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Pathophysiology of Microwave Radiation: Effect on Rat Brain

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Abstract The study aims to investigate the effect of 2.45 GHz microwave radiation on Wistar rats. Rats of 35 days old with 130±10 g body weight were selected for this study. Animals were divided into two groups: sham exposed and experimental (six animals each). Animals were exposed for 2 h a day for 45 days at 2.45 GHz frequency (power density, 0.21 mW/cm^2). The whole body specific absorption rate was estimated to be 0.14 W/kg. Exposure took place in a ventilated plexiglas cage and kept in an anechoic chamber under a horn antenna. After completion of the exposure period, rats were killed, and pineal gland and whole brain tissues were isolated for the estimation of melatonin, creatine kinase, caspase 3, and calcium ion concentration. Experiments were performed in a blind manner and repeated. A significant decrease ($P<0.05$) was recorded in the level of pineal melatonin of exposed group as compared with sham exposed. A significant increase ($P<0.05$) in creatine kinase, caspase 3, and calcium ion concentration was observed in whole brain of exposed group of animals as compared to sham exposed. One-way analysis of variance method was adopted for statistical analysis. The study concludes that a reduction in melatonin or an increase in caspase-3, creatine kinase, and calcium ion may cause significant damage in brain due to chronic exposure of these radiations. These biomarkers clearly indicate possible health implications of such exposures.

Keywords Melatonin · Caspase-3 · Creatine kinase · Calcium ion concentration · Microwave

Introduction

Exposure of radio frequency electromagnetic field (RF-EMF) to brain and consequent tumor promotion has become a subject of debate because of the availability of 3G (third

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generation mobile communication) spectrum for wireless communication. The frequency range of 3G spectrum varies between 1,800 and 2,700 MHz, where microwave oven, mobile phone, and its towers are the main source of RF-EMF. Present study has been carried out at 2.45 GHz microwave frequency. It is generally accepted that mobile phones are used very close to the head, where the emitted radiations are absorbed by the brain [1]. Some authors have shown that the microwave frequency range between 800 and 1000 MHz can penetrate the cranium, and nearly 40% of these can reach the deep brain [2, 3], where penetration depth may be up to 4–5 cm into the human brain [4, 5]. In confirmation with this, several studies have suggested that microwave exposure may affect brain functioning and behavior [6–10]. In the present study, caspase-3, creatine kinase, melatonin, and calcium ion concentration have been undertaken in brain, exposed to electromagnetic fields. A recent study of Kumar et al. [11] on reproductive pattern has shown a reduced melatonin, increased caspase-3, and creatine kinase in sperm, thereby posing a significant adverse health effect due to 2.45 GHz microwave exposure. These parameters control biochemical functioning in the biological system, where creatine kinase (CK) activity plays a key role in energy metabolism of tissues with intermittently high and fluctuating energy requirements, such as skeletal, cardiac, and neuronal tissues like brain and retina [12]. CK is an enzyme, which catalyzes the reversible transfer of the phosphoryl group from phosphocreatine to ADP to regenerate ATP [13]. CK or phosphocreatine system exerts several integrated functions in brain cells, such as temporary energy buffering, metabolic capacity, energy transfer, and metabolic control [14]. This system is recognized as an important metabolic regulator during health and disease [12]. Moreover, melatonin is a hormone that plays an important role in central nervous system especially in pineal gland. Decreased melatonin has many biological effects where calcium ion efflux from the pinealocytes leads to a reduced melatonin by decreasing cyclic AMP (cAMP), which is the key element of calcium ions [15] and essential for cell growth and survival. The pineal gland and its hormone melatonin play a central role in deciding the post-effects of such exposure. Furthermore, an intracellular membrane bound Ca^{2+} is found to be effected by electromagnetic field exposure [16], where enhanced efflux of calcium is due to the released intracellular bound calcium in response to RF-EMF radiations [17, 18]. Such events may also lead to tumor promotion, caused by an increased apoptosis. Apoptosis or programmed cell death is an important biological event in tumor promotion, which may be enhanced due to DNA fragmentation [19–21]. These authors have reported an increased level of DNA strand break (single and double) due to microwave exposure in rat brain at various frequencies (2.45 and 50 GHz). It is thus suggestive that changes in levels of enzymes or hormone may occur due to influence of microwave exposure and free radical generation, leading to enhancement in the level of reactive oxygen species (ROS) [22].

