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Detection of Oxidative Stress Induced by Mobile Phone Radiation in Tissues of Mice using 8-Oxo-7, 8-Dihydro-2'-Deoxyguanosine as a Biomarker

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***Abstract*—**We investigated oxidative DNA damage caused by radio frequency radiation using 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxodG) generated in mice tissues after exposure to 900 MHz mobile phone radio frequency in three independent experiments. The RF was generated by a Global System for Mobile Communication (GSM) signal generator. The radio frequency field was adjusted to 25 V/m. The whole body specific absorption rate (SAR) was 1.0 W/kg. Animals were exposed to this field for 30 min daily for 30 days. 24 h post-exposure, blood serum, brain and spleen were removed and DNA was isolated. Enzyme-linked immunosorbent assay (ELISA) was used to measure 8-oxodG concentration. All animals survived the whole experimental period. The body weight of animals did not change significantly at the end of the experiment. No statistically significant differences observed in the levels of oxidative stress. Our results are not in favor of the hypothesis that 900 MHz RF induces oxidative damage.

***Keywords*—**Mice, Mobile phone radiation, oxidative stress, 8-oxo-7, 8-dihydro-2'-deoxyguanosine

I. Introduction

THE electromagnetic fields (EMF) were, are and will be a part of our life. There is now a very extensive scientific literature on the biological effects of RF resulting from mobile phones. However, a strong debate over the issue still exists; in many cases there are studies demonstrating effects and also others which have found no effect. On the one hand, several recent reports [1]-[2] presented adequate epidemiological evidence to suggest a link between prolonged mobile phone usage and development of brain tumor.

However, on the other hand, other researchers [3] concluded that the epidemiological literature pertaining to the use of mobile phones and head and neck tumor is not consistent and on balance does not provide enough evidence of an association.

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Furthermore, other findings [4]-[6] implicated the extensive use of mobile phones by males in potential harmful effects on their fertility and wellbeing of their offspring. In contrast, no significant changes in sperm motility in vitro were found [7].

A total of 101 publications on genotoxicity studies (effect on chromosomes, DNA fragmentation, and gene mutations) of RF-EMF were exploited [8], of which 49 reported genotoxic effects, 43 did not and 9 find that RF induces genotoxic events by itself but enhances the genotoxic action of other physical or chemical agents. These studies have been performed with a variety of different test systems – some studies used more than one test system. Examples showing no effects or no significant effects include the more recent studies [9]-[12].

Biological mechanisms that can explain the link between exposure to RF and possible harmful effects are still lacking. One of the proposed mechanisms is the stimulation of oxidative stress (formation of free radicals and DNA oxidatively generated damage) [5], [13]-[15]. The purpose of the present research was to investigate the extent of oxidative DNA damage caused by RF by measuring the level of 8-oxodG generated in mice tissues after exposure to 900 MHz mobile phone frequency. 8-oxo-7, 8-dihydro-2'-deoxyguanosine is a promutagenic DNA lesion induced by the reaction of hydroxyl radicals (•OH) with guanine at the C8 site in DNA [16]. 8-oxo-7, 8-dihydro-2'-deoxyguanosine is considered as a key biomarker of oxidative stress and carcinogensis [17] since it is a miscoding lesion causing misincorporation during replication and subsequently G to T transversions [18].

II. Materials and Methods

A. Animals

Thirty-six eight-week-old male BALB/c mice (average weight 26 g), obtained from the Animal House Production Unit of Yarmouk University, were used in this research (18 RF-exposed and 18 control, in triplicate of 6). The animals involved in this study were maintained and used in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals Prepared by Yarmouk University, Animal Ethical Committee. Mice were randomly placed in groups of six animals in plastic cages for 24 h before an

experiment in the room in which they would be exposed to mobile phone RF fields. The animal house was maintained on a 12/12 h light/dark cycle, at an ambient temperature of 22°C and a relative humidity of 65%. During the experiments, the animals had free access to water and normal chow.

B. In vivo RF exposure system

A Global System for Mobile Communication `signal source generator (DLW-3000 xp, Harvest General Trade Corp. Taiwan, Republic of China, Chung Ho City, Taipei) was used to generate the RF at 900 MHz. The antenna was located at about 5 cm next to the cage. Electromagnetic Radiation Monitor model 8616 and Isotropic probe model 8623D (Narda Microwave Corporation. Hauppauge, New York, USA) were used to measure the RF within the area where animals were restricted. The field within the exposure area was adjusted, on average, to be 25 V/m. This simulates the actual exposure levels from the current GSM mobile phones to the direction of the human tissues. Furthermore, the high frequency structure simulator (HFSS) software [19] was used to simulate the exposure environment. Fig.1 shows the uniformity of the electric field distribution within the area where animals were found. It is worth noting that the value of 25 V/m is equivalent to a specific absorption rate (SAR) value of 1.0 W/kg at 900 MHz [20].

