

Histopathological, immunohistochemical, and stereological analysis of the effect of *Ginkgo biloba* (Egb761) on the hippocampus of rats exposed to long-term cellphone radiation

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Summary. Cellular phones are major sources of electromagnetic radiation (EMR) that can penetrate the human body and pose serious health hazards. The increasingly widespread use of mobile communication systems has raised concerns about the effects of cellphone radiofrequency (RF) on the hippocampus because of its close proximity to radiation during cellphone use. The effects of cellphone EMR exposure on the hippocampus of rats and the possible counteractive effects of *Ginkgo biloba* (Egb761) were aimed to investigate. Rats were divided into three groups: Control, EMR, and EMR+Egb761. The EMR and EMR+Egb761 groups were exposed to cellphone EMR for one month. Egb761 was also administered to the EMR+Egb761 group. Specifically, we evaluated the effect of RF exposure on rat hippocampi at harmful EMR levels (0.96 W/kg specific absorption rate [SAR]) for one month and also investigated the possible impact of Egb761 using stereological, TUNEL-staining, and immunohistochemical methods. An increase in apoptotic proteins (Bax, Acas-3) and a decrease in anti-apoptotic protein (Bcl-2) immuno-reactivity along with a decrease in the total granule and pyramidal cell count were noted in the EMR group. A decrease in Bax and Acas-3 and an increase in Bcl-2 immunoreactivity were observed in rats treated with Egb761 in addition to a decrease in TUNEL-stained apoptotic cells and a higher total viable

cell number. In conclusion, chronic cellphone EMR exposure may affect hippocampal cell viability, and Egb761 may be used to mitigate some of the deleterious effects.

Key words: Apoptosis, Cellphone EMR, *Ginkgo biloba* extract (Egb761), Hippocampus, Rat

Introduction

The cellular phone is one of the fastest growing technological developments in modern life (Dogan et al., 2012). Cellphones are indispensable communication tools and have transformed from a status symbol to a necessity because of their countless perks. Since 1997, worldwide cellphone subscriptions have increased to over 5.6 billion, and about 70% of the global population has a cellphone (Gupta et al., 2015). As a result of the wide and growing use of mobile communication, increasing concerns have centered on the interaction of electromagnetic radiation (EMR) with the human body, particularly with brain tissues (Narayanan et al., 2010), as cellphones are a major source of EMR (Anderson, 2003; Faruque et al., 2011). These concerns mostly stem from the potential impacts of the penetration of EMR into the human body, as this may represent a serious health hazard (Anderson, 2003; Faruque et al., 2011).

Specifically, wireless technology takes advantage of EMR in the microwave frequency ranges of 300 MHz to 300 GHz (Andersen et al., 2002; Kundi et al., 2004). Cellphones transmit and receive electromagnetic waves

at frequencies of 800-1900 MHz (Barnett et al., 2007). Electromagnetic field (EMF) energy absorbed by biological organisms is converted into heat in the human body, which can cause a rise in temperature and other thermal effects (Behari, 2010). The interaction between the body and EMF energy radiation is expressed by a defined parameter-specific absorption rate (SAR), which is directly related to the electric and magnetic field strength distribution within human tissues (Hossain et al., 2015). In particular, the close proximity of the antenna of a cellphone handset to a user's head exposes the brain to EMFs. In fact, approximately 40-45% of a cellphone's radiofrequency (RF) output power energy was found to be absorbed by the user's head (Khullar et al., 2013). In one experiment, exposure to GSM 900 EMR caused structural damage to rat brains, and this damage was postulated to be caused by albumin leakage from the blood-brain barrier (BBB) into the brain (Salford et al., 2003). RF fields were reported to increase the permeability of the BBB, allowing large plasma proteins like albumin to leak into the brain and cause structural damage (Eberhardt et al., 2008).

