AG 2331 Harmburg, Germany) available at the Institute of Modern Physics laboratory, Lanzhou, China and digital output was recorded.

Amplification and electrophoresis

Each reaction was carried out with a combination of one of the selective primers spanning the 16-kb rat genome sense, 5 - GTTTTCCCAGTCACGACGC-3 , and antisense, 5 - TTTCACACAGGAAACAGCTATGAC-3 (Takara Biotechnology Co., Dalian). The PCR were carried out in a final volume of 15 L starting

from 1 L DNA sample, 1 L of each primer 7.5 L of Premix Ex-taq (Takara Biotechnology Co., Dalian) using biometra thermocycler The cycling parameters were 94°C 10 min; 94°C 1.5 min; 50°C 1 min; 72°C 2 min; 29 more cycles to step 2 and 72°C 10 min; 4°C pause. 5 L formamide loading dye was added to the PCR products, this is essential to achieve sufficiently high 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 ddH_2O; 200 L Ammonium Pensulfate (APS) and 10 L tetramethylethylenediamine (TEMED) in an Hoefer VE (Vertical Electrophoresis System, Sweden) connected to an electrophoresis power supply (Amersham Pharmacia Biotech, Sweden) and run at 120 V for 2 h. The staining was started by transferring the gel into $AgNO_3$ solution in the dark and shaking for 7 min, washed twice in distilled water for 1 min. Stained using very sensitive staining solution (3.5 g NaOH, 0.0475 g $Na_2B_4O_7.10H_2O$ 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 multilimage light cabinet connected to computerized system using the Chemilmager software (Alpha Innotech Corporation, USA). Densitometric analysis of the tracks was carried out with Image J gel analyzer software (National Institute of Health, USA).

Single cell gel electrophoresis (Comet assay)

Comet assay also referred to as SCGE was used to determine DNA damages in terms of single strand breaks (SSB) in brain, lung and spleen of the animals after exposure to MW radiation. This assay has been used extensively in toxicological studies for DNA damages (Ali and Epplen, 1991) and found to be sensitive especially for assessing SSB DNA damages. Whole tissue was washed two to three times with phosphate buffered saline (PBS) (1.37 mM NaCl, 4.3 mM Na_2HPO_4, 2.7 mM KCl, 1.4 mM KH_2PO_4, pH 7.4) to remove the red blood cells. 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 L pipette. The comet assay was performed as described by Lai and Singh (1995) with minor modifications. Normal melting point agarose (NMA) and low melting point agarose (LMA) were suspended in PBS at 37°C. Then, 100 L of 1% NMA was added to comet slides and allowed to solidify. 22.5 L of the cell suspension was mixed with 67.5 L, 1% agarose (3:1) added on the solidified gel and covered with slip for 5 min in the refrigerator. After removing the cover slips, the slides were submersed in the lysing solution (2.5 M NaCl, 100 mM $EDTANa_2$, 10 mM Tris–HCl, pH 10; 1% Triton X-100 and 10% DimethylSulfoxide (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 the (Amersham Pharmacia Biotech UK) power supply and adjusting the current to 302 mA by modulating the buffer level. After electrophoresis, the slides were neutralized by washing 3 times with neutralizing buffer (400 mM Tris–HCl, pH 7.4) for 5 min each. The 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 L of 10 g/ml ethidium bromide (EB). They were then examined using the 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. For each cell, two slides were prepared and each 50 randomly chosen cells (total 100 cells) were scored. DNA damage was evaluated by calculating the Olive moment and % DNA tail using comet score software 1.5 (TriTek Cor., Virginia). 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.