Methodology

Material

Caspase 3 assay kit, colorimetric (catalog number CASP-3-C) was purchased from Sigma, USA. Creatine assay kit (catalog number K635-100) from BioVision Research Products (Mountain View, CA, USA), enzyme-linked immunosorbent assay (ELISA) melatonin kit (catalog number E90908Ra) form Uscn Life Science Inc (Wuhan, China). The rest of the chemicals were purchased from Thomas Baker Chemicals Limited, Marine Drive, Mumbai, India.

Animals Exposure

Thirty-five-day-old male Wister rats (130±10 g) were used in the present study. The animals were maintained as per guidelines and protocols, approved by the Institutional Animal Ethics Committee (IAEC-JNU/83/675-687; code number 12/2008). The animals were housed in clean polypropylene cages and maintained in a controlled temperature with constant 12-h light and 12-h dark schedule. The animals were fed on standardized normal diet (Tetragon Cheime Private Limited, Bangalore) and water ad libitum.

Exposure Chamber

Rats were placed in a Plexiglas cage ventilated with holes of 1 cm diameter. The dimension of each house in exposure cage was identical and made in such a way that animals are comfortably placed. The exposure cage with all six animals were kept in an anechoic chamber in a far field region from the horn antenna (Fig. 1). In the exposure chamber, all six animals were facing horn antenna. No animals blocked the radiations falling on other animals. Animals were divided in two groups: exposed and sham exposed (n=6 in each group). All the experiments were performed and repeated in a blind manner. Exposure schedule was randomly exchanged for sham-exposed and exposed groups by keeping the temperature and the humidity at the pre set level. Exposure time was scheduled in between 10 AM to 5 PM. Anechoic chamber is lined with radar absorbing material (attenuation, 40 db) to minimize the reflection of scattered beam. Temperature in chamber was maintained around 25–27 °C throughout the experiment by air circulation. In the position of animal placement, a horn antenna was placed, and the field was measured, which is homogeneous in the vertical plane of midline of the beam. Rats were exposed to 2.45 GHz radiations source at 50 Hz modulation frequency (input, 1,080 W; output, 700 W). Microwave oven [Haier India Co. Ltd, made in China (model HR-18MS1)] was used as a source of exposure, connected to 40-db attenuation and to the horn antenna. Exposure was given for 2 h a day for 45 days at 0.21 mW/cm^2 power density. The whole body specific absorption rate (SAR) was estimated to be 0.14 W/kg. The emitted power of microwaves was measured by a power meter, which is a peak sensitive device (power sensor) [RF power sensors 6900 series and IFR 6960 B RF (radio frequency) power meter; made of Aeroflex, Inc., Wichita, KA, USA]. Every day, the cage was placed in the same position

