900 MHz pulse-modulated radiofrequency radiation induces oxidative stress on heart, lung, testis and liver tissues

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Abstract. Oxidative stress may affect many cellular and physiological processes including gene expression, cell growth, and cell death. In the recent study, we aimed to investigate whether 900 MHz pulse-modulated radiofrequency (RF) fields induce oxidative damage on lung, heart and liver tissues. We assessed oxidative damage by investigating lipid peroxidation (malondialdehyde, MDA), nitric oxide (NO_x) and glutathione (GSH) levels which are the indicators of tissue toxicity. A total of 30 male Wistar albino rats were used in this study. Rats were divided randomly into three groups; control group ($n = 10$), sham group (device off, $n = 10$) and 900 MHz pulsed-modulated RF radiation group ($n = 10$). The RF rats were exposed to 900 MHz pulsed modulated RF radiation at a specific absorption rate (SAR) level of 1.20 W/kg 20 min/day for three weeks. MDA and NO_x levels were increased significantly in liver, lung, testis and heart tissues of the exposed group compared to sham and control groups ($p < 0.05$). Conversely GSH levels were significantly lower in exposed rat tissues ($p < 0.05$). No significantly difference was observed between sham and control groups. Results of our study showed that pulse-modulated RF radiation causes oxidative injury in liver, lung, testis and heart tissues mediated by lipid peroxidation, increased level of NO_x and suppression of antioxidant defense mechanism.

Key words: Radiofrequency radiation — Oxidative stress — Malondialdehyde — Glutathione — Nitric oxide

Abbreviations: GSH, gluthatione; MDA, malondialdehyde; RF, radiofrequency; ROS, reactive oxygen species.

Introduction

Oxidants can be generated in numerous exogenous ways such as chemicals and radiation exposure through redox-catalysis and encompass reactive oxygen species (ROS), reactive nitrogen species (RNS), sulfur-centered radicals and various others (Abuja and Albertini 2001). Lipid peroxidation is the deleterious consequence of oxidative damage which involves hydrogen abstraction from fatty acids by free radicals. Malondialdehyde (MDA), the end product of the lipid peroxidation is a highly reactive three carbon dialdehyde and one of the most intensively aldehydes formed during lipid per-

oxidation. RNS, such as nitric oxide (NO), a free radical and peroxynitrite ($ONOO^-$) that is an extremely potent cellular oxidant produced with the reaction of NO with superoxide ($O_2^{\bullet-}$) act together with other ROS to damage cells, causing nitrosative stress (Squadrito and Pryor 1998). In a healthy human body, the generation of pro-oxidants in the form of ROS and RNS are effectively kept in balance by the various levels of antioxidant defense (Devasagayam et al. 2004). However, when it gets exposed to adverse environmental agents, this delicately maintained balance is shifted in favor of pro-oxidants resulting in oxidative stress. Sub-lethal levels of oxidative stress may activate and inactivate transcription factors, membrane channels, and metabolic enzymes and promote calcium-dependent and phosphorylation signaling pathways (Winterbourn and Hampton 2008).

Scientists have been conducting experimental studies on whether radiofrequency (RF) radiation emitted from cellular

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mobile phones induces oxidative stress on biological tissues or cells and others developed theoretical explanations how these fields could impair free radical re-combinations (WHO 2006 Geneva). Moreover, some authors suggested that mechanism of RF/MW (microwave) radiation damage may be attributable to the induction of oxidative stress leading to free radical generation and radical scavengers' alterations in biological tissues or cells as a result of the interaction of the tissues or cells with RF/MW fields (Cleary et al. 1996; Diem et al. 2005). According to the current assumption electromagnetic (EM) fields may initiate oxygen- and nitrogen-free radical intermediates and several types of tissue injury may be associated with oxidative stress process induced by these fields. EM fields may affect biological cells by interacting electrons during redox reactions (Blank 2008).

Although some authors pointed out the possibility that RF radiation could affect the concentration of free radicals through the radical pair recombination thus increase the oxidant stress on cells or tissues, studies on some specific tissues are very limited. So we aimed to investigate whether low intensity 900 MHz-modulated RF radiation induces oxidative damage on lung, heart, liver and testis tissues by assessing the levels of lipid peroxidation and glutathione (GSH) on these tissues. We also measured total NO (NO_x) levels to determine biochemical damage in tissues as an additional parameter to lipid peroxidation and antioxidant level.

