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RESEARCH ARTICLE

Biochemical alterations in cerebellum of Swiss albino mice after 10 GHz microwave exposure

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Animals of 6-8 weeks of age from an inbred colony were selected and were divided into 2 groups- (i) sham exposed (without energizing the system) (ii) microwave exposed (for 30 days - 2hours/day). The mice were exposed to 10 GHz microwaves daily for 2 hours per day for 30 days with a power density 0.25 mW/cm^2 (milliwatt per centimetre square) and an average whole body specific absorption rate (SAR) was calculated (0.1790 W/Kg). After the exposure period the animals were sacrificed at all follow up intervals viz., 1-30 and cerebellum was excised to study various biochemical parameters (Lipid peroxidation- LPO, Protein) and antioxidant enzyme activity (Superoxide dismutase-SOD, Catalase- CAT and Glutathione-GSH), were estimated. Lipid peroxidation significantly increased at the autopsy intervals with depletion in protein content of the brain after microwave exposure whereas significant decrease in antioxidant enzyme activity of SOD and GSH with increased Catalase. Free radicals causing oxidative stress may be responsible for altered biochemical levels of the brain of Swiss albino mice after microwave exposure.

Introduction

In this study, the effect of EMF on the cerebellum was investigated, since it is known that the cerebellum has substantial connections with the brain cells that point a cognitive role of cerebellum, besides control of muscle movement, equilibrium and posture of body (Kaplan et al., 2013). For this reason, we aimed to study the effect of 10 GHz (X-Band) EMF in the cerebellum of *Swiss albino* mice. The X-band is used primarily for aeronautical radio-navigation and radiolocation where X stands for "extended". Monitoring of this band indicates that the signals, when present, can be very strong but the use of the band is very variable, like X-band is used in radar applications including continuous wave, pulsed, single polarization, dual polarization and phrased arrays. It is also used in traffic light crossing detectors. The X- band has an advantage of giving a reasonable energy density in a waveguide of convenient size from a moderately powered microwave generator. Moreover, 10-GHz (Giga Hertz) frequency is present in the central region of the frequency range which is commonly considered hazardous. It is one of the easiest microwave bands to get on primarily because of its proximity to frequencies heavily used by different radars and the resulting equipment availability. An appreciable proportion of energy is absorbed as this particular frequency also penetrates tissues to a depth of about 0.5 mm in biological body (Lawrence, 1968). There is widespread scientific and public interest in possible health hazards from exposure to electromagnetic fields (EMFs) associated with radiofrequency (RF) and microwave (MW) radiation. Daily, over three billion people in more than 200 countries are exposed consciously to EMFs (Fragopoulou et al., 2010). Scientific studies performed to date suggest that exposure to RF fields at intensities far less than the levels required to produce measurable heating can cause effects in cells and tissues. Non thermal effects occur when the intensity of the RF field is sufficiently low that the amount of energy involved would not significantly increase the temperature of a cell, tissue or an organism, yet some physical and biochemical changes are still induced (Cleveland, 1999). There is evidence that microwaves may produce

adverse biological effects in the nervous system (Sharma et al., 2014; Zhao et al., 2012), even at low levels of radiation power (Jauchem, 2008).

Researchers have pointed enhancement of the presence of free radicals after EMF exposure (Kumar et al., 2010 and Yoshikawa et al., 2000). It is also known that microwaves generate free radicals that accelerate the aging process in human tissue, and promote adult chronic diseases and cancer. ROS level can increase dramatically, which may cause damage to cell structures and react with various biochemical reactions (Desai et al., 2009). When unbalanced it may lead to oxidation of poly-unsaturated fatty acids in lipids, amino acids in proteins and damage to DNA. Brain is considered abnormally sensitive to oxidative damage (Skaperet al., 1997 and Robbins, 2004) because it has abundant lipid contents, a high oxygen consumption rate and relatively scarce antioxidant enzymes compared with peripheral tissue (Kikuchi et al., 2003). It contains relatively low levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) (Ahaskar et al., 2007). It has been established that the overproduction of reactive oxygen species (ROS) comes through free radicals formation and may change the levels of SOD, GPx and CAT activity in the whole brain.