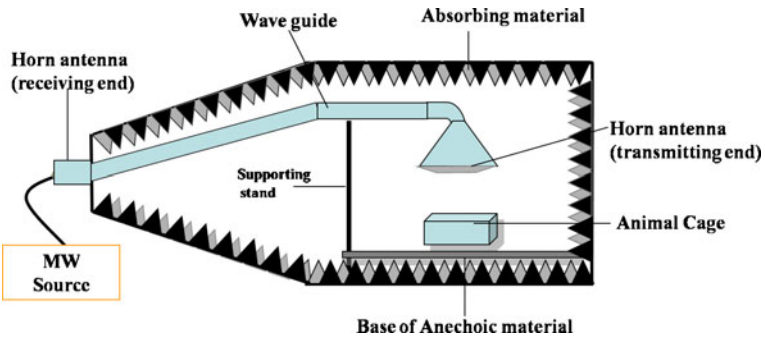

Fig. 1 Schematic layout of 2.45 GHz exposure setup

facing the horn antenna, and the same numbers of rat positions were reshuffled. Similar experiment was performed with sham-exposed animals without energizing the system. The same experimental setup and procedures were earlier adopted by Kumar et al. [11], Paulraj and Behari [21] and Kesari and Behari [29].

Sample Preparation and Tissue Homogenate

Melatonin Assay

Immediately after exposure, animals were killed by an overdose of anesthesia, and the brain was collected. Pineal gland was removed from brain and homogenized in ice-cold buffer. Melatonin level in pineal gland was estimated by kit (Uscn Life Science Inc.). Fifty microliters each of dilutions of standard, blank, and samples were added to precoated wells with polyclonal antibody specific for rat melatonin followed by addition of 50 μl detection reagent A to each tube. After incubation for 1 h at 37 °C, wells were washed with wash solution three times. One hundred microliters of detection reagent B was added to each well and incubated for 30 min at 37 °C. Plate was washed again followed by addition of substrate solution to each well than after incubation for 15 min at 37 °C. The color development was stopped by the addition of stop solution and the intensity of the yellow color measured by spectrophotometer (450 nm). Concentrations of the unknown samples were calculated by comparison with a standard curve.

Estimation of Creatine Kinase

The CK level was estimated using ELISA kit. Whole brain was homogenized and washed with ice-cold imidazole buffer (0.15 M NaCl and 0.03 M imidazole, pH 7.0 in ratio of 1:15). The supernatant was decanted after centrifugation at 500×*g*, and pellet was re-suspended in 0.1% Triton X-100 detergent solution by vortex for 20 s. The sample was centrifuged again at 500×*g*, and the supernatant was analyzed for CK activity. In the assay, creatine is enzymatically converted to sarcosine and specifically oxidized to generate a product that converts a colorless probe to an intensely red color product, which is detected calorimetrically (λ_{max}=570 nm).

Estimation of Caspase-3

The activity of caspase-3 was measured using the colorimetric caspase assay kit. Briefly, the homogenized whole brain was centrifuged at 300×*g* for 10 min on 4 °C. The pellet was then re-suspended in lysis buffer for 20 min and centrifuged at 20,000×*g* for 20 min on 4 °C, and the supernatant was collected. The assays were conducted in 96-well plates, and all the measurements were carried out in a microplate reader. The caspase-3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA), resulting in the release of *p*-nitroaniline (pNA) moiety. To assess the specific contribution of caspase-3 activity, Ac-DEVD-pNA substrate (2 mM) was added to each well. The plates were incubated overnight at 37 °C to measure caspase-3 activity. Absorbance was measured with a microplate reader (Spectromax M_2) at 405 nm. Caspase-3 activity was expressed in micromoles of pNA released per minute per milliliter of cell lysate at 37 °C.

Calcium Ion Estimation

Brain calcium ion concentration was estimated by atomic absorption spectrophotometer (Thermo Scientific M Series) in flame mode analysis. Brain was dissected and homogenized in phosphate buffer. Homogenized brain was centrifuged at 8,000 rpm for 10 min at 4 °C. Thereafter, the supernatant was collected in another tube, and equal dilution was maintained with experimental and sham-exposed group. Finally, the samples were filtered with 0.45-μm membrane filter. The standard solution for analysis was made by calcium salt in Milli-Q water.

Statistical Analysis

All experimental results were compared with sham-exposed group and expressed as mean±standard deviation (SD). The samples were processed in triplicate for each animal. The mean of three samples (per animal) were taken, and the final average mean on six animals were presented in data. The analysis was done using GraphPad Prism software and one-way analysis of variance (ANOVA) by considering P value significance ($P<0.05$).