RESULTS

Plates 1 to 5 show the electrophoresis gels while the densitometry analyses of various lanes are shown in Figures 1 and 2. A multilocus monomorphic band profile was demonstrated with direct amplification of length polymorphic (DALP) of rat DNA in the tissues studied. The hybridization profile of DNA of all tissues show sharp bands (molecular weight 58 bp) in both the control and the exposed samples as shown in all the Plates. However, for the exposed tissue samples, insertions of additional band below and above this particular band (40 - 200 bp), gel track analysis of the control (lane a) of all the plates and some of the tails DNA obtained before exposure to microwave radiation (Plate 1) consistently have sharp peak marked 1 at the same molecular weight. In the exposed animals there are always additional peaks either before or after the particular peaks which are marked 2, 3, 4, etc.

Single strand break detected from comet assay

Our findings showed that low SARs exposure to 2.45 GHz MW radiation could result in single strand breaks of DNA in blood leucocytes, brain, lung and spleen cells of rats. The results of mean ± Standard Deviation (SD) of % DNA in tail and Olive moment obtained are presented in Figures 3 and 4 respectively.

DISCUSSION

The results obtained from the electrophoresis direct genome analysis of DNA of different tissues demonstrate that MW is capable of inducing genotoxic effects in living tissues. The gel electrophoresis in Plates 1 - 5 and the densitometric analysis of various lanes in Figures 1 and 2 show multilocus monomorphic band profile demonstrated with DALP of DNA in the tissues studied. In the exposed sample analyzed, additional peaks are observed before or after a particular peak. Since this fragment is not present in the control or even in the tail DNA of the same animal before exposure to radiation, this suggests that probably in the unexposed animals, the copy number of these repeat sequences is not sufficient to form a distinct band. Radiation exposure may have led to the amplification of these tandem sequences generating more copies of sequences in this region. Multilocus monomorphic band profiles (Ramel, 1989) are particularly useful because any loss or gain of band due to sequence rearrangement or sporadic mutation can be easily detected. In this work both control and exposed animals have similar profile with exception of additional fragment specific to all exposed animals, which is not present in

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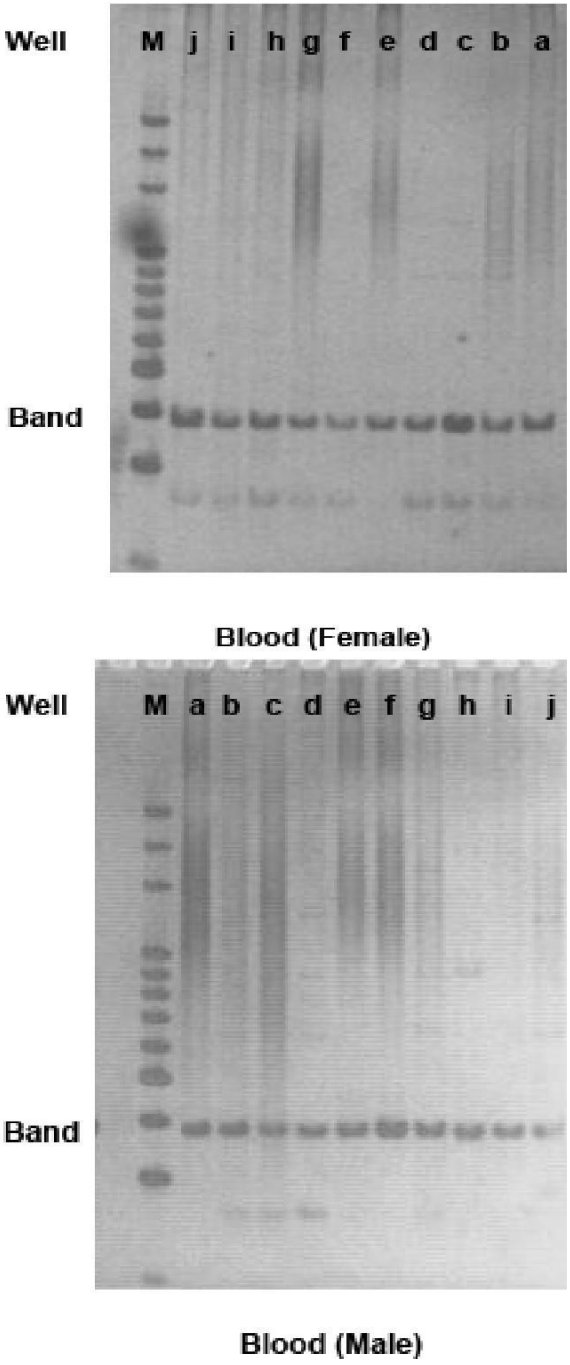