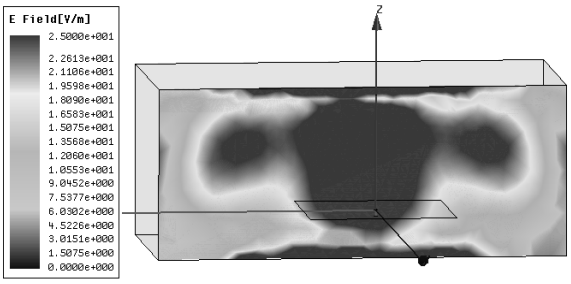

Fig. 1 HFSS analysis output of the electric field distribution inside the cage that was used during the exposure period

The control group was constructed so that the signal generator was switched off and placed close to the cage in which the animals were kept for the same period of time. All mice were observed for mortality and morbidity on daily basis. The body weight of each animal was recorded at the beginning and the end of the experiments.

C. Collection of tissues

On day 30, body weights of the animals were recorded. Twenty four h after the exposure period mice were killed humanely, brains and spleens were excised by a non traumatic technique. Blood serum samples were collected. All specimens were kept frozen at – 80 ° C for later use.

D. DNA extraction

Nuclear DNA was carefully extracted by a non enzymatic non-organic method [21] as modified later [22]. Prior to DNA extraction, the phenol step was omitted from the extraction procedure to reduce artifactual 8-oxodG formation [23]. The homogeneous solution was incubated at 56°C for 10 min, and 200 µl of 100% ethanol (Gainland Chemical Company, GCC, UK) were added to precipitate DNA. Briefly, 150 mg of brain tissue (50 mg of spleen) were chopped on ice cold glass and homogenized in 0.5 ml Tris-HCl-KCl-$MgCl_2$ (TKM) buffer (10 mM Tris-HCl [pH 7.6, Sigma, USA], 10 mM KCl, 10 mM $MgCl_2$, 2 mM ethylenediamine tetraacetic acid [EDTA, PARK Scientific Limited, Northampton, UK] for 30 sec at 4 °C using a homogenizer. The homogenate was suspended in 0.5 ml TKM buffer containing 2% Triton x-100 (PARK Scientific) and the tube was inverted several times to lyse the cells, then centrifuged for 10 min at 1000 g. The supernatant was discarded and the pellet was washed twice with TKM buffer. Then, the pellet was suspended in 100 µl TKM buffer containing 0.75% sodium dodecyl sulfate (SDS, PARK Scientific), mixed vigorously and incubated at 55 °C for 5 min. 300 µl of 6.0 M NaCl were added after incubation, mixed well and spun for 5 min at 12000 g. Two volumes of 100% ethanol were added to the separated supernatant and the pellet was washed twice with 70% alcohol and air-dried. After that the pellet was resuspended in 200 µl Tris- EDTA (TE) buffer pH 8.0 and incubated at 65 °C for 15 min. The quality of DNA samples was checked by agarose gel electrophoresis and purity assessed by recording the A260/280 and A 260/230 ratios. Final DNA concentrations were 50 to 200 ng/ml determined by minigel electrophoresis. The DNA samples were stored at -20 °C until later use.

E. DNA digestion

Following isolation, DNA samples were concentrated using DyNA Vap evaporator (Labnet, USA). The DNA was digested to nucleosides. Briefly, to 50 µg of DNA dissolved in 135 µl of water, 15 µl of 200 mM sodium acetate and 6 units of nuclease P1 (US Biological, USA) were added. The mixture was incubated for 1 h at 37 °C under Argon to prevent exposure to atmospheric air. Fifteen µl of Tris-HCl buffer (1 M, pH 7.4) and 2 units of alkaline phosphatase (Sigma) were added and the mixture was incubated for another 1 h under Argon. The sample was incubated at 37°C for 30 min to remove the phosphate groups from the nucleotides. Incomplete hydrolysis of DNA is usually responsible for underestimated values for 8-oxodG.

F. Measurement of 8-oxodG by ELISA

Some of the DNA samples were either contaminated or not sufficient for this assay, therefore only 13 to 15 values appear in table II. The levels of 8-oxodG were determined by the highly sensitive enzyme-linked immunosorbent assay (ELISA) kit (Northwest Life Science Specialities, USA). This

competitive in vitro ELISA is specially designed for measurement of 8-oxodG in biological tissues believed to have low concentrations of this biomarker. The indirect antibody technique like ELISA offers a valid and comparatively simple alternative to direct but more technically demanding high-performance liquid chromatography with electrochemical detector (HPLC-EC) method for quantitation of oxidative damage [7], [23]-26]. Previous results suggest good correlation between HPLC and ELISA [27].