The adult mammalian brain with its highly differentiated post mitotic neurons is considerably more vulnerable to microwaves than any other organs with rapid renewal system. The possible effect of RF exposure on nervous system has prompted investigations with animal models mostly focusing on biochemical and morphological alterations. Neuronal damages to the cortex, hippocampus, cerebellum, and basal ganglia due to RF exposure have also been reported earlier (Mausset et al., 2001 and Salford et al., 2003). Hence it was thought appropriate to make a detailed investigation on a specific part of the brain, the cerebellum. We therefore investigated the effects of 30 days exposure of microwaves on biochemical parameters and antioxidative enzymes in cerebellum.

MATERIALS AND METHODS

Experimental animals

The animal care and handling was carried out according to the guidelines set by the INSA (Indian National Science Academy, New Delhi, India). Male Swiss albino mice, 6-8 weeks old weighing 23+2g, procured from an inbred colony were used for the present study. The mice were maintained under controlled conditions of temperature and light (light: dark, 12:12 h) and provided with standard mice feed (procured from Hindustan Levers Ltds., India) and water ad libitum. The research was approved by the Departmental Ethical Committee (DEC) for animal use.

Instrument and method for irradiation by microwaves

Mice were divided into two groups-

Sham exposed: Mice of this group which served as control were kept in a plexiglass cage and placed symmetrically along the pyramidal horn antenna aperture connected with klystron power supply without energizing the system for 2 hours/day for 30 consecutive days.

Microwaves exposed: Mice of this group were exposed to the 10 GHz radiation source through the antenna for 2hours/day for 30 days at pulsed density 0.25 mW/cm^2. Four mice at a time were placed in a plexiglass cage which was well ventilated with holes of 1 cm diameter. The dimensions of the cage were (4.5×9×9cm) in which the animals were placed and the head part facing the horn antenna. The horn antenna was kept in H (Magnetic field) plane configuration. Therefore, electric field was perpendicular to the ground surface. Field was almost uniform because the dimensions of the cage were of the order of wavelength. At near field distance from the horn antenna, it was found that the power density measured was 0.25 mW/cm^2 which was maximum. Mice were exposed with 10 GHz continuous source through the antenna for 30 days, 2hrs/day (Fig. 1). Every day the cage was placed in the same position facing the horn antenna. The whole microwave exposure system was procured from Wavetech, Faridabad, Haryana, India.

Specific Absorption Rate (SAR)

The emitted power of microwaves was measured by a power meter which is a peak sensitive device (RF power sensors 6900 series and IFR 6960B RF power meter; made of Aeroflex Inc., Wichita, Kansas, USA). The power density at the cage location was 0.25 mW/cm2 and the SAR was calculated as 0.1790 W/kg (Watt/ Kilogram) (estimation done according Durney et al., 1984).

REMOVAL OF BRAIN TISSUE

The mice were sacrificed by cervical dislocation. An incision was made at the sides of the jaws to separate the upper and the lower palates. The upper palate was cut in the middle and after having cleared of the surrounding tissue, the brain was excised and separated from the spinal cord at the decussation of the pyramids. The intact whole brain was

then removed carefully and homogenate was prepared and used for quantitative estimation for various biochemical and enzymatic changes.

BIOCHEMICAL ASSAY

Lipid peroxidation (LPO) assay- LPO was measured by the method of Buege and Aust (1978). A 10% tissue homogenate of the desired tissue (1 g) was prepared in 9 ml of 1.15% KCl. Tissue homogenate (0.8 ml) was mixed with 1.2 ml solution of TCA (15% w/v)– TBA (0.375% w/v)–HCl (0.25N) prepared in a 1:1:1 ratio. This final mixture was heated in a water bath for 30 min at 80°C and cooled. After centrifugation the absorbance was recorded at 532 nm using a UV–vis double beam spectrophotometer. A standard curve was prepared by using TMP. After comparison with a standard curve the LPO level was expressed in nmol TBARS g/tissue (TBARS, thiobarbituric acid reactive substances).

