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Original article

Effect of Mobile Phone Microwaves on Fetal Period of BALB/c Mice in Histological Characteristics of Hippocampus and Learning Behaviors

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Abstract

Objective(s)
The possible risks of radio-frequency electromagnetic fields (EMF) for the living organisms and human body are a growing concern for our society. In this study, we examined the possibility of changes in working memory and hippocampal histological characteristics effects in mice brain following whole body exposure to microwave radiation.

Materials and Methods
During gestation period, we exposed mice for 4 hr to Global system for mobile communications (GSM), Specific Absorption Rate (SAR) of 200 mW/kg. Pregnant control mice were sham-exposed or free in a cage without further restraining. Three month after exposure, animals were prepared for behavioral (Radial Arm Maze (RAM) and Morris Water Maze (MWM)) and histological studies.

Results
The results showed that microwave exposed mice were slower than sham, and control in finding the platform. Analyses of error rates in RAM and MWM performance revealed significant differences which emphasize the effect of acute exposure to pulsed microwaves in deficit of spatial reference memory in the mice. However in this study exposed group didn't show any statistically significant loss of hippocampal CA1, CA3 neurons versus controls or sham.

Conclusion
We conclude that there is evidence from the current study that exposure to MW radiation under parameters examined caused decrements in the ability of mice to learn the spatial memory task.

Keywords: Mobile microwave, Behavior, Spatial reference memory, learning, Mice

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Introduction

The voluntary exposure of the brain to microwaves from hand-held mobile phones by one-fourth of the world's population has been called the largest human biologic experiment ever (1). In the near future, microwaves will also be emitted by an abundance of other appliances in the cordless office and also in the home. The possible risks of Radio-Frequency Electromagnetic Field for the human body are a growing concern for our society (2). The possible risks of microwaves for the human body has attracted interest since the 1960s (i.e., before the advent of mobile phone), when radar and microwave ovens posed a possible health problem (3).

Continuous and pulsed microwave exposure of brain has been reported to modulate electrophysiological activity *in vivo* (4, 5) and *in vitro* (6-9) and influence neurotransmitter systems as well as signal transduction pathways (10-12).

Today's mobile phones, with a total power output of about 1W, are estimated to produce insignificant local heating (equivalent to about a 0.1 $^{\circ}$C rise in temperature in the brain), which is unlikely to produce any deleterious effects (13). Microwaves produce thermal effects on biological systems at high power levels. The energy absorption at high power levels probably lead to nonspecific stimulation of hypothalamic-hypophyseal axis with liberation of corticosterone which causes sequestration of cells, an effect induced by any known stressor (14). The exposure to Electro Magnetic Field emitted by mobile phone is found to speed up response times in simple reaction time and vigilance tasks and to decrease cognitive performance in attention, perception and memory performance. It may be due to a facilitator's effect on brain functioning, especially in tasks requiring attention and manipulation of information in working memory. This could be associated with an effect on the angular gyrus which acts as an interface between the visual and speed centers and lies directly under and on the same side as the antenna. Such effect could be consistent with mild localized heating, or possibly a non-thermal response, which is nevertheless power-dependent. It is not clear whether these findings have implications for health or not (15,16). In an experimental study with pregnant rats exposed *in vitro* to the Global System for Mobile Communication (GSM), field did not induce any measurable cognitive deficits in offspring (17). The influence of pulsed electromagnetic fields (EMFs) of digital GSM mobile phones on working memory in healthy subjects is found to be speeding up response times (18). From histological point of view there are some researches which show controversial results. Fritze and colleagues did not find obvious abnormalities in microwave exposed rat brains and even Wu reports decrease of lesion volume in the hippocampal CA3 region after exposure of guinea pig (19). The purpose of this study was the investigation of the possibility of changes in working memory and hippocampal histological characteristics effects in mice brain following whole body exposure to microwave radiation.

Materials and Methods

Animals

All experiments were carried out in accordance with the local university "Guide for the Care and Use of Laboratory Animals". The animals used in this study were female Blab/C (25-30 g), maintained in individual cages with a 12 hr light-dark cycle (lights on from 8:00 to 20:00 hr), under controlled temperature (21±1 $^{\circ}$C) and humidity. Animals were given free access to water and food.

