

The neuroprotection role of heat shock protein 70 (HSP70) against microwave radiation induced DNA damage in male Wistar rat brain

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Abstract : This investigation aims to study the effect of radiofrequency (RF) radiation on DNA damage in brain cells of male Wistar rats using the comet assay and to investigate the role of HSP70 as a protective molecular chaperone that increases stress tolerance of brain cells. Male Wistar rats (118±20g) were divided into three groups. The 1st (exposed) group was subdivided into three subgroups and exposed for 15 min to activated cell phone emitting a frequency radiation of 900 MHz, at non-thermal specific absorption rate (SAR) of 2.9×10^{-3} W/Kg. The 2nd (exposed) group was also subdivided into three subgroups but was exposed for 30 min to the cell phone. The third group was the sham-exposed (control). Animals in each group were sacrificed after 1, 3 and 7 days recovery period. The comet assay parameters showed significantly increased DNA damage in brain cells after 1 and 7 days in the first group and after 7 days in the second group. The HSP70 showed significantly increased levels after 7 days in both exposure groups. Meanwhile, HSP70 showed significantly decreased levels after 1 day in the second group. The results of the present study demonstrate a damaging effect of RF radiation on DNA of the brain cells. This damaging effect initially inhibits the synthesis of HSP70; But after a 7 day recovery period, the levels of HSP70 increase significantly possibly due to powerful capacity of the cells for recovery.

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Introduction

The cellular response to stress is represented at the molecular level by the induced synthesis of a set of highly conserved proteins, heat shock proteins (HSPs) (Park et al., 2000) which protect cells and organisms against oxidative stress and often prevent cell death. While prolonged exposure to conditions of extreme stress is harmful, and can lead to cell and tissue death, induction of HSP synthesis can result in stress tolerance and cytoprotection. The cytoprotective effect of HSPs has been attributed to one of the major HSPs, HSP70 (Lee et al., 2001) that is particularly relevant as it is the major protein induced transcriptionally by various forms of stress including lethal heat shock, anoxia, heavy metals, ionizing and non-ionizing electromagnetic radiation (Santoro, 2000, Calini et al., 2003).

In recent years, there has been growing concerns about the potential effects of radiofrequency electromagnetic fields (RF-EMFs; 10MHz—300GHz) on human health, especially the influence on DNA damage (Panagopoulos et al., 2007, 2010; Yan et al., 2007; DeJuliis et al., 2009), because of the wide use of mobile phones. The proximal distance of mobile phone to the head has raised anxieties about the biologic effects of microwave radiation (300MHz – 300 GHz) on the brain (Lai and Singh, 2005; Paulraj and Behari, 2006; Luukkonen et al., 2009). It has been

demonstrated that mobile phones affect neural function in human. These effects range from changes in the permeability of the blood brain barrier (Grigor'ev, 2005; Nittby et al., 2009) to changes in electroencephalogram (EEG) pattern (Croft et al., 2002) suggesting that exposure to an active cell phone affects the resting EEG in humans. Functions such as sleep (Hamblin and Wood, 2002), attention (Lee et al., 2001) or learning and memory (Koivisto et al., 2000) have been also shown to be "mobile phone sensitive". Cell phone exposure may also lead to brain cancer and that link is via the heat shock response (French et al., 2000).

Although many studies have reported that RF-EMF does not induce genotoxic effects (Maes et al., 2001; McNamee et al., 2003; Scarfi et al., 2006; Zeni et al., 2008), several independent studies (Sykes et al., 2001; Baohong et al., 2005; Lixia et al., 2006; Yao et al., 2008; Luukkonen et al., 2009), have provided evidence for DNA damaging alterations. Since RF-microwave EMF is non-ionizing, it is not considered to induce direct alterations in DNA, the mechanism is unclear. Previous studies have shown that RF-EMF increased the formation of reactive oxygen species (ROS) which have been shown to induce DNA damage (Moustafa et al., 2001; Stopczyk et al., 2005; Yao et al., 2008). Oxygen free radicals may play a role in mechanism of biological effects induced by RF-EMF. In aerobic cells, ROS

are generated as a by-product of normal mitochondrial activity. If not properly controlled, ROS can cause severe damage to cellular macromolecules, especially DNA (Barzilai and Yamamoto, 2004). There may be some association between the overproduction of ROS and DNA damage induced by RF-EMF.

The comet assay is considered a sensitive assay for detecting DNA single strand breaks, double strand breaks, alkali labile sites, incomplete excision and repair sites (Fairbairn et al., 1995). So, the present study aims to investigate whether RF-EMF induces DNA damage in male Wistar rat brain. The comet assay was used to measure DNA damage after two different exposures to GSM radiation (15 and 30 min) and the animals were sacrificed after a recovery period of 1, 3 and 7 days. The stress response to RF-EMF was also determined by measuring the levels of HSP70 in male Wistar rat brain.