The hippocampus is an integral part of the brain's limbic system. Its glucocorticoid and mineralocorticoid receptors regulate behavior and the hypothalamic-pituitary-adrenal (HPA) axis, and it is also the site of long-term potentiation of the cellular mechanisms believed to underlie learning and memory. Damage to hippocampal neurons may lead to impaired learning and memory as well as behavioral disturbances and therefore negatively impact hippocampal functioning (Daniels et al., 2009).

The microwaves emitted by cellphones contain almost all bands of the electromagnetic spectrum. In particular, cellphones can produce extremely low-frequency EMF (Repacholi and Greenebaum, 1999), which may cause DNA damage or changes in oxidative metabolism (Ferreira et al., 2006). EMF can influence neuronal functions, including synaptic plasticity regulation, neurotransmitter release, neuronal survival, and learning and memory (Sakatani and Hirose, 2002; Salford et al., 2003; Manikonda et al., 2007). It can also induce cell death and can inhibit the differentiation of neural stem cells into neurons during embryonic development (De Iuliis et al., 2009).

Furthermore, EMR may produce oxidative stress (OS) and accelerate neuronal degeneration, causing molecular damage to vital structures and functions. For instance, as a result of radiation, apoptotic signals could be generated in cell membranes through lipid peroxidation (Ozben, 2007; Motawi et al., 2014). In this respect, the effects of cellphone EMR may be mediated via OS, which could induce apoptosis. Even so, apoptosis plays a fundamental role in normal tissue homeostasis during embryogenesis and throughout the lifespan of multicellular organisms (Ameisen and Capron, 1991). However, environmental stressors can modulate many cellular functions, such as apoptosis and proliferation of cells (Tian et al., 2002).

Antioxidants are increasingly being recognized as important health promoters for mitigating cardiovascular problems, cancer, and aging. Antioxidants reduce the effects of free radicals formed in the body following exposure to environmental pollutants as well as the effects of free radicals that are naturally produced and present if somatic defense mechanisms are weakened (Ilhan et al., 2004).

The physiological effectiveness of Egb761 (*Ginkgo biloba* extract) is based on its antioxidative activity and protection against lipid peroxidation (Bridi et al., 2001). The antioxidative activity of flavonoids, which are found in this extract, greatly depends on the chemical structure and the relative orientation of the various moieties of the molecules that form such flavonoids (Tredici, 1991). The antioxidative effect of Egb761 is also based on the presence of ginkgolides B and C and bilobalide (Pitchumoni and Doraiswamy, 1998; Bridi et al., 2001; Motawi et al., 2014).

The current study aimed to investigate the possible harmful effects of EMR (0.96 W/kg SAR) emitted by cellphones on the cornu ammonis (CA) and dentatus gyrus (DG) regions of the hippocampus as well as the prevention of such harmful effects through administration of the antioxidant Egb761 in rats following long-term exposure to cellphones in calling mode.

Materials and methods

Animals

The experimental protocol was approved by the Animal Ethics Committee of Gaziosmanpasa University (2015 HADYEK-42). Twenty-one male Wistar albino rats at four months of age were purchased from the Experimental Medicine Research Center at Gaziosmanpasa University. The rats were randomly divided into three groups: a control group (n=7), an EMR treatment group (n=7), and an EMR+Egb761 treatment group (n=7). Animals were housed in standard plastic cages (650 x 500 x 450 mm) on sawdust bedding at 22-24°C and 50-65% relative humidity under a 12-hr light/dark cycle with free access to food and water.

Experimental protocol

After a rest period, the rats from the EMR and EMR+Egb761 groups, contained in polycarbonate cages, were placed at a central point between two cellphones (Nokia 3310, NHM-5NX) in talk mode. The phones having 0.96 W/kg SAR value were positioned 100 cm from the cages; the two phones were thus located at a distance of 265 cm from one another, including the length of the cages. Rats were exposed daily to cellphone RF for two hours every morning and afternoon for 30 days. Egb761 (100 mg/kg/day) (TriPharma Drug, Turkey) was also intraperitoneally (IP) administered to the EMR+Egb761 treatment group from the first day of

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the study until the last. After Egb761 administration, rats were exposed to mobile phone radiation. As rats were free to move in the cages, they received radiation from all directions. Control rats received no cellphone RF exposure or Egb761 and lived freely in their cages under routine laboratory conditions.