Exposure system

Transverse electromagnetic transmission line chamber (TEM-cells) used for the RF EMF exposure of mice was designed by dimensional scaling from previously constructed cells (20).

TEM-cells are known to generate uniform electro-magnetic fields for standard measurements (21, 22). A genuine GSM mobile phone with a programmable power output was connected via a coaxial cable to

the TEM-cell and no voice modulation was applied.

The TEM-cell was enclosed in a wooden box ($15 \times 15 \times 15$ cm) which supports the outer conductor and central plate (3). The outer conductor was made of brass net and attached to the inner walls of the box. The center plate, or septum, was constructed of aluminum (3).

The TEM-cell was placed in a temperature-controlled room, and temperature in the TEM-cells was kept constant by circulation room air through holes in the wooden box. The mice were placed in plastic trays ($12 \times 12 \times 7$ cm) to avoid contact with the central plate and outer conductor. The bottom of the tray was covered with absorbing paper to collect urine and faces.

The mice (n=60) were assigned to three groups (n=20 each): (i) free moving control group; (ii) sham-exposed (4 hr placement in the off exposure system); (iii) exposed group: the peak output power of 1,000 mW/cell from the GSM mobile telephone was fed for (4 hr/day) from day 1 to the end of gestation. This exposure resulted in average whole-body, the Specific Absorption Rate (SAR) of 200 mW/kg, respectively.

Three month after exposure, animals in each group were prepared for behavioral studies. The spatial learning and memory capabilities of mice were assessed in the Morris Water Maze and in the radial-arm maze testing (17, 23).

Morris Water Maze (MWM)

It consisted of a circular pool (diameter 160 cm; height 60 cm) filled water to a height of 30 cm. The water (21°C) was made opaque with powdered milk. The pool was located in an experimental room surrounded by cues external to the maze (e.g. stool, computer, animal cages, sink, etc) which could be used by the mouse to guide and use for its learning. The pool was virtually divided into four quadrants of equal surface and different starting points were identified. A circular platform (diameter 11 cm) was placed in the pool, 1 cm underneath the water surface. It was not visible for the mice. For each trial, the mouse was released from the side of the pool, facing the wall at a randomly assigned starting point, and given 60 sec to reach the submerged platform. Each mouse completed four consecutive trials per day. When the mouse did not find the platform within 60 sec, the experimenter would have placed it there for 20 sec before the next trial was run.

The delay to reach the platform by the mouse was recorded for each trial. At the end of the four trials, the mouse was removed from the pool, towel dried, and returned to its cage. During 5 consecutive days, the platform was placed in the north-west quadrant. Each day, each mouse was given four trials, the starting point differing for each trial. Different starting points were assigned randomly each day. When the last trial of the last day was completed, the platform was removed and the mouse was given a probe trial for 60 sec.

Typically, mice that have learned the specific location of the escape platform exhibited a spatial bias and spent significantly more time in the target quadrant.

Radial-Arm Maze (RAM)

RAM training and testing were run using a grey PVC radial maze placed in an experimental room with several different visual cues around the mazes (e.g. animal cages, stools, trash basket, curtain, etc). the radial maze, elevated 68 cm above floor level, had eight arms (60 cm long and 10 cm wide) radiating from a central octagonal platform (diameter 40 cm), food pellets were placed 3 cm from the end of each arm. Guillotine doors controlled access to the arms only in one way. All mice were habituated to eat calibrated food pellets in the 5 training days before the period of testing on the first day, the food pellet only in one arm was accessible.

On the second day, two adjacent arms contained four pellets per food well. For the following 3 days, three adjacent arms were accessible with two pellets placed in each food well. Following training, all mice were tested once a day. The procedure used allowed determination of two types of memory (working and reference memory) in the same session. On a single trial, the mouse

was placed on the central platform with all guillotine doors open. Four arms were baited according to two different patterns:
1, 3, 4, 6 and 2, 5, 7, 8. The baited arms were always the same for each mouse, but changed from one mouse to another.