Materials and Methods

Animals

Male Wistar rats weighing about 118±20g were obtained from Helwan Farm for Vaccine and Biological Preparations. The animals were housed in cages 5 animals in each cage in the laboratory for one week before the beginning of the experiment. The animals were maintained on 12h dark/light cycle and were given food and water *ad libitum*.

Experimental design

After the acclimation period, animals were randomly divided into two exposed groups and one sham-exposed (control) group:

Group 1: exposed 15 min (n=15).

Group 2: exposed 30 min. (n=15).

Group 3: sham-exposed (control) (n=15).

Animals in groups 1 and 2 were kept for a recovery period of 1, 3 and 7 days (with 5 animals in each) after which they were sacrificed.

Method of exposure

During irradiation, each animal was placed in its own restrainer rocket Plexiglas (15 cm length, 6 cm diameter) and a cone (3 cm length) in which the rat inserted its head. A cell phone in the "on" mode (spiking mode) was placed against the cone directly above the rat's head. The end of the cone was opened and holes were made in the rocket to facilitate breathing and minimize body temperature elevation. A Plexiglas disk was placed at the back to prevent the rat from backing out of the rocket. The cell phone was manufactured by Nokia (model 6300 type RM-217, GSM 900MHz, SAR 1.6 W/Kg) in the "on" mode (spiking mode) was placed with its antenna above the head of the rat. Control animals were

treated identically as the exposed ones; but the cell phone is 'switched off' during of the sham-exposed.

Preparation of brain samples

Rats from each group were anesthetized by anesthetic ether and then decapitated and their brains were dissected out. Whole brain was washed three times with phosphate buffer solution (PBS: NaCl, 8.0g, KCl, 0.2g, Na₂HPO₄.12 H₂O 2.8g, KH₂PO₄0.2g, pH 7.4), cut into pieces with stainless steel scissors, homogenized with the appropriate amount of PBS at 4°C and pH 7.4 in a glass homogenizer at 0-4°C, and then sifted through a 300-µm sieve. The slides were previously stained by trypan blue (3,3'-[3,3'-Dimethyl(1,1'-biphenyl)-4,4'-diyl]bis(azo)}bis(5-amino-4-hydroxy)-2,7-naphthalenedisulfonic acid, tetra-sodium salt), and under a microscope up to 90% of the brain cells were found to be alive. The cells were resuspended at approximately 10⁶ cells per ml in PBS and then used immediately for comet assay.

The comet assay

To measure the potential DNA damaging effect of microwave radiation in Wistar rat brain, the comet assay was carried out as described by Ge *et al.* (2005) with some modifications. At least 100 cells per slide subjected were analyzed (original magnification ×200) under a fluorescent microscope (BX51, Olympus) equipped with a green light excitation and at 590-nm barrier filter. Comets form as the broken ends of a negatively charged DNA molecule becomes free to migrate in the electric field toward the anode. For each cell, the length of DNA migration (comet tail length) was measured in micrometers from the center of nucleus to the end of the tail. The percentage of damaged DNA concentration in the comet tail was determined by measuring the total intensity of ethidium bromide fluorescence in the cells, which was taken as 100% and determining what percentage of this total intensity correspond to the intensity measured only in the tail.

Determination of HSP70 levels

The levels of HSP70 (pg/ml) in the brain samples were determined according to the method described by Oc *et al.* (2008) using ELISA Kit (DUOSet[®] IC, US).

Statistical Analysis

Data were expressed as a mean ± standard error (SE). Differences between the control and treated groups were tested using Student's t-test with the help of statistical software origin 7.5. Differences between control and exposed animals were considered statistically significant when P< 0.05.

Results

Table (1) shows the mean tail length and the mean % of damaged DNA (comet assay parameters) performed on rat brain cells after exposure to 900 MHz of RF radiation for 15 min and sacrificed after 1, 3 and 7 days recovery period. Mean tail length and mean % of damaged DNA showed significantly ($P<0.05$) increased levels after 1 and 7 days of the recovery period as compared to the control animals. No significant differences in the comet assay parameters were observed after 3 days of the recovery period.

After 7 days of the recovery period, the mean levels of HSP70 showed significantly ($P<0.05$) increased levels (Table.1). Meanwhile, HSP70 levels showed non significant decreased levels after 1 and 3 days recovery period as compared to the control group.

The data of the comet assay parameters (mean tail length and mean % of DNA damage) performed on rat brain cells after exposure to 900 MHz microwaves for 30 min and sacrificed after 1, 3 and 7 days recovery period are summarized in table (2). As shown in the table, there were non significant increased levels of the comet assay parameters after 1 and 3 days of exposure, while after 7 days recovery period, the comet assay parameters showed significantly ($P<0.05$) increased values as compared to the control ones.