At the end of the study, all rats were anesthetized and sacrificed, and their cerebral hemispheres were dissected and fixed with neutral formalin for 48 hours. After rinsing with tap water, the specimens were processed through graded alcohols and xylene and then embedded in paraffin. The paraffin blocks of the left hemisphere were cut transversally by a rotary microtome (LeicaRM2135, Germany) into serial sections of 25 µm thickness. Each section was collected on adhesive-coated slides and stained with hematoxylin and eosin (H&E) for stereological analysis. As the left hemisphere was used for stereological sections, we used the right hemisphere to calculate the apoptotic index and to perform immunohistochemistry analyses. For these purposes, thin 5 µm sections of the right hemisphere were collected on adhesive-coated slides for terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling (TUNEL) staining.

Tissue preparation

All histological procedures were uniform. Rats were euthanized via cervical dislocation under anesthesia and their brains were carefully removed. The brain tissues were fixed in 10% buffered formalin (pH 7.4) for 48 h. The tissues were washed off formalin, dehydrated in an upgraded series of ethanol, cleared in xylene and embedded in paraffin at 60°C. All brains were oriented in the same direction during embedding to minimize differences in the sectioning angles. To maintain consistency, a single researcher processed all specimens. The brain tissue sections were cut on a rotary microtome (Leica RM2135, Germany) at a thickness of 25 µm and stained with H&E. The hippocampal regions were analyzed under a light microscope. To avoid any observer bias, an independent person coded the slides before analyzing.

Stereological procedure

Stereological analyses were performed at a stereology workstation (Tokat, Turkey) with a digital camera, a personal computer, a digital microcator (Heidenhein Traunreut, Germany), a trinocular light microscope (Leica DM 2500, Germany), and a computer-controlled, motorized specimen stage (Ludl Electronics, US). A software program (Stereo Investigator PC, MBF Biosciences, Wiliston, US) was used to control, measure, and record the stereological data and capture images of the sections. This system reproduced microscopy images (using a 60×Objective) on a computer monitor at a final magnification that allowed granule and pyramidal cells in the hippocampus

to be recognized and their total number to be counted.

All stereological analyses were blinded with respect to the treatment group to obtain unbiased results. The optical fractionator technique was used to count cells (Odaci et al., 2004; Ragbetli et al., 2010). Briefly, the section procedure was initiated outside the hippocampus. After the first section of the hippocampus in the series was found, section sampling was begun from a random point.

The section sampled fraction (SSF) was 1/15, and the first section was selected randomly from the first 15 sections. Ten to 12 sections were sampled from each hippocampal region in a random, systematic manner. The unbiased counting frame size for estimating cells was 156 µm². The area sampling fraction (ASF) was 156/22,500 µm², and the thickness sampling fraction (TSF) for the cell count was 15/21.4 µm. Although the upper guard zone of the TSF was 5 µm, the thickness of the bottom guard zone varied depending on each section's thickness.