Materials and Methods

Animals and treatments

Two months old male Wistar albino rats were used in this study. The animals were acclimatized in the temperature-controlled conditions (22 ± 1°C) and humidity (50 ± 10%) with light/dark cycles of 12 h and had free access to food and drinking water. None of the animals died during the experiment. Rats were divided randomly into three groups; group I: control group ($n = 10$), group II: sham group ($n = 10$) and group III: RF radiation group ($n = 10$). The RF rats were exposed to 900 MHz pulsed-modulated RF radiation 20 min/day for three weeks. The sham rats were settled in the exposure cage and applied the same experimental procedure of the exposed group rats, but the exposure device was off. Sham- and RF-exposed animals were kept in the same conditions as the control ones when sham and RF exposures were not performed. At the end of the last day of the exposure, rats were sacrificed by injection of ketamine (50 mg/kg) and xylazine (5 mg/kg) combination. The investigation was performed following European Community guidelines and approval by the ethics committee of Gazi University Faculty of Medicine.

Radiofrequency radiation exposure system

A rectangular horn antenna (Ets-Lindgren, St. Louis, MO, USA) was used as a transmitting antenna which generates RF signals inside polymethyl methacrylate plastics cages (15 cm × 20 cm × 20 cm) where the rats were restrained. Antenna was fed by a properly selected RF power generator (Agilent Technologies, Santa Clara, CA, USA) that delivers 900 MHz carrier RF waves and connected to a power amplifier (Hittite, Chelmsford, MA, USA) which amplifies the RF signals. The signals were pulse-modulated by rectangular pulses (repetition frequency 217 Hz, pulse width 0.576 ms) by using a function generator (Thandar, Cambridgeshire, UK). Cages were placed symmetrically along the axis that is perpendicular to the antenna (Fig. 1) and constantly ventilated to eliminate the external stress and avoid the possibility of any temperature increase inside them.

The applied electric field was measured by using an electric field isotropic probe along the horn antennas axis. At the beginning of exposure, average power density was observed at a reference point which was the midpoint of the bottom of the cage wall facing horn the antenna. The maximum power density was on the axis of the antenna and it was observed that the power density was decreased uniformly with the distance from the antenna's axis. Whole body specific absorption rate (SAR) was approximately calculated according to the formulary of SAR = $\sigma/\rho \cdot |E_{RMS}|^2$ (W/kg). Where σ is the mean electrical conductivity of the whole body (S/m), ρ is the mass density (kg/m^3) and E_{RMS} is the root mean square value of the electric field (V/m). The whole body average SAR was calculated to be 1.20 W/kg.

Measurement of tissue lipid peroxidation and GSH levels

Tissue samples were obtained after measuring lesion areas and frozen immediately by liquid nitrogen then kept in
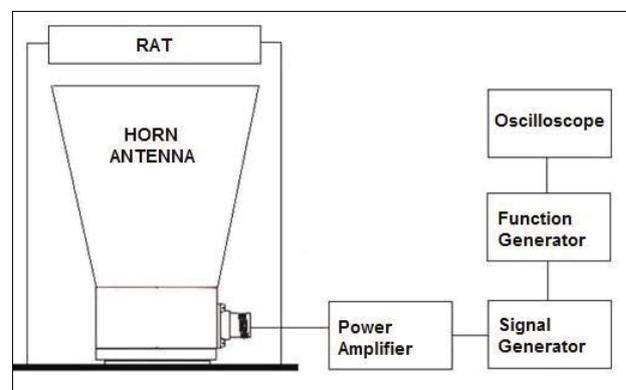
Figure 1. Schematic diagram of the RF exposure system.

–70°C deep-freeze until the assay. Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS). Samples were homogenized in ice-cold trichloroacetic acid (1 g tissue in 10 ml 10% trichloroacetic acid) in a tissue homogenizer (Heideloph Diax 900, Germany). Following centrifugation of the homogenate at $3000 \times g$ for 10 min (Hermle Z 323 K, Germany), 750 μl of supernatant was added to an equal volume of 0.67% (m/v) thiobarbituric acid and heated at 100°C for 15 min. The absorbance of the samples was measured at 535 nm. Lipid peroxide levels are expressed in terms of MDA equivalents using an extinction coefficient of 1.56×10^5 l·mol^{-1}·cm^{-1}.