Results

Melatonin

An average concentration of melatonin in the pineal gland of microwave-exposed group was found significantly lower ($P<0.001$) as compared with the average concentration of melatonin in the sham-exposed group. Data presented in Table 1 are for all the biochemical studies between exposed and sham exposed presented in Fig. 2.

Creatine Kinase Activity

CK in central nervous system energy transport was examined by measuring ATP/ADP ratio. The mean value of CK showed a significant increase ($P<0.012$) in exposed group of brain as compared with sham-exposed group (Table 1 and Fig. 3).

Caspase-3 Activity

Brain caspase activity behaved similarly ($P=0.015$) in the exposed group as compared to the sham-exposed ones (Table 1 and Fig. 4).

Table 1 Data for biochemical studies between exposed and sham-exposed rats

Parameters	Sham exposed	Exposed	P value
Melatonin (pg/mg of protein)	81.03±8.01	53.56±9.20	<0.001
Creatine kinase activity (IU/mg of protein)	1.12±0.17	1.80±0.11	<0.012
Caspase 3 activity (μmol pNA/min/ml)	44.05±1.43	46.83±1.83	0.015
Calcium ion concentration (PPM)	0.17±.004	0.33±0.01	0.001

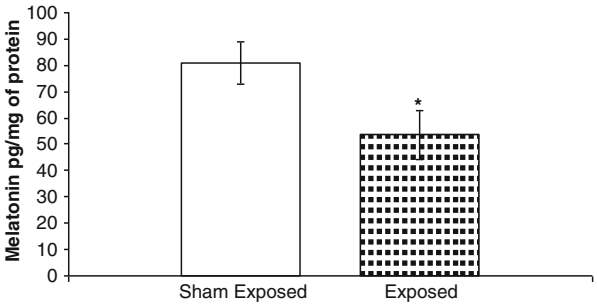

Fig. 2 Significant melatonin reduction in exposed group where *single asterisk* shows level of significance (*$P<0.001$). Statistical analysis was done by one-way ANOVA in mean±standard deviation

Calcium Ion Concentration

Calcium ion concentrations between sham exposed and exposed were measured in parts per minute (PPM). The result showed a significant increase ($P=0.001$) in exposed group as compared with sham exposed (Table 1 and Fig. 5).

Discussions

In the present investigations, creatine kinase, melatonin, caspase-3, and increase in calcium ion concentration were found to be affected in rat brain due to microwave exposure. Creatine kinase catalyses the reversible phosphorylation of ADP to ATP or creatine to creatine phosphate. Tomimoto et al. [23] postulated that alteration in CK activity may participate in neurodegenerative pathway, leading to neuronal loss, i.e., Alzheimer. The possible mechanism beyond increment of CK activity is followed by microwave exposure, leading to generation of hyperproduction of ROS [11]. CK is associated with increased ROS, and it shows possibility of oxidative stress in pathological environment. Microwave radiation is suggested to be causing the depletion in antioxidant enzyme, i.e., melatonin, which leads to an increase in CK activity. It is considered that CK and the creatine–creatine phosphate energy shuttle may play a role in brain development [24]. Any imbalance in this energy shuttle initiates the caspase-3 activity, causing apoptosis, whereby calcium concentration (efflux) is increased. Our findings are attributed to microwave radiation exposures causing toxicity, through cytosolic stress in rat brain.