Then, the total number of dentate granular cells and pyramidal cells in the hippocampus was estimated (West et al., 1991; Odaci et al., 2008). Cells were counted at the point in which the widest profile of the nucleus came into focus, and the optical dissector probes were systematically and randomly spaced throughout the hippocampus (Fig. 4) (Ragbetli et al., 2010). The total number of the cells in the hippocampus (N) was estimated as follows:

$$N = \Sigma Q(SSF)^{-1}(ASF)^{-1}(TSF)^{-1}$$

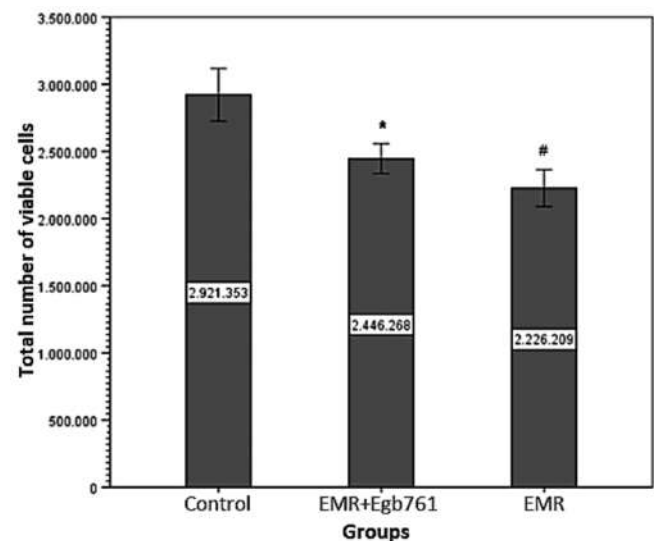


Fig. 1. The total number of pyramidal and granule cells was estimated for the all group rats' hippocampus DG and CA region. The total viable number of the cells of the EMR+Egb761 rats was significantly lower than that of the Control (* $P=0.015$). The total viable number of the cells of EMR+Egb761 group rats was increased; but it was not significant different from the others. H&E staining technique was used to count viable cells in the stereological technique (* $P>0.05$).

In this formula, ΣQ is the total dissector neuron number. SSF is the section sampling fraction; ASF is the area sampling fraction, and TSF is the thickness sampling fraction. Total neurons were estimated from the number of counted neurons and the sampling probability (Gundersen, 1986; Gundersen and Jensen, 1987; West et al., 1991; Odaci et al., 2004). The convenient number of sampled cells and the precision of total cell number estimates were verified using the coefficient of error (CE) (Gundersen and Jensen, 1987; West and Gundersen, 1990). The mean CEs for the number of counted cells per sample as well as further details on the counting procedures and stereological parameters of the groups were given in Table 1.

Immunohistochemical analyses

Sections of 5 μm thickness were placed on slides with adhesive and were deparaffinized with xylene and hydrated with 99% and 96-70% ethanol and distilled water. Then, the sections were boiled in a 10 mM sodium citrate buffer with a pH of 6.0 and maintained at a sub-boiling temperature for 5 min for antigen unmasking; at following, the sections were immediately cooled with distilled water, incubated for 15 min in 3% hydrogen peroxide, and then washed with phosphate buffered saline (PBS, 3 $\times$ 5 min). The sections were encircled with a hydrophobic pap pen. After incubation in blocking serum (non-immune) at room temperature (15 min), primary antibodies Bax, Bcl-2, and Acas-3 (1:50, Santa Cruz) were added to each section and incubated overnight at 4°C in a moist, dark environment. Next, the sample was stabilized at room temperature (30 min) and washed with PBS (3 $\times$ 5 min). The sections were then incubated for 30 minutes with biotinylated secondary antibodies and washed again with PBS (3 $\times$ 5 min). Afterwards, streptavidin secondary antibody was

added to each section. Sections were incubated for 30 minutes in a dark, humidified atmosphere and washed again with PBS (3 $\times$ 5min). Aminoethyl-carbazole (AEC) chromogen was applied to each section, which was then washed in water, counterstained with hematoxylin, and washed again. Finally, an aqueous mount solution (Invitrogen) was added to the sections mounted with coverslips. For the negative control, PBS was added to the sections instead of a primary antibody; the other steps were the same, and no immunostaining was performed.