Plate 1. Hybridization of the blood DNA in male and b – j (exposed animals). Lanes a-j have been tracked densitometrically for female (Figure 2A). (a) is control (b) 3.40, (i) 3.80 and (j) 4.30 Wkg^{-1}. 0.48, (c) 0.95, (d) 1.43, (e) 1.91, (f) 2.39, (g) 2.90, (h)
female rats. Note the appearance of other bands in lane

any of the control samples. Although it is not known at present whether exposure to a mutagenic agent or a specific class of mutagens increases the mutation rate in

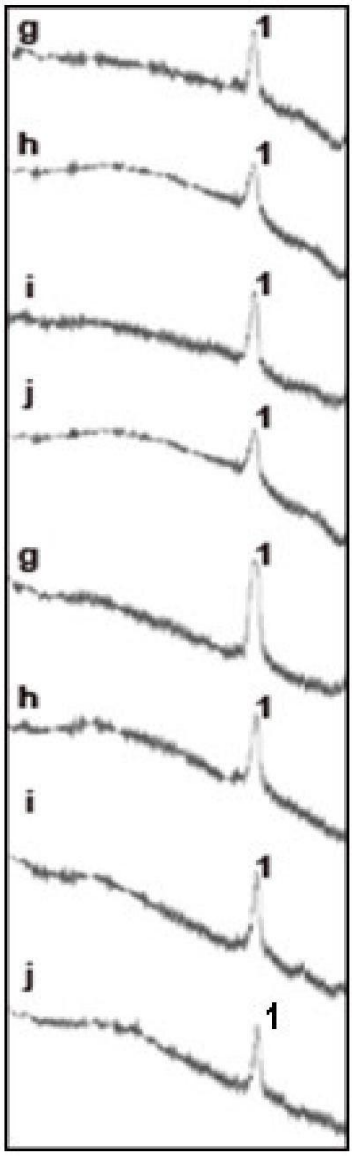

Figure 1. Densitometric track analysis of male and female Tails DNA before exposure having just peak 1.

the region of these tandem repeat units, it is known that stress induces amplification by extra replication of DNA segments in the non-coding repeat sequences (Yakus et al., 2008) . The observed DNA rearrangement after MW exposure from this can be attributed to magnetic fields interaction with biological matter created by MW field, production of reactive oxygen species (ROS) and not a result of thermal effects. Pilla (1979) observed the existence of a mechanism of interaction of weak electro-magnetic fields with biological systems accompanying cell heating. Also Maes et al. (1993) found an increase in