Glutathione (GSH) assay- The reduced GSH content of tissue samples was determined in brain by the method of Moron et al., 1979. A tissue sample was homogenised in the sodium phosphate–EDTA buffer then 0.6ml DTNB was added. The optical density of the yellow coloured complex developed by the reaction of GSH and DTNB was measured at 412 nm using a UV–vis spectrophotometer. The results were expressed as nmol GSH/100 mg of tissue.

Protein assay- Estimation of protein was based on the method proposed by Bradford (1976). 10% homogenate was prepared in NaCl and 0.1 ml of the sample was taken for the Bradford assay. Three repeats of the assay from each animal were carried out. The absorbance was read at 595 nm.

Superoxide Dismutase (SOD) - SOD was measured by the method of Marklund & Marklund (1974). 100mg of tissue was dissolved in 1 ml of NaCl for homogenate preparation, 0.1 ml of supernatant was taken for estimation. This tissue sample was then homogenised in 2.7 Tris buffer and 0.1 ml of pyragallal was mixed to this solution. Absorbance was measured at 420 nm.

Catalase (CAT) - Estimation of catalase enzyme was based on the method proposed by Aebi et al., 1984. Homogenate was prepared by adding 5 ml of phosphate buffer in 0.5 gms of tissue. Centrifuge for 10 mins at 10,000 rpm. 0.1ml of supernatant was mixed with 1 ml. PBS and 0.4 ml. H_2O_2. Absorbance was read at 420 nm.

STATISTICAL ANALYSIS

The results obtained in the present study were expressed as mean ± SD. The statistical difference between various groups was analysed by the one-way ANOVA and the significance was observed at the $p < 0.001$ (Highly significant), $p < 0.05$ (Significant) and $p < 0.01$ (Non-significant) level.

RESULTS

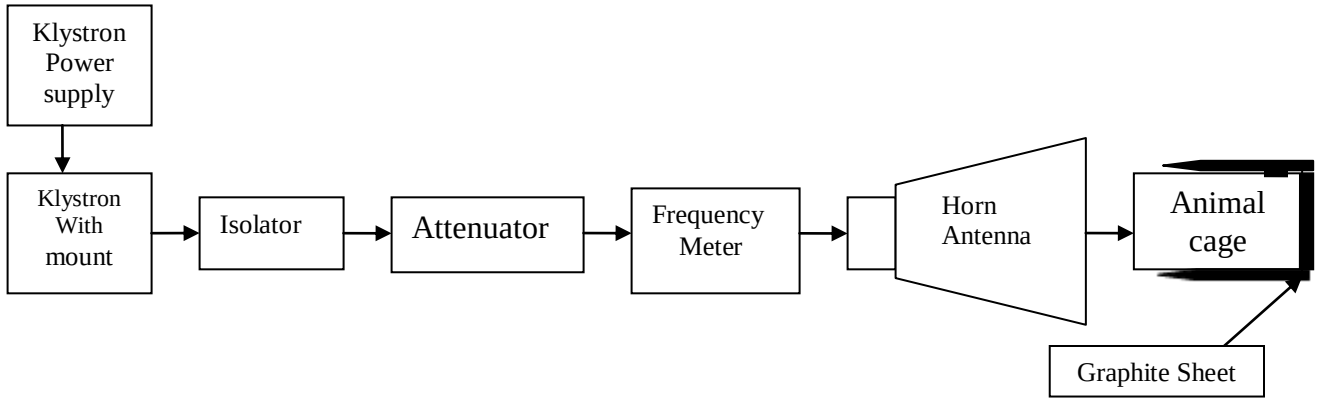

Figure 1. Schematic representation of 10-GHz microwave source

Biochemical Estimations

Lipid peroxidation

The intensity of lipid peroxidation measured by the levels of MDA in the cerebellum of brain tissue is shown in figure 1. The content of MDA in the cerebellum was significantly higher ($p< 0.001$) till day 7^{th} of exposure period which thereafter decreased ($p<0.05$) as compared to sham exposed (control).