Caspases play an important role as mediators of cell death in acute and chronic neurological disorders. Caspase-3 was chosen in the present study because of its central role

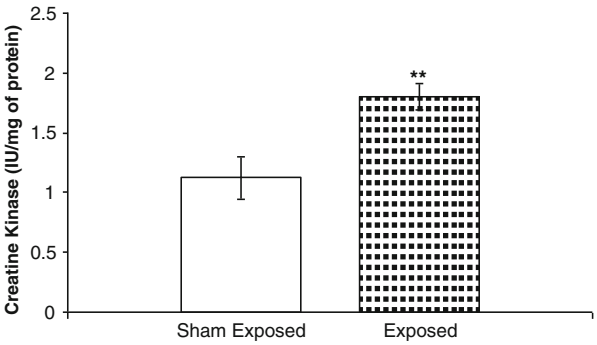

Fig. 3 Creatine kinase activity distribution in the whole brain fractions of exposed and sham exposed groups (**$P<0.001$, level of significance). Statistical analysis was done by one-way ANOVA in mean±standard deviation

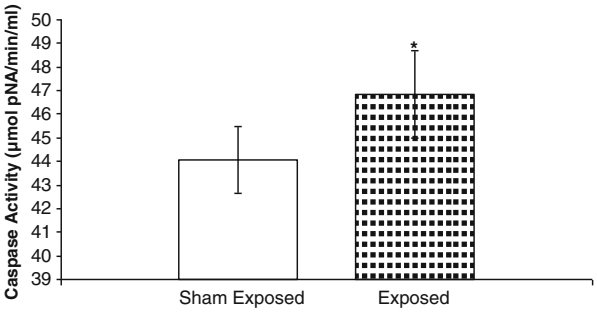

Fig. 4 Caspase-3 activities in whole brain was measured after overnight incubation and expressed in micromoles of pNA released per minute per milliliter, showing significant difference (*P=0.015, ANOVA) among exposed and sham-exposed groups

in cell death. Usually, caspase-9 and 3 are activated to execute apoptosis. Caspases are present as inactive precursors and are activated by initiator caspase through autoactive proteolysis [25]. The initiator caspases 8 and 9 with effectors caspase 3 is considered as the main executors of apoptosis [26]. The effector caspase 3 share both pathways: mitochondrial pathway through caspase 9 and death-receptor pathway through initiator caspase 8 [27].

Generally, cell death can be divided into programmed cell death (apoptosis) and necrosis. However, recently, in addition to necrosis, other non-apoptotic cell death forms have been recognized, such as autophagic cell death, mitotic cell death, and caspases-independent cell death [28]. We have earlier reported [20, 29] that an increased apoptosis due to microwave radiations at 2.45 GHz exposure effects both reproductive and brain system, which may also be a possible cause of tumor promotion. Apoptosis is executed by a family of zymogenic proteases known as caspases (cysteinyl aspartate-specific proteinases) that dismantle the cell in an orderly fashion by cleaving an array of intracellular substrates. An increased caspase-3 is a well-established indicator of apoptosis [11], and it has been shown that such effects at 2.45 GHz microwave exposure affect the reproductive pattern of male Wistar rats. Apoptosis is also based on the intracellular dominance of various proteins that induce or inhibit the apoptotic process, like caspase-3 and several other key enzymes [30]. However, an induction of the increased heat shock protein 27 activation by the radio frequency electromagnetic wave (RF-EMW) exposure might lead to inhibition of the apoptotic pathway that involves apoptosome and caspase 3. Caspases activated by apoptotic signals cleave various cellular substrates such as actin, poly(ADP-ribose) polymerase, fodrin, and lamin, which may be responsible for the morphological changes that occur in the cells. In this study, we measured a vital natural neuro-hormone, which was found to be decreased. Melatonin is the most potent known natural antioxidant that scavenges free radicals to protect cells throughout the body, especially the brain, heart, and immune