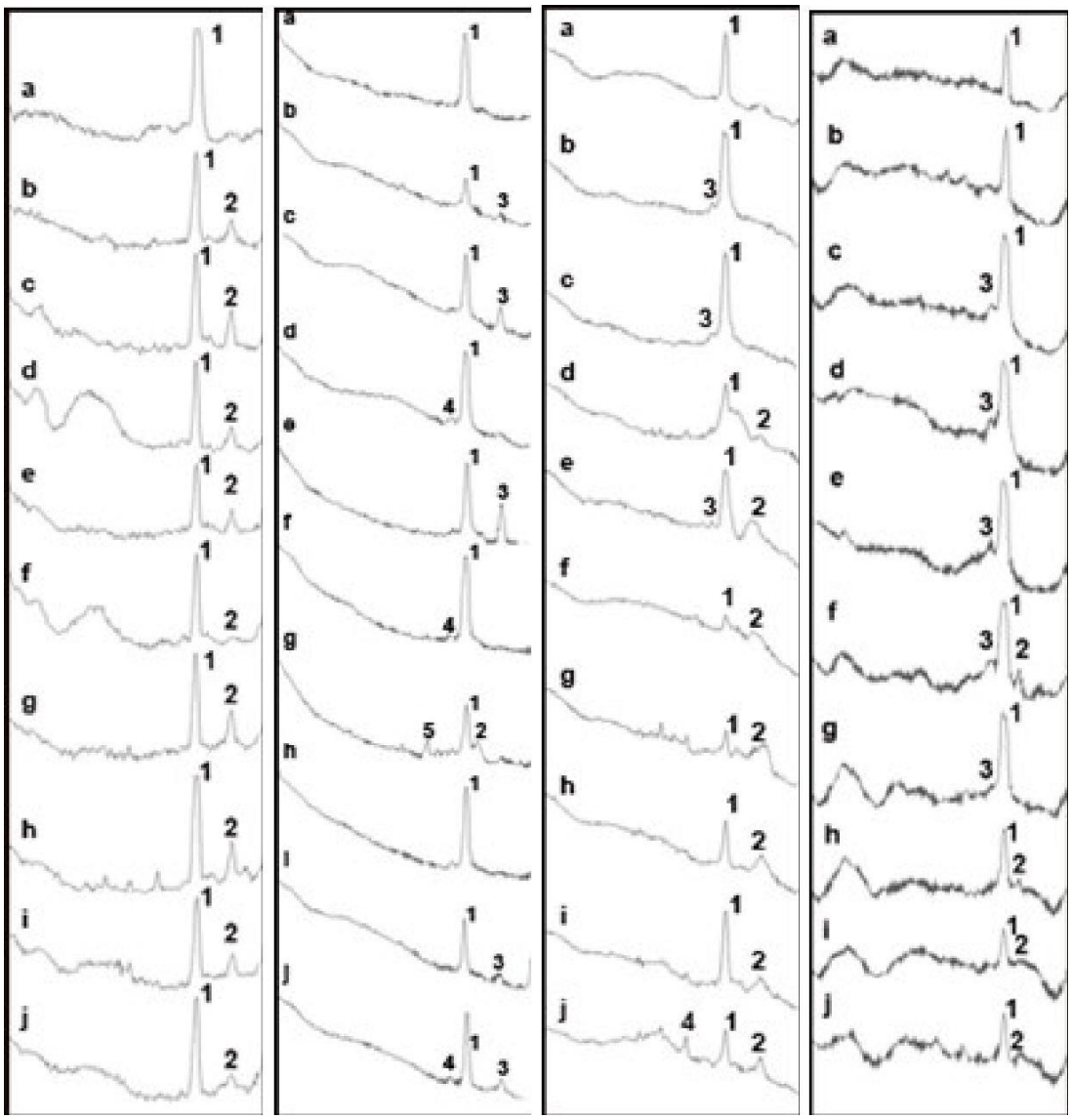

Figure 2. Densitometric track analysis of Tails DNA (A) female blood leucocytes DNA. (B) female brain DNA (C) male lung DNA, and (D) female spleen DNA after exposure. Lane (a) is the DNA of the control; lanes (b-j) are DNA from exposed animals. Peak 1 is present in both control and exposed while other peaks appear in all exposed animals.

the formation of 8- Hydroxyguanine **(**8- OH-Gua) in HL–60 leukemia cells and Rat-1fibroblasts exposed to 0.5and 1.0 mT MF for 24 and 72 h. Ehling (1989) has suggested that germ cell mutation in mice can be used as a standard for protecting the human genome. Our results also revealed that different tissue has different sensitivity to MW radiation as extra peaks were distinct in some tissues even at low SAR of 0.48 Wkg^{-1}, while in some others it was not distinct until after 1.91 Wkg^{-1}. For instance, in brain, blood leucocytes and lung DNA (Plates 2A - 2C) right from 0.5 Wkg^{-1} the extra peak was seen distinctly while in spleen it was not seen distinctly until lane f (Plate 2D), corresponding to 2.9 Wkg^{-1}. The effects studied are seen to be SAR-dependent and not sex dependent, as almost similar trend was observed in both sexes but different for different SARs. The differences in tissues exposure level may also account for the disparity in the results obtained by different researchers regarding MW effects.