Mean levels of HSP70 of rat brain exposed to 900 MHz microwaves for 30 min showed significantly ($P<0.05$) decreased levels after 1 day recovery period, non significantly decreased levels after 3 days and significantly increased levels after 7 days recovery period as compared to the control group (Table 2).

Table 1. Comet assay parameters (mean tail length and mean % of damaged DNA) and mean HSP70 levels performed on rat brain after exposure for 15 min to 900MHz RF radiation and sacrificed after 1, 3 and 7 days recovery period as compared to the control group.

Parameter	control	1 day	3 days	7 days
TL (μm)	1.585 $\pm$ 0.22	4.632 $\pm$ 0.90	3.585 $\pm$ 0.78	10.233 $\pm$ 1.05*
% of damaged DNA	1.270 $\pm$ 0.20	3.628 $\pm$ 0.42	3.135 $\pm$ 0.69	6.063 $\pm$ 0.51*
HSP 70 (pg/ml)	204.776 $\pm$ 3.62	186.87 $\pm$ 16.97	202.399 $\pm$ 17.66	302.458 $\pm$ 5.28*

Results are means $\pm$ SE of 5 animals.

* statistically significant difference between control and exposed group at ($P<0.05$).

Number of cells (100 cells per each animal).

Table 2. Comet assay parameters (mean tail length and mean % of damaged DNA) and mean HSP70 levels performed on rat brain after exposure for 30 min to 900MHz RF radiation and sacrificed after 1,3 and 7 days recovery period as compared to the control group.

Parameter	control	1 day	3 days	7 days
TL (μm)	1.642 $\pm$ 0.17	3.322 $\pm$ 0.73	3.269 $\pm$ 1.16	3.126 $\pm$ 0.24*
% of damaged DNA	1.333 $\pm$ 0.15	2.699 $\pm$ 0.64	2.184 $\pm$ 0.51	2.495 $\pm$ 0.13*
HSP 70 (pg/ml)	1.99.524 $\pm$ 1.66	153.856 $\pm$ 0.82*	160.098 $\pm$ 18.45	207.852 $\pm$ 1.72*

Results are means $\pm$ SE of 5 animals.

* statistically significant difference between control and exposed group at ($P<0.05$).

Number of cells (100 cells per each animal).

Discussion

The need for research regarding biological effects of electromagnetic fields is justified by the dramatic increase of RF-EMF sources and therefore, the population exposed during recent years (Zotti-Martelli et al., 2005). Mobile phones emit RF radiation into the heads of their users and the brain is one of the energy absorbing structures in the body. This resulted into a variety of neurological effects such as headaches, change in sleep patterns, modification in the electroencephalogram (EEG) and increase in blood pressure (Ilhan et al., 2004). Because DNA damage is closely related to every aspect of physiological and pathological activity of cells, one of the most active areas of RF radiation investigation is the assessment of direct and indirect effects on DNA (Brusick et al., 1998).

The comet assay is a sensitive method for detecting DNA damage in eukaryotic cells. It has become one of the standard methods for assessing genome damage in genotoxicity tests as well as in fundamental research of DNA damage and repair (Garaj-Vrhovac et al., 2009).

The present study, provides evidence that RF radiation from GSM cell phone exposure results in damaging effect on DNA of the brain cells, with remaining observable effects 7 days after the exposure. The study also shows that 15 min exposure to GSM has no effect on comet assay parameters after 3 days recovery period and after 1 and 3 days recovery period of the exposure time 30 min. These observations suggest that exposure to RF radiation can induce damaging effect on DNA in rat brain. DNA damage is closely related to human risk particularly DNA damage in brain cells could affect neurological functions and possibly lead to neurodegenerative diseases (Lai and Singh, 1996). The exact mechanism by which RF radiation induced DNA damage is still unclear. As is well known reactive oxygen species (ROS) are reactive and readily damage biological molecules, including DNA (Barzilai and Yamamoto, 2004). Stopczyk et al. (2005) found that oxidative stress after exposure to microwave may be the reason for many adverse changes in cells. The study of Moustafa et al. (2001), indicated that acute exposure to the RF-EMF of commercially available cellular phones may modulate the oxidative stress of free radicals by enhancing lipid peroxidation and reducing the activation of superoxide dismutase and glutathione peroxidase. A number of studies have indicated that exposure to RF-EMF could lead to DNA damage through free radicals and interaction with transitional metals (e.g: iron) (Zmyslony et al., 2000; Jajte et al., 2001; Lai and Singh 2004; Phillips et al., 2009). Iron

have also implicated to play a role in the genotoxic effects of RF-EMF exposure.