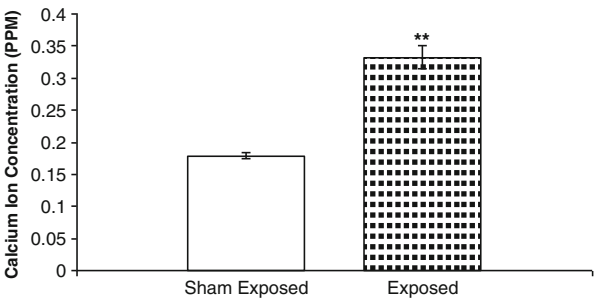

Fig. 5 Mean values of calcium ion among the exposed group was significantly higher (**P<0.001). Statistical analysis was done by one-way ANOVA in mean±standard deviation

system. It is a primary signal of the daily cycle system. Cells in the brain, heart, circulation system, central nervous system and peripheral nervous system components, and testes are linked with melatonin receptors by which cell cycle activity is regulated. Moreover, the daily sleep/wake cycle, blood pressure, heart rate cycle, and hormone production activity directly or indirectly are regulated by melatonin through the autonomic system. Serotonin is converted into melatonin at night by the pineal gland. This indicates that melatonin may also change in shift workers (long-term night shifts and work schedules in which employees change or rotate shifts) [31]. Henshaw et al. [32] have shown the critical role of magnetic field exposure and melatonin disruption due to microwave exposure, melatonin/serotonin cycle in brain, and possibly other vital organs.

Evidence that electromagnetic radiation reduces melatonin in human beings commenced with the work of Wang [33], who found that workers highly exposed to RF-EMW had a dose–response increase in serotonin, and leads to a reduction in melatonin. Recently, Kumar et al. [11] have also reported a decreased melatonin in 2.45 GHz exposed Wistar rats. Decreased melatonin has many biological overtones where calcium ion efflux from the pinealocytes has the effect of reducing melatonin through reduction of cAMP, which is the key element of calcium ion concentration [15]. Another aspect of neurochemical effect of radio frequency radiation (RFR) is the efflux of calcium ions from brain tissue. Calcium ions play an important role in the functions of the nervous system, such as the release of neurotransmitters and the actions on neurotransmitter receptors. Changes in calcium ion concentration could lead to alteration in neural functions. Calcium ion is generally a regulatory signal of cellular functions, such as muscle contraction [34] microtubule assembly, stimulus secretion coupling in glandular cells, and hormone-mediated regulation of cyclic nucleotide levels [35].

Cell membrane is considered as the primary site for EMF interaction. The mobilization of cellular calcium ion (Ca^{2+}) by electromagnetic radiation is an important biological response in the regulation of cellular activities [36]. Bawin et al. [37] reported an increase in efflux of calcium ions from chick brain tissue after 20 min of exposure to a 147 MHz RFR (1–2 mW/cm^2). Increase in calcium ion efflux was observed in the chick brain irradiated at 0.1 and 1.0 mW/cm^2. From our laboratory [17, 18], it is reported that an increased level of calcium ion concentration is caused due to microwave exposure on developing rat brain. RF-EMW may also alter intracellular calcium homeostasis by acting on plasma membrane calcium channels [38]. Rao et al. [39] have recently provided evidence suggesting that RF-EMW affects plasma membrane. They studied the effects of RF-EMW on calcium dynamics in stem-cell-derived neuronal cells and discovered a significant increase in intracellular calcium spikes in response to non-thermal RF-EMW. Paulraj and Behari [40] have also reported that an increased level of calcium ion concentration may affect several enzymes like protein kinase C due to non-thermal microwave radiation. Recently, Kesari et al. [41] have shown several biochemical changes due to radio frequency exposure at 900 MHz in different regions of the rat brain. These data suggest an impact on cellular permeability due to this radiation exposure affecting cell signaling and possibly causing tumor promotion.

Conclusion

Our results suggest that a 2.45-GHz exposure decreases the melatonin activity and increases that of ceatine kinase, caspase-3, and calcium ion, which affect the brain physiology. Our findings of these parameters are clear indications, pointing toward tumor promotion. The

results, in general, have wide ranging implications affecting normal physiological functioning of the exposed group.

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Conflict of interest The authors have no conflicts of interest. They alone are responsible for the content and writing of the paper.

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