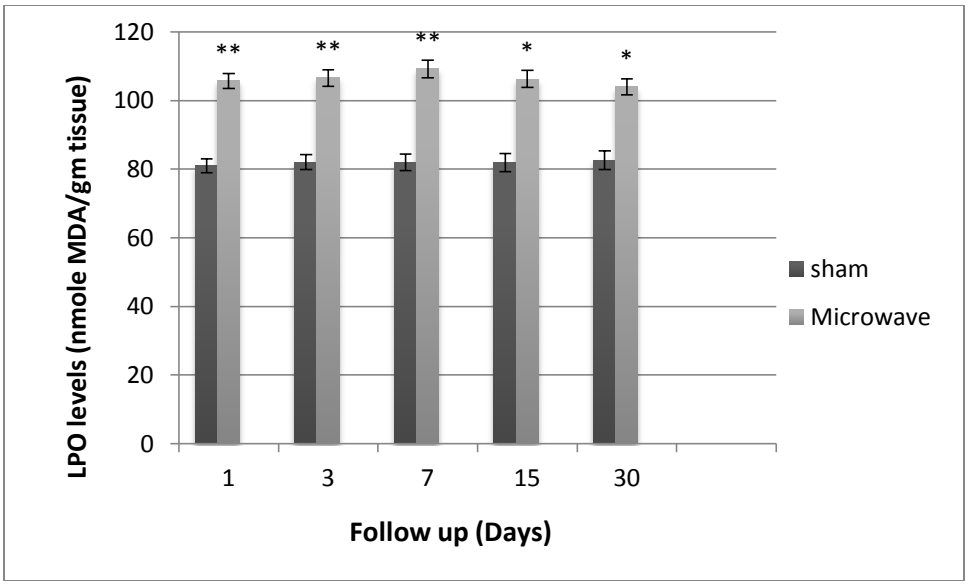

Figure 1: Variations in LPO levels (nmole MDA/gm tissue). Values are mean ± SD. Where $p<0.01$ is non significant (n); $p<0.05$ is significant (*); $p<0.001$ is highly significant (**).

Protein

Microwave radiation induced a statistically significant ($p < 0.05$) decrease in protein content at all the follow up autopsy intervals in cerebellum of mice (Figure 2). The protein content initially decreased gradually and continuously after irradiation up to the 7th day. Thereafter, recovery was evident though the protein level was still significantly below the sham level.

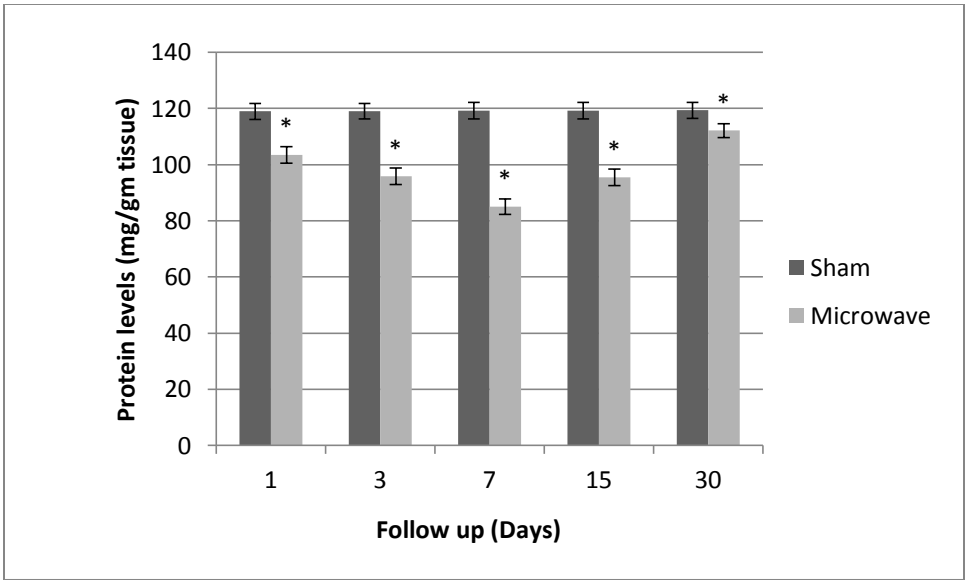

Figure 2: Variations in Protein levels (mg/gm tissue). Values are mean ± SD. Where $p<0.01$ is non significant (n); $p<0.05$ is significant (*); $p<0.001$ is highly significant (**).