Several reports have indicted that EMF enhances free radical activity in cells (Lai and Singh, 2005; Oral et al., 2006; Simkó, 2007; Phillipis et al., 2009) particularly via the Fenton reaction (Lai and Singh, 2004). The Fenton reaction is a process catalyzed by iron in which hydrogen peroxide, a product of oxidative respiration in the mitochondria, is converted into hydroxyl free radicals which are very potent and cytotoxic molecules. This supports the view that RF-EMF affects DNA via indirect secondary process, since the energy level associated with EMF exposure is not sufficient to cause direct breakage of chemical bonds within molecules, the effects are probably indirect and secondary to other induced biochemical changes in cells.

Since the brain is exposed to rather high levels of EMF during cell phone use, the consequences of EMF induced genetic damage in brain cells are of particular importance. Brain cells have high levels of iron. Special molecular pumps are present on nerve cell nuclear membranes to pump iron into the nucleus. Iron atoms have been found to intercalate within DNA molecules. In addition, nerve cells have a low capacity for DNA repair and DNA breaks could easily accumulate. Another concern is the presence of iron particles in body tissues, particularly in the brain. These particles could enhance free radical activity in cells and thus increase the cellular-damaging effects of EMF. These factors make nerve cells more vulnerable to EMF. Thus, the effect of EMF on DNA could conceivably be more significant on nerve cells than other cell types of the body (Phillips et al., 2009). Since nerve cells do not divide and are not likely to become cancerous, the more likely consequences of DNA damage in nerve cells include changes in cellular functions and in cell death, which could either lead to or accelerate the development of neurodegenerative diseases. Double-strand breaks, If not properly repaired are known to lead to cell death. Cumulative DNA damage in nerve cells of the brain has been associated with neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases. However, another type of brain cell, the glial cell, can become cancerous as a result of DNA damage. Mausset-Bonnefont et al. (2004) showed an increase in the glial reactivity after 3 days recovery period of acute exposure to 900 MHz EMF. The authors concluded this increase in glial reactivity was due to an astrocyte hypertrophy as a result of the effect of microwaves. These observations are in agreement with the results of the present study, since the increased DNA damage was observed after 1 and 7 days of exposure to EMF.

It has been shown that exposure to mobile phone signals can influence cellular processes such as proliferation (French et al., 1997, Velizarov et al., 1999), cell morphology (Donnellan et al., 1997) and the level of heat shock protein expression (Kwee et al., 2001; Leszczynski et al., 2002). The results demonstrated here showed that HSP70 levels decreased 1 and 3 days after 15 and 30 min exposure to mobile phone radiation and then increased significantly after 7 days. This could be due to the possibility that after 1 and 3 days of recovery period, the basal content of HSP70 which protects brain cells from microwave exposure is consumed. Another thing is that some neurons do not appear to express HSP70 during stress.

There are two characteristics that determine a cell's response to a stress factor: (i) its original pre-stress level of HSP70 and (ii) its ability to rapidly accumulate the protein. Nollen et al. (1999) reported that there are basal levels of HSP70 which can protect a cell against harmful conditions without the need for additional synthesis of the protein. Although the protective system based on HSP70 exists in all tissues and organs, some cell types do not appear to express the protein. Among these are certain types of neurons (Sprang and Brown, 1987).

The results of the present study demonstrates three interesting findings: one is the finding of detectable DNA damage after 1 and 7 days from the 15 min exposure and after 7 days from the 30 min exposure. The second is significant decreased levels of HSP70 after 1 day from the 30 min exposure. The third is the significant increased levels of HSP70 after 7 days from both exposure durations. This means that exposure to GSM 900 MHz RF radiation at SAR 2.9×10^{-3} W/Kg induces a transient DNA damage in rat brain. The significant decrease in HSP70 levels after 1 day recovery from the 30 min exposure means that the disrupting action of RF radiation on DNA delayed the induction of this protein. The significant increase in the levels of HSP70 after both exposure durations means that brain cells induced the synthesis of the protein for protecting themselves from DNA damage and thus have a powerful capacity for recovery from damage induced by RF radiation.

In conclusion, this study confirms the existence of genotoxic effect of RF radiation on brain cells, this effect is not statistically significant but it becomes significant 7 days after the exposure. The data presented here show that HSP70 can be induced with exposure of brain cells to a GSM signal of 900MHz at SAR 2.9×10^{-3} W/Kg. But the cells have a powerful capacity for recovery from damage induced by RF radiation. The study also prove the potential for HSP70 to be used directly as cytoprotective agents in

wide variety of clinical situations involving neurodegenerative diseases.

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