Procedures followed manufacturer's instructions. First, the hydrolysate was filtered through a 10K microcentrifuge filter (Microcone, Millipore, MA, USA) at 4°C for 30 min. The type of filter and the prolonged centrifugation are required to produce a clear chromatogram with a smooth baseline.

50 µL each of a sample, a standard or phosphate- buffered saline (PBS, blank) were added to each well of a microtiter plate precoated with 8-oxodG. Then, 50 µl of 8-oxodG primary antibodies were added to each well except blanks. The plates were shaken gently, sealed and incubated at 4°C overnight. Following washing steps to remove the antibodies bound to 8-oxodG, 100 µl of secondary antibody were added to each well and the plate was incubated at room temperature for 1 h. After repeating the plate wash, 100 µl of chromogen ((3, 3', 5, 5') tetramethyl benzidene) were added to each well and incubated in the dark for 15 min.

Plates were read at 450 nm using a microplate reader (Stat Fax 3200, Awareness Technologies, USA). The concentration of 8-oxodG in each sample was determined by generating standard curves for each lot of assay reagents from standardized samples contained in each ELISA kit.

Statistical analysis: All experimental DNA samples were assayed in duplicates, and the mean value of ΔC_t was used in the statistical analyses. Results are expressed in ng/ml.The one-way ANalysis Of VAriance (ANOVA) test was employed to statistically evaluate the collected data using PASW statistics 18 software package, (formerly called SPSS).

III. RESULTS

All animals (RF-exposed and control) survived the whole experimental period. Furthermore, the body weight of animals did not change significantly at the end of experiments as compared to the starting weights. Also, when the RF- exposed and control groups are compared, RF exposure did not cause statistically significant ($p = 0.05$) changes in the body weight of mice (Table I).

TABLE I
MEAN PERCENT MICE WEIGHT DIFFERENCES OF THE THREE EXPERIMENTAL TRIALS (6 MICE EACH) AT THE END OF EXPOSURE PERIOD (30 DAYS) TO RADIO FREQUENCY RADIATION AT 900 MHz

Experiment Number	Mean Percent Weight Difference	
	Exposed	Control
1	14.95	16.63
2	16.44	18.70
3	14.13	17.32

Fig. 2 demonstrates the complete digestion of the DNA samples after treatment with nuclease P1. Statistical analysis indicated no large variability within the data collected from any group. Therefore, corresponding data for animals of each group were pooled.

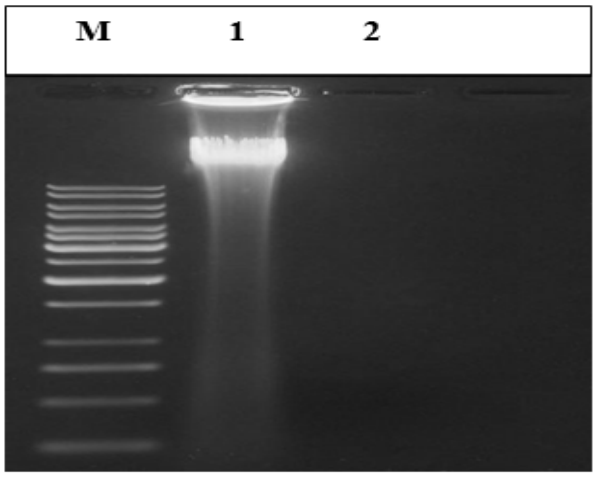

Fig. 2 Genomic DNA samples in agarose gel stained with ethidium bromide. Lane M: 1kb DNA ladder (0.1µg/µl). Lane 1: DNA sample from mouse brain before digestion. Lane 2: DNA sample from mouse brain after treatment with nuclease P1 (Magnification is 2X)

In respect to the levels of oxidative stress, expressed as ng/ml of 8-oxodG in the reaction mixture, statistical analysis showed no significant differences (P=0.05) within the three experiments of a tissue under study. The only exception to this was the first experiment of serum (P=0.003). Here again, the results of the three experiments for each tissue were pooled (Table II). Fig. 3 compares the concentrations of 8-oxodG in the brain, spleen and the serum.