It is shown that there are significant increases in the tail of DNA and Olive moment of the exposed as compared to the control animals. From the results, the brain has the highest DNA damage as observed from comet assay, this suggests that brain cells has high exposure level (high

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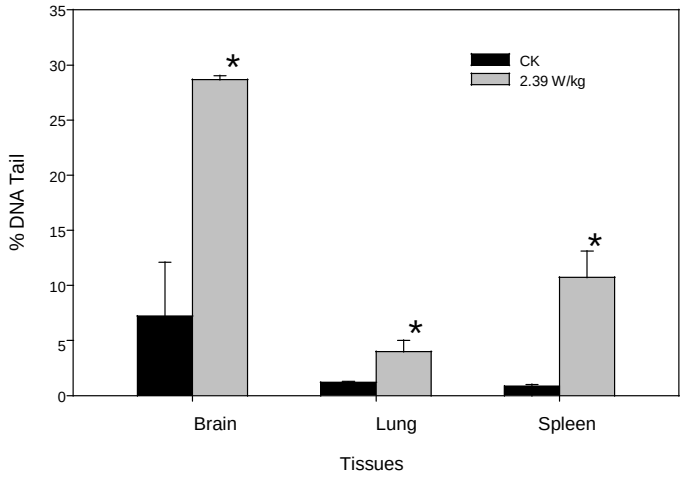

Figure 3. The mean ± SD of % DNA in tail before and after exposure to 2.45 GHz MW radiation. The error bars indicate the standard error of the mean (SEM) for N = 6 independent experiments. *indicates statistically significant difference compared with control ($p < 0.05$).

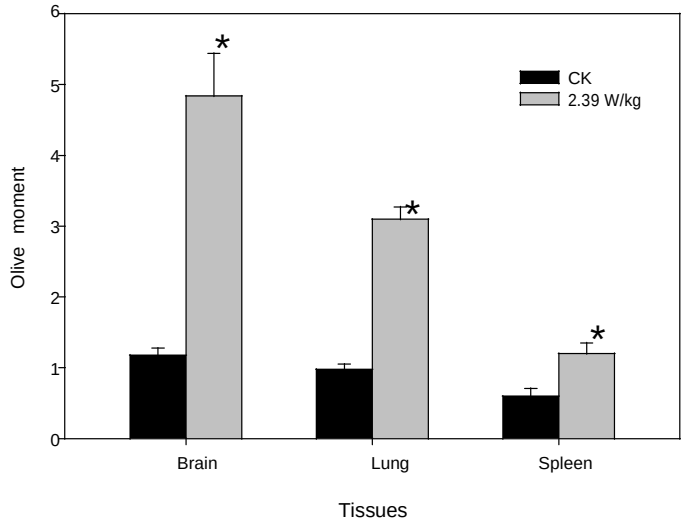

Figure 4. The mean ± SD of Olive moment before and after exposure to 2.45 GHz MW radiation. The error bars indicate the standard error of the mean (SEM) for N = 6 independent experiments. *indicates statistically significant difference compared with control ($p < 0.05$).