ANTIOXIDANT ENZYME ACTIVITY

Compared to the sham exposed group, those exposed to 10 GHz microwaves showed a sharp deficit in GSH activity till day 3rd($p<0.001$). Thereafter it increased gradually upto the last follow up interval day 30th. However it was much below the sham levels (Figure 3). A sharp fall in SOD activity was noticed at 24 hrs after completion of 10 GHz microwave exposure which continued till 7th day post treatment where maximum fall was noted. Thereafter, SOD levels started to increase continuously upto the last interval studied ie 30 days (Figure 4). This implies that irradiation from microwaves resulted in the generation of superoxide radicals ($p<0.001$). On the other hand, the exposed group of animals showed highly significant increase in CAT activity as compared to the sham exposed with maximum increase in CAT noted on day 7 (Figure 5).

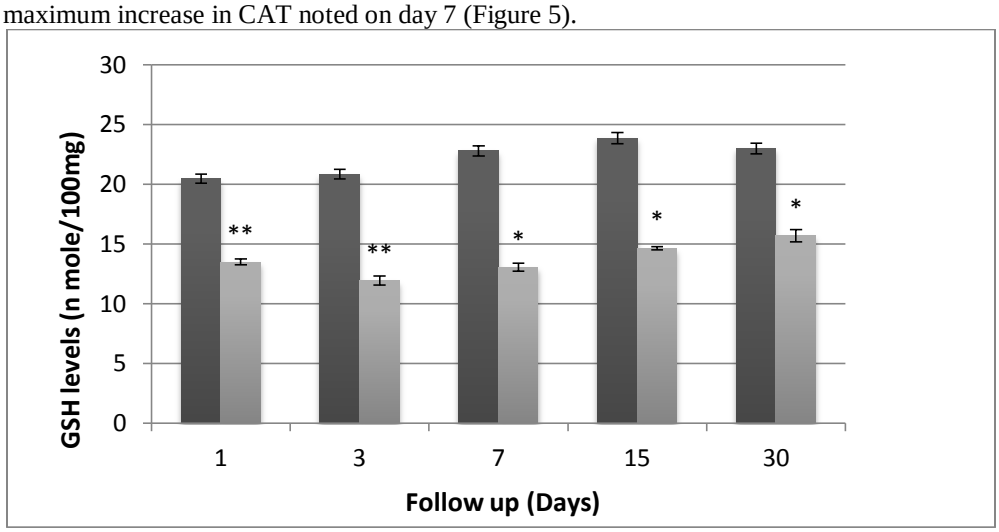

Figure 3- Variations in GSH levels (n mole/100mg). Values are mean ± SD. Where $p<0.01$ is non significant (n); $p<0.05$ is significant (*); $p<0.001$ is highly significant (**).