TABLE II
UNITS POOLED DATA OF OXIDATIVE STRESS EXRESSED AS NG/ML OF 8-OXODG, FOR THE THREE EXPERIMENTAL TRIALS, IN THE THREE TISSUES FROM RF-EXPOSED AND CONTROL BALB/C MICE FOR 30 DAYS (900MHZ, SAR OF 1.0 W/KG)

Brain		Spleen		Serum	
Exposed	Control	Exposed	Control	Exposed	Control
1.051	1.157	1.201	1.190	1.173	0.949
1.073	1.107	1.206	1.199	0.993	0.970
1.066	1.072	1.214	1.090	0.976	0.950
1.135	1.207	1.234	1.197	0.984	0.922
1.052	1.191	1.141	1.135	0.993	0.983
1.132	1.109	1.184	1.251	0.906	0.989
1.127	1.211	1.270	1.279	1.007	0.960
1.104	1.204	1.184	1.169	0.976	0.991
1.143	1.083	1.230	1.232	1.001	0.976
1.123	1.164	1.132	1.244	1.001	0.955
1.123	0.894	1.278	1.251	0.987	0.968
1.124	1.017	1.148	1.086	0.966	1.018
1.059	1.037	1.266	1.281	1.022	0.957
N.D.	0.940	1.164	1.275	0.995	0.921
N.D.	1.328	1.074	N.D.	0.984	1.009

N.D: NOT DETERMINED

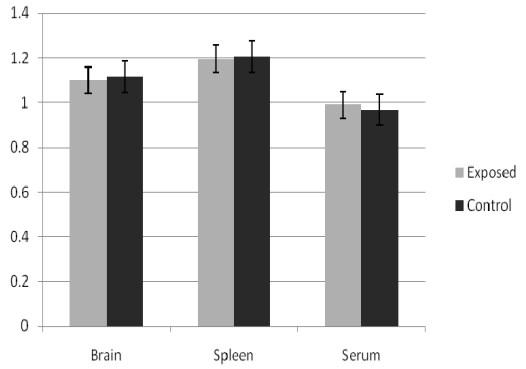

Fig. 3 Average levels of 8-oxodG expressed as nanogram per milliliter reaction mixture in the three tested samples. Error bars indicate the standard error of mean (SEM) for N = 3 independent experiments

IV. Discussion

The duration period for mobile phone use and its frequency are important factors. In this study, we have estimated average time of analogue like mobile phone device for an individual in a day as half h. The GSM telephones use frequencies between 850 and 950 MHz. The biomarker 8-oxodG is an oxidized metabolite of 2'-deoxyguanosine released after digestion of DNA repairing enzyme. Measurement of 8-oxodG is an effective method in determining the effect of endogenous oxidative damage to DNA and as a factor of initiation and promotion of carcinogenesis [17], [28]. With respect to oxidative stress induced by mobile phone radiation in tissues, to our knowledge, only few studies have been done, therefore, our results may constitute a reference for future studies. It is interesting to note that investigators often report artificially high levels of 8-oxodG (up to 20-fold) in DNA samples that have been exposed to phenol/chloroform solutions and/or air during processing [23], [29]. In the present study, these conditions have been avoided to provide more quantitative and realistic results. Additionally, we have used relatively large amounts of DNA (100 μg) to reduce the contribution of artifactual 8-oxodG formation during DNA extraction that has been reported previously [30]-[32].

It is conceivable that RF radiation-induced oxidative stress caused mitochondrial dysfunction through damage to mitochondrial DNA (mtDNA). In turn, reduced mitochondrial function might further exacerbate the production of reactive oxygen species (ROS), which could induce further oxidative damage to mtDNA. This vicious cycle might proceed until mitochondrial failure and apoptosis occurred. Indeed, recent data showed that exposure to RF decreased cerebral cytochrome oxidase activity and activated apoptosis signaling pathways in primary cultured neurons [33]. On the contrary, incomplete hydrolysis of DNA is usually responsible for underestimated values for 8-oxodG [34]. This possibility is excluded in the present study as shown in Fig. (2).

In New Zealand White rabbits, whole body exposure (15 min/day for a week) to1800 MHz GSM-like RF, the formation of radical molecules in hepatocytes did not appear to significantly increase as evidenced by sensitive detection for 8-oxodG in cellular DNA by (HPLC-EC) [35]. The results of this work are consistent with our finding. In contrast, other researchers [36] exposed primary cultured cortical neurons to pulsed electromagnetic fields at a frequency of 1800 MHz modulated by 217 Hz at an average SAR of 2.0 W/kg. After 24 h exposure, they found that RF induced a significant increase in the levels of 8-oxodG in the mitochondria of neurons. Similarly, exposure of purified human spermatozoa to RF-EMR tuned to 1.8 GHz and covering a range of SAR from 0.4 W/kg to 27.5 W/kg significantly elevated the mitochondrial generation of radical molecules or ROS (increased 8-oxodG concentration) and DNA fragmentation [5]. The latter two studies have employed in vitro system whose data can not be extrapolated to the in vivo system without reservations.