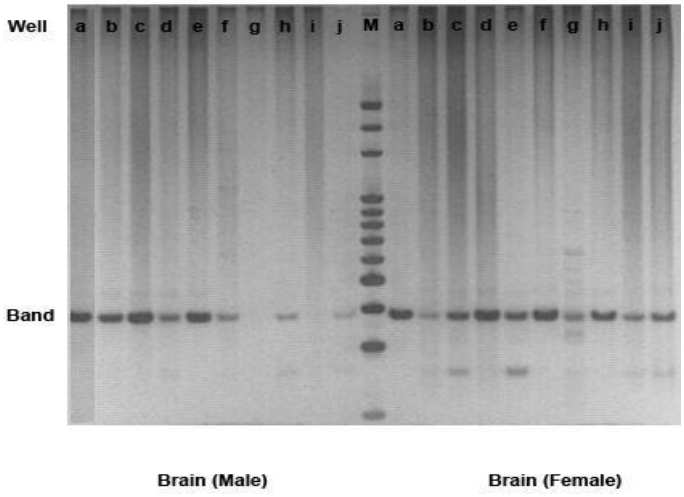

Plate 2. Hybridization of the brain DNA in male and female rats. Note the appearance of other bands in lane b – j (exposed animals). Lanes a-j have been tracked densitometrically for female (Figure 2B).

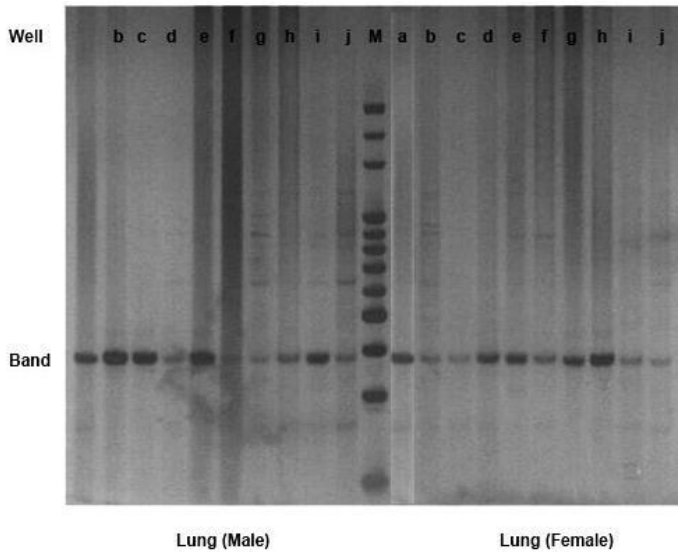

Plate 3. Hybridization of the lung DNA in male and female rats. Note the appearance of other bands in lane b – j (exposed animals). Lanes a-j have been tracked densitometrically for male (Figure 2C). (a) is control (b) 0.48, (c) 0.95, (d) 1.43, (e) 1.91, (f) 2.39, (g) 2.90, (h) 3.40, (i) 3.80 and (j) 4.30 Wkg^{-1}.

average SAR) to MW radiation compared to other tissues (Lai and Singh, 1996) in their studies reported that acute exposure (for 2 h) to both pulsed and continuous wave (CW) 2.45 GHz radiations (2 mW/cm^2, SAR 1.2 Wkg^{-1}) produced a significant increase in the DNA single and double strand breaks in rat brain. Maes et al. (1993) observed that acute 30–120 min exposure to 2450 MHz at SAR 75 Wkg^{-1} and constant temperature, 36.1°C increased dicentric and acentric chromosomal fragments and micronuclei formation in human lymphocytes.

Mitchell et al. (1988) showed a decrease in motor activity in rats after 7 h of exposure to CW 2450 MHz (10 mW/cm^2, average SAR 2.7 Wkg^{-1}). According to other studies (Dizdaroglu, 1992), MW radiation may cause both single and double strand breaks in the DNA molecule and this damage can be correlated to mutagenic and carcinogenic changes (Dizdaroglu et al., 2008). The present study is in agreement with these studies showing a significant difference in DNA single strand breaks in the exposed animals various cells. DNA damage is closely