The mice remained in the maze until all four pellets of food had been eaten or until 5 min had elapsed. Reference memory supposes information that remains constant over time to be stored and used appropriately (i.e. the never-baited arms) and difficulties in reference memory are thus reflected by choices of arms which were never baited. Working memory (WM) supposes information that is pertinent only within a short period of time to be stored and used appropriately, and impairments in working memory are indicated by repeated entries into arms which have already been visited within the trial (23).

Histological study of hippocampal neurons

After the completion of behavioral experiment, mice were re-anesthetized and their brains were extracted, fixed in 10% buffered formalin for 1 week, dehydrated with alcohols, and embedded in paraffin. Seven-micrometer thick coronal sections were cut at 1-mm intervals through the samples on a rotary microtome and mounted on gelatin-coated glass microscope slides. After drying at room temperature, the sections were de-paraffinized in xylen, rehydrated, and stained with cresyl violet. An observer blinded to experimental conditions analyzed the coronal section underlying the area (~ 3.5 mm posterior to bregma) from all mice in each group for determination of treatment efficacy on selectively vulnerable hippocampal CA_1 and CA_3 neurons. To reduce counting errors associated with false positive identification of dying neurons, the total number of CA_1 and CA_3 morphologically intact neurons (i.e., those with a clearly defined cell body and nucleus) were counted using a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan) with a 40× objective. The data were reported as the percent of total neurons in the ipsilateral (injured) CA_1 and CA_3 regions relative to the contra lateral hippocampus.

Data analyses

The acute neurological, probe trial histological, the trial block (eight 4 trial blocks in the radial-maze), and swim speed data were analyzed by ANOVA. When the overall ANOVA revealed a significant effect, the data were further analyzed with the Bonferroni/Dunn post hoc test to determine specific group differences. The data are presented as the mean ± standard error (SE) and are considered significant when corresponding *P* values are <0.05.

Results

Spatial learning acquisition

Effect of exposure to mobile phone on reference and working memory in an eight-arm radial maze

Three months after exposure, mice were trained to perform an eight-arm radial maze task. By baiting five of the eight arms, the mice were allowed to find these baited arms (only once for each mouse). So, it is possible to measure RM-entering into the baited arms, and WM capacities-(entering baited arms only one time). ANOVA with repeated measures was applied to analyze reference memory (RM) errors (i.e. entries into unbaited arms) as well as WM errors (i.e. repeated entries into baited arms) in six series of trials for all three groups. Exposed animals demonstrated a decrease of RM errors number with training (4.2 ± 0.7 in the first block of trials; 2.8 ± 0.2 in the sixth block of trials) (Figure 1A). In particular, exposed animals made more RM errors comparing to sham and control in the second, fifth and sixth blocks of trials.

A decrease in the number of WM errors with trials was revealed when ANOVA has been applied on the data from all the trials in all three groups (Figure 1B); F (5.2= 83.12), ($P< 0.001$). Moreover, a strong dependence of WM errors on the group factor was found (F: 4, 15), ($P< 0.02$). During learning session the mice of control and sham groups demonstrated 60% decrement of WM errors, respectively.

Histology

Quantification of hippocampal neurons CCl produced no significant reductions in normal appearing (i.e., morphologically intact) CA1 and CA3 neurons in the hippocampus ipsilateral to the impact. Exposed group did not differ from the sham group, and from the

other in the percentage of normal appearing CA1 neurons. The mean ratio of morphologically intact CA3 neurons was not significantly reduced in exposed group, versus sham and control (P> 0.05). Furthermore, as elaborated in Table I, no additive effect of treatments was observed (P= 0.44, versus control).

Exposed group expressed no significantly fewer normal appearing neurons compared to the sham and controls. (P> 0.05)

Table 1. Effect of microwave exposure on hippocampal cell survival quantified from day 1 to 19 of gestation in the three groups of the study..

Groups	cell survival	
	CA_1	CA_3
Exposed group	87.4 ± 3.4	89.6 ± 4
Sham	98.2 ± 6	94.3 ± 1.2
Control	92.7 ± 4	93.4 ± 2.3

Mean (± SE) normal appearing neurons expressed as the percentage of the contra lateral hippocampus.