The GSH levels were determined by Ellman method with some modifications. Briefly, after centrifugation of the homogenates at $3\,000 \times g$ for 10 min, 0.5 ml of supernatant was added to 2 ml of 0.3 M $Na_2HPO_4\cdot2\,H_2O$ solution. A 0.2 ml solution of dithiobisnitrobenzoate (0.4 mg/ml 1% sodium citrate) was added and after mixing, the absorbance at 412 nm was measured using a spectrophotometer (UV 1208, Shimadsu, Japan) at room temperature immediately. The GSH levels were calculated using an extinction coefficient of 13 600 l·mol^{-1}·cm^{-1}.

Determination of NO_x (Griess assay)

NO_x levels were obtained from Elisa reader by vanadium chloride (VCl$_3$)/Griess assay. Before NO_x determination, tissues were homogenized in five volumes of phosphate buffer saline (pH = 7) and centrifuged at $2000 \times g$ for 5 min. After centrifugation, 0.25 ml of 0.3 M NaOH was added to 0.5 ml supernatant. The incubation of the samples for 5 min at room temperature was followed by addition of 0.25 ml of 5% (w/v) $ZnSO_4$ for deproteinization. This mixture was then centrifuged at $3000 \times g$ for 20 min and supernatants were used for the assays. Nitrate standard solution was serially diluted and the plates were loaded with samples (100 μl). Then Vanadium III chloride (VCl3) (100 μl) and Griess reagents sulphanilamide (SULF) (50 μl) and N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD) (50 μl) were added to each well. After incubation in 37°C for 45 min, samples were measured at 540 nm using ELISA reader (Miranda et al. 2001).

Statistical Analysis

All data were presented as mean values ± SD. Differences among three groups were analyzed by one way analysis of variance (ANOVA). Mann-Whitney U test was used for pairwise comparisons among groups. The accepted level of significance was set at $p < 0.05$. Data were analyzed with statistical package (SPSS) version 13.0.

Results

The MDA levels of heart, liver and lung tissues

Tissue MDA level of liver (Table 1) and lung (Table 2) tissues was significantly higher ($p < 0.001$) in RF-exposed group in comparison with sham and control tissues. A significant increase was observed also in heart MDA level of RF-exposed rats compared to sham and control rats ($p < 0.05$) (Table 3). The most dramatic increase in the MDA level of the tissues was observed in testis tissue. MDA level of testis was marginally higher in RF exposed group than sham and control groups ($p < 0.001$) (Table 4). No significant difference was observed in terms of tissue MDA level between sham and control groups in each tissue.

NO_x levels in heart, liver and lung tissues

Liver NO_x level was significantly higher in RF-exposed group than sham and control groups ($p < 0.001$) (Table 1). The level of NO_x increased significantly in RF-exposed lung tissues ($p < 0.01$) (Table 2). NO_x level of heart increased in RF-exposed group compared to sham and control groups ($p < 0.001$) (Table 3). Tissue level of NO_x was quite high in RF-exposed testis tissues ($p < 0.001$) (Table 4). No statistically difference was seen between sham and control groups.

The GSH levels of heart, liver and lung tissues

GSH level of liver decreased following to RF exposure in exposed group compared to sham and control groups ($p < 0.001$) (Table 1). Lung GSH level reduced significantly in RF-exposed group ($p < 0.01$) (Table 2). A significant decrease in GSH level of heart was observed in RF-exposed group in comparison with sham and control groups ($p < 0.001$) (Table 3). Exposure to RF radiation reduced GSH level of testis tissues about 50% compared to those of sham and control groups ($p < 0.001$) (Table 4). There was no statistically significant difference in GSH levels between sham and control groups.

Discussion

Increasing evidence has indicated that RF radiation has the potential of inducing oxidative stress in biological systems *via* free radicals by enhancing lipid peroxidation and reducing antioxidant levels. In the recent study, we showed that chronic exposure to 900 MHz pulse-modulated RF radiation can cause lipid peroxidation, nitrogenic stress and antioxidant suppression in various tissues. MDA levels of liver, heart, lung and testis tissues increased significantly in

Table 1. Mean liver tissue levels of MDA, total NO and GSH in control, sham and RF-exposed groups

Group	MDA (nmol/g)	NO_x (μmol/g)	GSH (nmol/g)
control	9.02 ± 1.1	12.44 ± 1.96	3.36 ± 0.45
sham	9.31 ± 0.91	11.35 ± 1.42	3.14 ± 0.31
RF-exposed*	14.78 ± 1.2	16.00 ± 2.99	2.09 ± 0.26

The values are means ± SD; $n = 10$. * $p < 0.01$, RF-exposed *vs.* control or sham groups.