It should be remembered that the specificity of the monoclonal antibody used in ELISA measurement of 8-oxodG has been questioned due to possible cross-reactivity of the antibodies raised against oxidized nucleosides including 8-oxodG with the corresponding normal nucleosides at a frequency comprised between 10^{-4} and 10^{-5} [26], [37]-38]. Usually, ELISA measurements are performed on whole DNA after its extraction from cells. In the present study, DNA has been enzymatically digested into 2'-deoxyribonucleosides. No attempts were made to check in model studies how increasing amounts of 2'-deoxyguanosine may interfere or not with the ELISA detection of very low amounts of 8-oxodG as it would be the case in cellular DNA. Thus, further investigations are needed to validate the ELISA measurements that are reported in this study. Another drawback of ELISA measurements deals with the semi-quantification of oxidatively generated damage to DNA due to the lack of suitable calibration of the immunoassay. This would prevent accurate comparison of the yields of 8-oxodG that were recorded in different samples. Still, the low levels of 8-oxodG measured in the present investigation could be due to the low accumulation of 8-oxodG resulting from an efficient repair system in the tissues under study. This possibility can be ruled out by using cells in which base excision repair devoted to 8-oxodG is reduced as it is the case in Ogg1 knockout mice [39]. Currently, we are working on the immunodetection of 8-oxodG after shorter periods (every 30 min for four h) of exposure to 900 MHz radiofrequency. Alternatively, small changes in the levels oxidized purine bases can be followed by the modified alkaline comet assay which involves a pre-incubation step of released DNA with bacterial formamidopyrimidine DNA glycosylase that converts 8-oxodG and a few other modified purines into DNA strand breaks [40].

Our data demonstrated that exposure of mice to RF at whole body SAR of 1.0 W/kg did not affect survival of animals. This finding is consistent with [41] who reviewed 18 studies and reached the same conclusion. Furthermore, the

results concerning the absence of significant differences between the body weights of RF-exposed and control mice agree with those of others [42] who reported that exposure to RF, at levels seen from base stations, for 8 days, 7 h per day, did not cause any differences in mean weights of sham and exposed rats. Similarly, adult male Wistar rats sub-chronically exposed to radiation emitted from a conventional cell phone (for 1 h daily during 11 weeks, 1835–1850 MHz at 0.04–1.4 mW/cm^2) did not show significant changes in total body weight and absolute and relative testicular and epididymal weights [43]. Also, intermittent exposure of C57BL mice heads to 849 MHz or 1763 MHz of mobile phone RF (7.8 W/kg for a maximum of 12 months), did not show any differences the body weights of 3 groups-sham, 849 MHz RF, and1763 _ MHz- exposed [44]. In contrast, electromagnetic field exposure (GSM like 900 MHz electromagnetic fields for 24 h per day, 7 days per week) of female AKR/J mice had a significant effect on body weight gain, with higher values in exposed than in sham-exposed animals [45]. However, survival rate and lymphoma incidence did not differ between exposed and sham-exposed mice. In this regard, it was reported [46] that exposure of adult male Djungarian hamsters (*Phodopus sungorus*) for 24 h/day for 60 days to radio frequency electromagnetic fields (RF-EMF) at 383, 900, and 1800 MHz, modulated according to the TETRA (TErrestrial Trunked RAdio) (383 MHz) and GSM standards (900 and 1800 MHz), may result in metabolic changes which eventually cause body weight increases in exposed animals.

V. Conclusions

The present results indicate that 900 MHz RF radiation did not induce significant levels of oxidative damage under the present experimental conditions. However, it is difficult to directly extrapolate our data (which were obtained using rodent cells) to humans for different exposure conditions and SAR values because the entire body of a mouse is exposed whereas for a person using a mobile phone, only the skin near the region that is close to the telephone would be exposed. Our data are not consistent with the hypothesis that oxidative damage to important macromolecules is a potential common pathophysiological mechanism underlying exposure to cell phone radiation [47]-[49]. However, it should be recalled that measurement of 8-oxodG alone does not reflect the total amount of damage induced in DNA by oxidative stress [50]. Thus, future work would be much more compelling with different frequencies and exposure periods using some other well-accepted markers for oxidative injury, such as markers for lipid or protein oxidation studies. Studies including multiple oxidative damage markers to different macromolecules using different types of tissues from different species are being carried out in our laboratory.

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