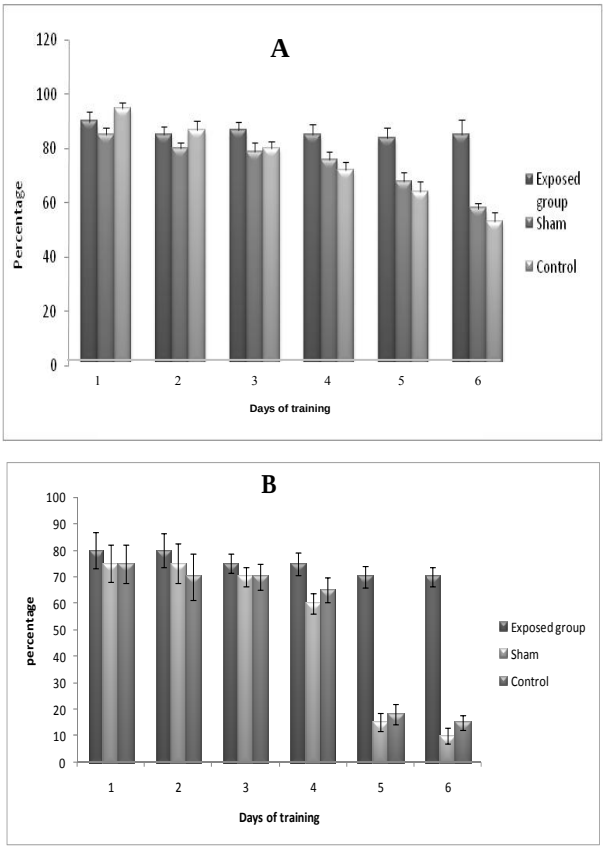

Figure 1. Effects of microwave exposure on RM (A) and WM (B) in a radial maze. The mice were trained in the eight-arm radial maze three month after exposure. Entries into un-baited arms were defined as RM errors. Repeated entries into baited arms, which had been previously visited during a trial, were defined as WM errors. Data are presented as Mean ± SEM (P < 0.05 is considered statistically significant)

Effect of exposure by mobile phone on MWM performance

Analysis of spatial learning acquisition revealed significant Group (F:5, 35)= 24.34, (P< 0.0001) and Day (F:4, 14)= 29.66, (P< 0.0001) effect. Exposed group was significantly impaired relative to sham group. Sham group facilitated spatial learning and memory as evidenced by shorter time to locate the target platform versus the exposed group (P< 0.001 and 0.0001, respectively). Moreover, as depicted in Figure 2, a significant difference in spatial acquisition was observed between exposed group and control group (P< 0.001). However, no significant difference in swim speed (from 29.3 ± 3.4 cm/s to 35.4 ± 1.8 cm/s) or visible platform acquisition (Figure 2) was observed between the control and sham groups.

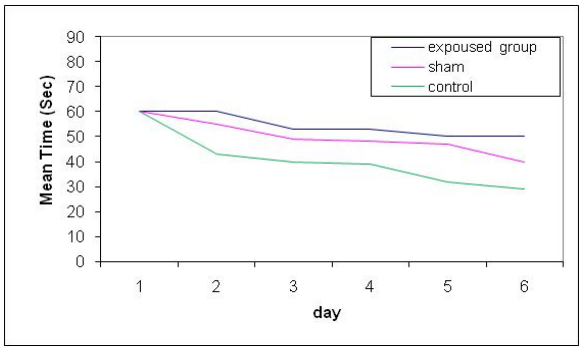

Figure 2. Mean time (in seconds) to locate either a hidden (submerged) or visible (raised) platform in a water maze. Exposed group, had significant difficulty with the cognitive task vs. sham and controls. No difference was observed in locating the visible platform.

Discussion

Previous studies have shown that microwaves of mobile phone might induce or promote cancer, sleep disorders and memory changes (24). It may be due to a facilitator effect on brain functioning, especially in tasks requiring attention and manipulation of information in working memory (18).

To discern the effects of hyperthermia on working memory, previous studied recorded the ability of rats to discriminate between objects following microwave radiation exposure.

We presented here; for the first time, evidence for neuronal damage during gestation caused by non thermal microware exposure.

The hippocampus in the brains of exposed mice contained damaged neurons. Although our study comprises few animals, the combined results are highly significant and exhibit an obvious dose-response relationship.