Table 3. Mean heart tissue levels of MDA, total NO and GSH in control, sham and RF-exposed groups

Group	MDA (nmol/g)	NO_x (μmol/g)	GSH (nmol/g)
control	6.07 ± 1.11	7.83 ± 1.39	1.84 ± 0.1
sham	5.93 ± 0.86	6.96 ± 1.37	1.72 ± 0.29
RF-exposed*	7.3 ± 0.93	13.09 ± 2.6	1.01 ± 0.11

The values are means ± SD; $n = 10$. * $p < 0.01$, RF-exposed *vs.* control or sham groups.

Table 2. Mean lung tissue levels of MDA, total NO and GSH in control, sham and RF-exposed groups

Group	MDA (nmol/g)	NO_x (μmol/g)	GSH (nmol/g)
control	10.95 ± 2.56	16.21 ± 1.63	3.24 ± 0.27
sham	11.45 ± 2.16	15.23 ± 1.56	3.22 ± 0.34
RF-exposed*	23.62 ± 4.14	19.97 ± 2.71	2.99 ± 0.25

The values are means ± SD; $n = 10$. * $p < 0.01$, RF-exposed *vs.* control or sham groups.

Table 4. Mean testis tissue levels of MDA, total NO and GSH in control, sham and RF-exposed groups

Group	MDA (nmol/g)	NO_x (μmol/g)	GSH (nmol/g)
control	6.48 ± 0.93	9.92 ± 1.72	4.1 ± 0.37
sham	6.49 ± 0.92	11.87 ± 2.07	4.14 ± 0.42
RF-exposed*	15.11 ± 1.37	32.99 ± 3.83	2.25 ± 0.39

The values are means ± SD; $n = 10$. * $p < 0.01$, RF-exposed *vs.* control or sham groups.

RF group compared to sham and control groups. Similarly tissue NO_x levels were significantly higher in RF-exposed tissues. However, GSH levels showed significant reduction in exposed tissues. The results of our study verified the hypothesis that RF radiation may cause oxidative damage in biological tissues. Moreover, we observed that the magnitude of the damage depended on type of the tissue.

The change in MDA and GSH levels in exposed rats reflected pathophysiological effects of pulse modulated RF fields on liver tissue. Oxidative stress may accelerate the peroxidation reactions of lipids in liver (Yasa et al. 1999). It has been reported that patients diagnosed with degenerative liver diseases have higher level of lipoperoxide in their liver tissues and several forms of liver diseases have been shown to be associated with oxidative tissue injury (Suematsu et al. 1981). Dasdag et al. (2008) reported increased levels of MDA and of the total oxidant status in liver tissue of the Wistar albino rats exposed to 900 MHz RF radiation. However, they found no significant endpoints in terms of catalase, myeloperoxidase, total antioxidant capacity levels. Koyu et al. (2005) investigated the effects of MW radiation on the liver oxidant/antioxidant system, and the possible protective effects of caffeic acid phenethyl ester (CAPE) on liver tissue. They observed increased level of lipid peroxidation and decreased activity of GSH-Px in the liver tissue of the 1800 MHz RF radiation exposed rats. Moreover the authors noted that CAPE prevented the oxidative injury in liver tissue due to RF radiation exposure. The significant increase in lipid peroxidation level in exposed rat liver tissues may be due to increase of NO_x level in the recent study. Overproduction of NO is one of the most important sources of oxidative

damage in liver (Beckman et al. 1990). The reaction of NO with $O_2{}^{•-}$ can produce the $ONOO^-$ which can cause lipid peroxidation.

The results of our study also showed that the levels of MDA and NO_x may be increased in heart tissue due to RF radiation exposure. These indicate that RF radiation may act as a stressor on heart tissue as well. Our results were consistent with those of Ozgüner et al. (2005), who observed increased level of NO and MDA in rat myocardial tissue as a result of the exposure to 900 MHz RF radiation. NO is a major intracellular messenger synthesized by NO synthetase in vascular endothelium and converted into more stable forms nitrite ($NO_2{}^-$) and nitrate ($NO_3{}^-$) in plasma. It mediates a variety of biological functions including cardiovascular homeostasis, yet overproduction of NO has been implicated as a cytotoxic factor in a variety of pathophysiological processes. Olsen et al. (1977) observed bradycardia in isolated and perfused rat hearts that were maintained in 20°C and exposed to 960 MHz continuous MW radiation.