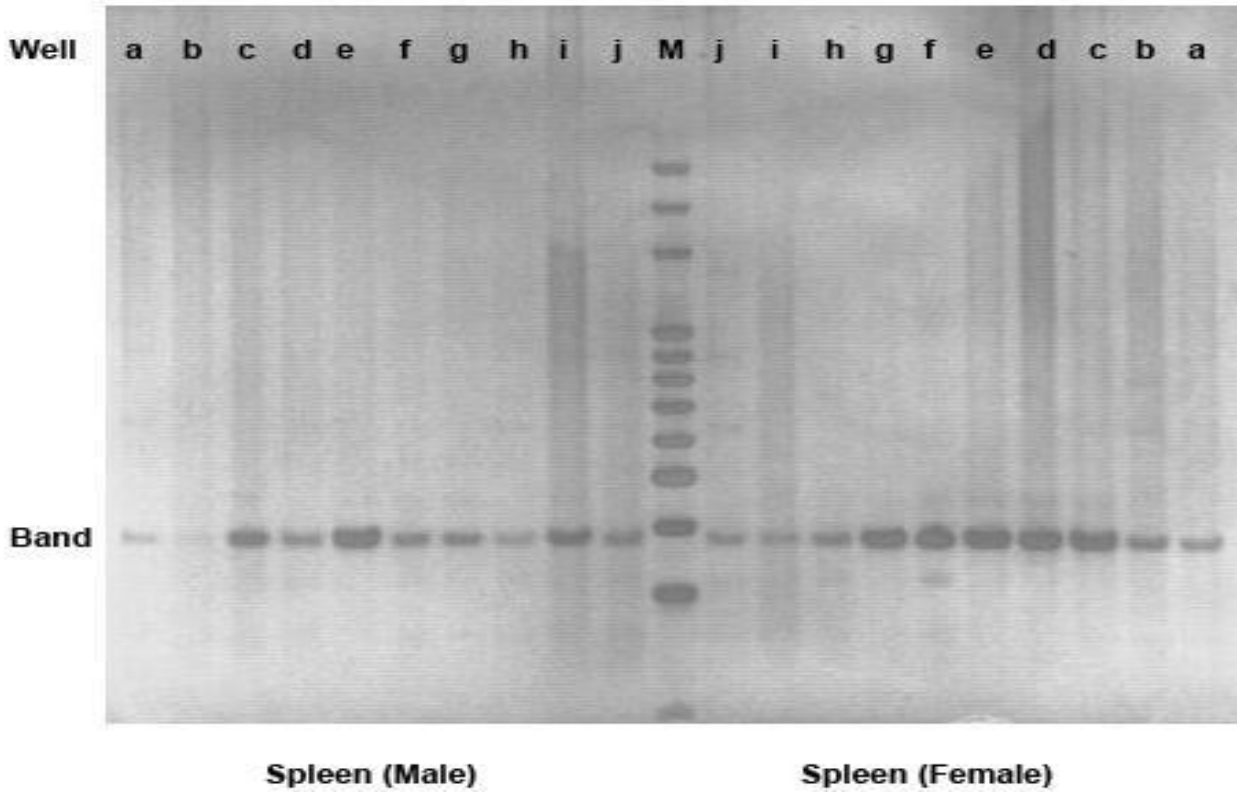

Plate 4. Hybridization of the spleen DNA in male and female rats. Note the appearance of other bands in lane b – j (exposed animals). Lanes a-j have been tracked densitometrically for female (Figure 2D). (a) is control (b) 0.48, (c) 0.95, (d) 1.43, (e) 1.91, (f) 2.39, (g) 2.90, (h) 3.40, (i) 3.80 and (j) 4.30 Wkg^{-1}.