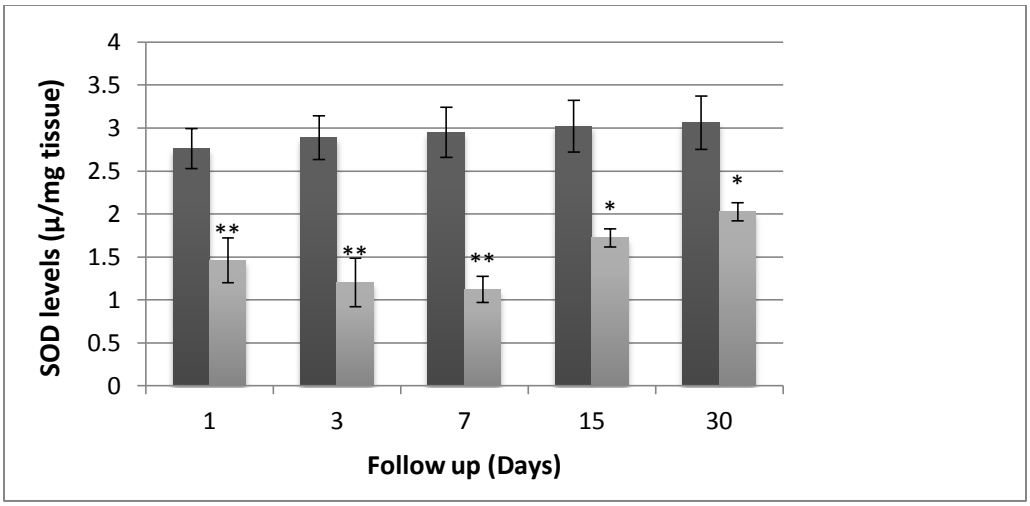

Figure 4- Variations in SOD levels (µ/mg tissue). Values are mean ± SD. Where $p<0.01$ is non significant (n); $p<0.05$ is significant (*); $p<0.001$ is highly significant (**).

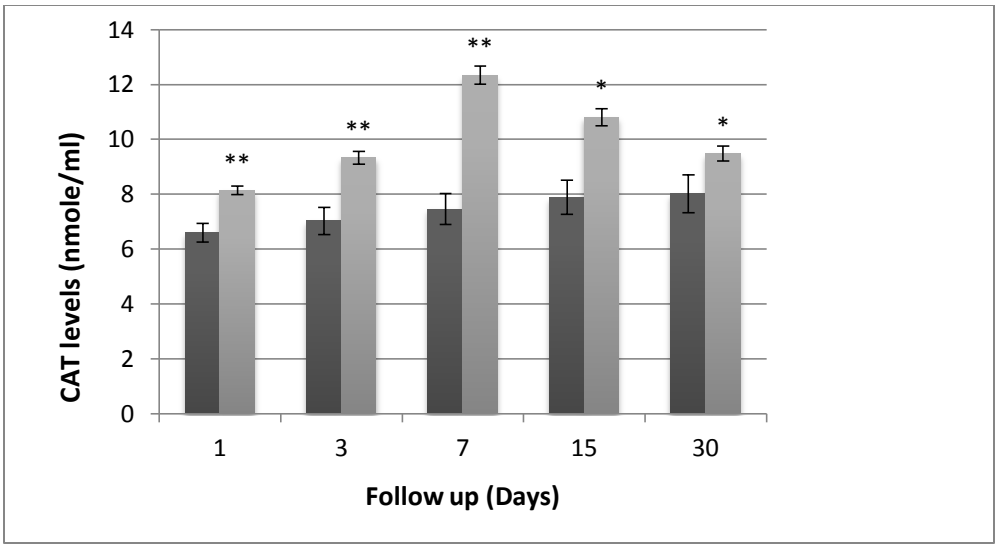

Figure 5- Variations in Catalase (n mole/ml). Values are mean ± SD. Where $p<0.01$ is non significant (n); $p<0.05$ is significant (*); $p<0.001$ is highly significant (**).

DISCUSSION

The possible risk of electromagnetic radiation (EMR) for nervous system is regularly published from the middle of 20th century. The brain which naturally functions for thinking, emotions and memory is a highly sensitive and complex organ of nervous system that responds to a wide range of extremely low frequencies (ELF) of the electromagnetic radiations. Therefore, this study provides some important findings in cerebellum of *Swiss albino* mice at 10 GHz microwave radiation. In several studies electromagnetic field exposures causes the overproduction of free radical formation and effects such as genotoxicity and oxidative stress (Mailankot et al., 2009).

The peroxides via lipid peroxidation damage cell membrane and other components of the cell. MDA is the breakdown product of the major chain reactions leading to the oxidation of polyunsaturated fatty acids, and thus serves as a reliable marker of oxidative stress- mediated lipid peroxidation in the mice brain (Koylu et al., 2006). The preservation of cellular membrane integrity depends on protection or repair mechanisms capable of neutralizing oxidative reactions such as lipid peroxidation (Sisodia et al., 2008). Megha et al., 2012 also reported increase in MDA level in rat brain exposed to 900 MHz and 1800 MHz for 30 days. Similar studies have been reported by Sokolovic et al., 2008 where exposure to 900 MHz at SAR 0.043-0.135 W/Kg can induce brain damage by lipid peroxidation. Kerman and Senol (2012) reported that in the EMR exposed group (900 MHz) neural tissue MDA levels increased while SOD, CAT, and GSH-Px activities were reduced which could be reversed by oral administration of melatonin. In the present study, there was considerable increase in TBARS content after EMF exposure.