Lung is a major ROS production organ having a large surface that is constantly in contact with pollutants. This makes lung to need a strong antioxidant defense system to protect it against oxidative/nitrosative stressors. The oxidant/antioxidant imbalance in lung may activate redox-sensitive transcription factors which regulate the protective antioxidant genes. GSH is a major extracellular protective antioxidant against oxidative/nitrosative stresses, which plays a key role in the control of pro-inflammatory processes in the lung. The decrease GSH level in the lung of exposed rats showed that this protective mechanism was reduced due to RF radiation exposure. The low level of GSH in lung tissues

of the RF-exposed rats may be associated with increase of the production of lipid peroxidation and NO. It is known that increased level of ROS can alter the GSH redox state. Moreover, increased level of lipid peroxidation impairs membrane function and inactivates membrane-bound receptors and enzymes that result in increase in the tissue permeability which has been implicated in the pathogenesis inflammatory lung disorders (Rahman and MacNee 2000).

The most dramatic increase in lipid peroxidation and decrease in antioxidant levels of the RF-exposed rats were observed in testis tissue. The obtained results indicated that 900 MHz pulse-modulated RF radiation exposure is quite detrimental on testis tissue and RF fields induce an unbalance between production and the neutralization of prooxidant and antioxidant processes and cause oxidative damage in testis. Similar to our study, Mailankot et al. (2009) demonstrated increased lipid peroxidation and reduced GSH content in the testis and epididymis of 10–12 weeks old male Wistar rats exposed to GSM mobile phone radiation 1 h/day for 28 days. They also observed reduced percentage of motile sperm. De Iuliis et al. (2009) found increased level of ROS and higher DNA damage due to RF radiation exposure. Agarwal et al. (2009) reported significant increase in ROS level and significant decrease in ROS-TAC score in unprocessed ejaculated human semen which were taken from each patient that were exposed to cellular phone radiation for 1 h.

Due to rich polyunsaturated fatty acid content human spermatozoa plasma membranes are highly sensitive to ROS-induced damage. It is considered that lipid peroxidation is the key factor in ROS-induced sperm damage leading to decrease in sperm count, loss of motility, abnormal morphology and reduced capacity of sperm oocyte penetration and infertility (Sikka 1996; Storey 1997). There is strong clinical evidence that men diagnosed with infertility have significantly higher levels of ROS (Pasqualotto et al. 2000). Some of the reports have suggested a possible linkage between mobile phone usage and male infertility (Baste et al. 2008). Decreased sperm count and capacity of quails were reported by McRee et al. (1983) as a result of MW exposure. Cleary et al. (1996) showed that RF radiation may reduce fertilizing capacity of spermatozoa. Agarwal et al. (2009) suggested that RF EMR-induced oxidative stress in semen may be responsible for decreased sperm motility and viability.

We observed an enormous increase in the level of NO_x in testis tissue due to pulse-modulated RF radiation exposure for 21 days. Tissue level of NO_x was marginally higher in RF-exposed testis tissues (32.99 μmol/g) than sham (11.87 μmol/g) and control tissues (9.92 μmol/g). Although a continent amount of NO in seminal plasma is essential for normal reproductive function and sperm motility, at supra physiological levels it may lead to testicular abnormalities by inhibiting sperm production and motility (Rosselli et al. 1995; Donnelly et al. 1997; Balercia et al. 2004) through formation of

ONOO⁻ (Kisa et al. 2004). Human studies showed that infertile males have higher concentration of NO in seminal plasma than the healthy males (Nobunago et al. 1996). Furthermore, high amount of NO in seminal plasma is an important factor in sperm DNA damage (Saleh et al. 2002).

In the recent study, we showed that 900 MHz pulse-modulated RF radiation is capable of inducing oxidative stress. This induction was mediated by increase of lipid peroxidation and the reduction of GSH. The study also gave evidence that RF radiation might enhance NO production. The results of our study are evident that pulse-modulated RF emissions may act as an environmental stressor and cause oxidative and nitrogenic damage in lung, liver, testis and heart tissues. Oxidative stress is known to underlie many human diseases including atherosclerosis, heart failure, myocardial infarction, chronic liver and lung diseases, infertility, etc. Our results suggested that mobile phone use should be limited.

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