related to human health risk. Particularly, DNA damage in brain cells could affect neurological functions and alsopossibly lead to neurodegenerative diseases (Ramel, 1989) . Integrity of genetic information is fundamental for living systems. It is therefore vital for cells that DNA damage induced by reactive oxygen species (ROS) such as free radicals or by other mutagens is effectively recognized and repaired efficiently (Dizdaroglu et al., 1998). 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 (Guy, 1987). It is obvious that MW is not able to induce genotoxic effects by direct interaction with DNA, because their intrinsic quantum energy (E = hv) is too low to dislodge an electron from a molecule. Among the putative mechanisms by which MW affect DNA, it is primarily by increasing free radical life span and the concentration of free radicals in cells (Hook et al., 2004; Yakus et al., 2008). Oxidative damage to DNA caused by free radicals, especially by the highly reactive hydroxyl radical, generates a multiplicity of modifications, which include modified bases and sugars, DNA- protein cross-links, base-free sites and strand breaks (d'Ambrosio, 2002). Among the many modified bases generated by free radicals in DNA, 8-OH-Gua is the most investigated lesion as a marker of cellular oxidative stress relevant to mutagenesis and carcinogenesis. This is because this compound has been shown to cause G -T transversions, and because it is readily measured as its nucleoside 8-OH-dG by a method using high-performance liquid chromatography (HPLC) with electrochemical detection (ECD) (Sargentini and Smith, 2002; Simko, 2004). However, 8-OH-Gua is not the only product resulting from oxidative damage to DNA. There is a plethora of other products that are formed with yields comparable to that of 8-OH-Gua (d'Andrea et al., 2003) 2. 2, 6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) 3 and 4, 6-diamino-5-formamidopyrimidine (FapyAde) are typical products in DNA resulting from hydroxyl radical reactions with purines (Evans et al., 2004). These lesions are often mutagenic and genotoxic, and have been implicated in the etiology of many diseases, including cancer and aging (Pilla, 1979). Due to various tissues or cell types having different average exposure levels (different average SAR) during exposure to electromagnetic field, contradictory results among the mammalian cell types have been reported (d'Ambrosio, 2002).

Conclusion

The effects of 2.45 GHz MW exposures at various low SAR

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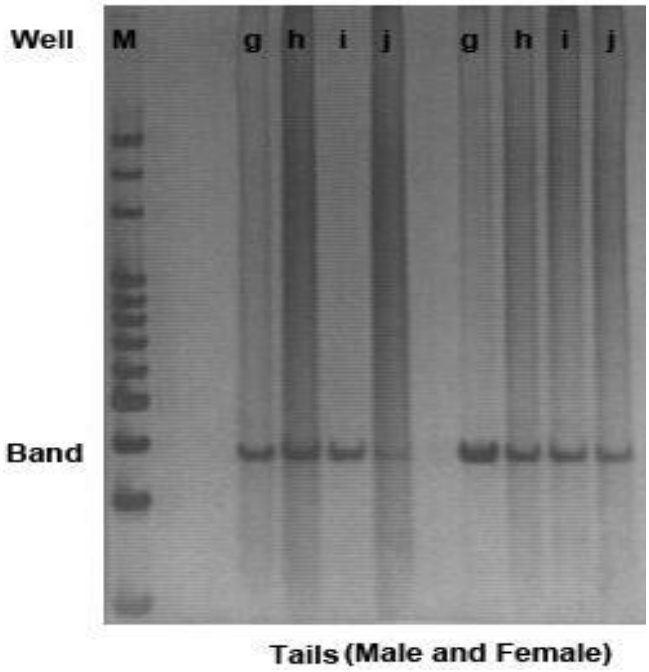

Plate 5. Hybridization of the control tails DNA before exposure to microwave in male and female rats. Lanes g - j for both sexes have been tracked densitometri-cally (Figure 1).

have been studied on the direct DNA genome analysis using very novel methods and very sensitive staining method. The results from DNA direct genome was equally affirmed by single cell gel electrophoresis. Single strand breaks were observed in the various cells studied via comet assay and consistent appearance of extra bands in the sequence of the exposed animals. These findings showed that exposure to 2.45 GHz MW radiation at SAR even as low as 0.48 Wkg^{-1} is potentially genotoxic as it produced single DNA strand breaks. These findings make us agree with Neil's (1999) extensive arguments and criticisms of the ICNIRP (1998) guidelines on MW and other non-ionizing radiation exposure limits. Mean-while, in order to minimize exposures and the eventual health detriments, strict compliance with all aspects of the guidelines personnel protection programs and medical surveillance is recommended, just like those recommended by the IAEA (1996) for ionizing radiation.

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