Previous report indicates that microwave induced hyperthermia can impair learning and memory (3).

In our study, an obvious impairment of reference and working memory in the water-maze test was observed in mice. Data from this experiment showed that acute exposure (4 hr) of mice to pulsed EMF at an average whole body SAR of 200 mW/kg, significantly affected the learning function for locating the platform in a water maze, which may indicate that reference memory was affected by microwave exposure. Although microwave-exposed mice displayed some memory of the location of the platform during the learning trials, their escape time and distance to land on the submerged platform were longer than those of the sham and controls, and the swimming speed of the mice was significantly affected by microwave-exposure. The mechanism by which pulsed microwave could affect spatial reference learning is not known. However, neruroanatomic and neurochemical processes associated with water maze performance are well studied. Cholinergic innervations to the cerebral cortex and hippocampus play different and important roles in learning and memory in the water maze (25-27). Deficit in water maze performance could be caused by a decrease in cholinergic activity in the brain. Lai *et al* (28--30) have found that acute microwave exposure decreased cholinergic activities in frontal cortex and hippocampus of the rat, and both cholinergic and endogenous opioid neurotransmitter system in the brain were involved in the pulsed microwave induced spatial learning deficit in the radial-arm maze (28-31).

It has been shown that central cholinergic pathways, especially those of the frontal cortex and hippocampus, were involved in place learning (32,33). Decrease in frontal cortical and hippocampal cholinergic activity may be responsible for the water maze learning deficit seen after acute exposure to pulsed microwaves.

A similar observation was made in the radial maze, in which working and reference memory were tested side by side on each trial. Previous results (34) showed that radial arm maze performance could be disrupted by RF exposure at relatively low SARs. The current study attempted to confirm their reported results.

In our study the microwave-exposed mice learned significantly slower than the sham and control animals. Multivariate analysis showed a significant defect in swimming speed associated with learning, between exposed and sham groups, but sham and control groups did not have a significant increase in time spent in the target quadrant in radial maze or swimming speed in water maze. Koivisto *et al* (35, 36) reported that exposure to 902 MHz radio frequency energy emitted by cellular telephones was associated with a facilitation of brain functioning during memory tasks, including faster response times, thus suggesting an improvement in cognitive function. Kraus *et al* (37) reported that exposure to similar field caused a decrease in cortical activity in human subjects performing auditory memory tasks. D'Andrea (38) noted that the difficulty in drawing conclusions about studies of the effects of MW irradiation on cognitive function, including working memory. Our findings and those of other studies about possible working memory changes following radio frequency radiation are similar. These results are in broad agreement with the other results which reported that exposure under examined parameters causes decrements in the ability of mice to learn the spatial memory task.

Previous reports indicate different non-thermal effects. Some in vivo studies of microwave exposure did not reveal any major abnormalities in the rat brain (39) and many studies have implicated that decreased lesion volume has been shown after exposure (40). However, in this study experimental group did not show significant loss of hippocampal CA3 neurons versus controls or sham. The

present study demonstrates that 4 hr microwave 200 mW/kg exposures in the fetal brain during gestation period to mobile phone does not induce substantial changes in quantity of hippocampal neurons. Our findings are, therefore, a strong argument against any major histological effects of microwaves emitted by mobile phone.

Appropriate end points for this study were determined by the following arguments:

Functional or behavioral changes in an organism often occur prior to structural changes. Functional changes in the central nervous system are among the earliest to become observable. Furthermore, the sensitivity of CNS-functions to interfering and environmental factors during development has been demonstrated in many studies on animals and human beings (41).

The situation of the growing brain might deserve special concern from society since biologic and maturational processes are particularly vulnerable during the growth process. In the long-term, however, it may result in reduced brain reserve capacity which might be unveiled by other later neuronal disease or even the wear and tear of aging. We cannot exactly show that after a long period of time (years) of mobile phone daily use, generations of users may suffer negative effects or not, perhaps as early as young or middle ages.

Conclusion

The intense use of mobile phones during gestation is a serious consideration. An important observation was decrease of the working and reference memory. This effect is, therefore, a strong argument against a biological impact of GSM microwave exposure.

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