Decrease in protein content after exposure to radiation has been reported by Verma et al., 2002 which might be due to either decline in the rate of protein synthesis or increase in the comsumption of protein. The reduction in the protein biosynthesis could be attributed to any of the following factors: (a) Activation of RNAase, or (b) depletion of mRNA or effect on the formation and /or maturation of RNAase. Radiation may also include local defects in the microstructure of protein molecules, which becomes the centre of thermal denaturation and cross linkage, thus causing tissue damage (Calabro et al., 2012 and Calabro and Magazu 2010). Brain cells are reported to be active sites of protein synthesis. Fragopoulou et al., 2012 reported that long term irradiation from both EMF sources altered significantly ($p<0.05$) the expression of 143 proteins in total (as low as 0.003-fold down regulation up to 114-fold overexpression). Increased protein concentration in the present study may be due to improved ribosomal activities, which enhance protein synthesis.

Moreover, EMF exposure seems to be associated with free radical and especially ROS overproduction (Phillips et al., 2009, De Iouliis et al., 2009 and Simko et al., 2007), as concluded in the present study. Microwave interaction with the brain may cause an overproduction of reactive oxygen species (ROS), resulting in significant increase in free radical formation which may enhance the probability of damage to the biological system. Damage to body occurs when intracellular antioxidant mechanism are overwhelmed by ROS. 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 and antioxidative enzyme (Kumar et al., 2010). Normally adequate level of cellular antioxidants, mainly superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) maintain the free radical scavenging potential in brain. ROS may result in peroxidation of lipids present in cells mainly polyunsaturated lipids present in cell membranes and may alter the levels of cellular proteins.

In the present study, decreased activities of the key antioxidants, GSH and SOD were found in the brain of mice exposed to 10 GHz. SOD plays a key role in defence mechanism against free radical activity. Normally, SOD enzyme works in parallel with GSH, playing an important role in the reduction of hydrogen in the presence of GSH forming GSSH (glutathione disulfide) and protects cell proteins and cell membranes against oxidative damage (Rush et al., 1985). In biological system, the mechanisms of tissue damages are thought to involve reactive oxygen species (ROS) produced due to exposure of electromagnetic fields in surrounding environments. Moustafa et al., 2001 reported decrease in plasma SOD activity in humans. A decrease in activity of SOD was related to accumulation of superoxide amino radicals in cells. The detoxification is mainly done by SOD. Hydrogen superoxide, a product of SOD activity, is also a strong inhibitor of SOD enzyme (Kula et al., 2002). The charge of detoxification is taken by CAT enzyme, which leads to increase its activity. The increased activity of catalase activity is increased to compensate the overproduction of reactive oxygen species which overproduced in brain by electromagnetic field. Zwirska-Korczala et al., 2005 also reported a decrease in activity of SOD, GSH and increase activity of CAT. Decreased activity of GSH in exposed group may be due to decrease in its formation, which requires NADPH and GR (Irmak et al., 2002). Free radicals are produced continuously and detoxified by SOD, glutathione (GSH), and catalase (CAT). With excessive free radical production and the resulting consumption of antioxidants, endogenous defence mechanisms become insufficient. The decreased activities of both SOD and GSH in the brain exposed to electromagnetic radiation indicate the highly reduced capacity to scavenge hydrogen peroxide produced in brain in response to acute stress.

The outcome of oxidative damage induced by electro-magnetic fields will therefore depend on various factors, including the oxidative status of the cell, capability of endogenous antioxidative enzymes and processes to counteract free radical build-up, availability of exogenous antioxidants, parameters of exposure (e.g., intensity and duration of exposure and possibly the wave shape).

Conclusion

It may be concluded that ROS overproduction by microwaves may alter the activity of antioxidant enzymes and increase the level of TBARS.

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