All hippocampal tissue sections were viewed under a light microscope with 40x magnification in order to identify the immune reactive cells. A semi-quantitative evaluation system, or scoring scale (H-Score), was used to assess the severity of staining resulting from antigen antibody reactions. A total of four values along this scale, from negative one to positive three, were assigned, corresponding with no staining (-), detectable weak staining (+), medium severity staining (++), and severe staining (+++). This scale was used to evaluate the immune staining of both pyramidal and granule cells. The final score was set as the average score of the blinded specimens. To estimate the final cell counts, a formula based on the H-Score [$\Sigma \text{Pi}(i+1)$] was used. In this formula, i references the staining intensity score and Pi the percentage of stained cells (Cheon et al., 2001; Godbole et al., 2007).

TUNEL staining

To evaluate the extent of apoptosis in the hippocampal pyramidal and granule cells, staining was performed using a TUNEL staining assay kit (*in situ* cell death detection kit, AP, Roche) according to the manufacturer's instructions. Briefly, 5 μm -thick sections of 4% neutral, buffered, formalin-fixed, and paraffin-

Table 1. Mean values of total cell numbers both granule cells of dentatus gyrus and pyramidal cells of cornus ammonis, CE of stereological analysis, mean dissector number, section thickness and number of steps for estimation of total neuron number in the DG and CA of all study groups.

		Control G	EMR+Egb761 G	EMR G
Total cell numbers	CA	1552151 $\pm$ 96511 ^a	1328578 $\pm$ 103638 ^{ab}	1140138 $\pm$ 112464 ^b
	DG	1369202 $\pm$ 100595 ^x	1117691 $\pm$ 63213 ^{xy}	1086071 $\pm$ 38812 ^y
Dissector particle number	CA	515	442	402
	DG	505	422	328
Section thickness (μm)	CA	21.64	20.94	20.17
	DG	21.44	21.74	21.00
Number of steps for counting	CA	243	230	209
	DG	212	189	162
Number of sampled sections	CA	13	11	12
	DG	12	10	12
CE	CA	0.04	0.06	0.05
	DG	0.05	0.07	0.07

$\pm$, SEM; CA, cornus ammonis; DG, dentatus gyrus; G, group; CE, coefficient of error. ^{ab} $p > 0.05$ vs. Control and EMR groups; ^b $p = 0.039$ vs. Control groups (Tukey HSD). ^{xy} $p > 0.05$ vs. Control and EMR groups; ^y $p = 0.029$ vs. Control groups (Tukey HSD)

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embedded tissues were deparaffinized in xylene and rehydrated using a graded ethanol series and distilled water. These sections were placed in a plastic jar containing 200 ml of 0.1 M citrate buffer (pH 6.0) and were subjected to 750 W microwave irradiation (1 min); at following, sections were cooled rapidly by immediately adding 80 ml of double distilled water. Sections were then transferred to PBS and immersed for 30 min at room temperature in Tris-HCl (0.1 M, pH 7.5, containing 3% bovine serum albumin and 20% normal bovine serum). After being rinsed with PBS and allowing excess fluid to drain off, 50 μ l of the TUNEL reaction mixture was added to the specimens. For the negative control, a 50 μ l label solution was added, and the sections were incubated for 60 min at 37°C in a

humidified atmosphere in the dark. After being rinsed with PBS (3×5 min) three times and dried, 50 μ l Converter-AP (vial-3) was added to the samples. They were then incubated in a humidified chamber for 30 min at 37°C and rinsed with PBS (3×5 min). Afterwards, 50-100 μ l substrate solution (Fast Red) was added, and the sections were incubated for 10 min at 15-20°C in the dark. After being rinsed with PBS (3×5min), the slides were counterstained with hematoxylin and mounted under a glass coverslip. For the TUNEL reaction, the hematoxylin-stained cells served as controls, and red-stained nuclei cells were considered apoptotic. Five slides from each group were used to count apoptotic cells. The apoptotic index was evaluated as a percentage of the apoptotic cells in relation to the control cells.

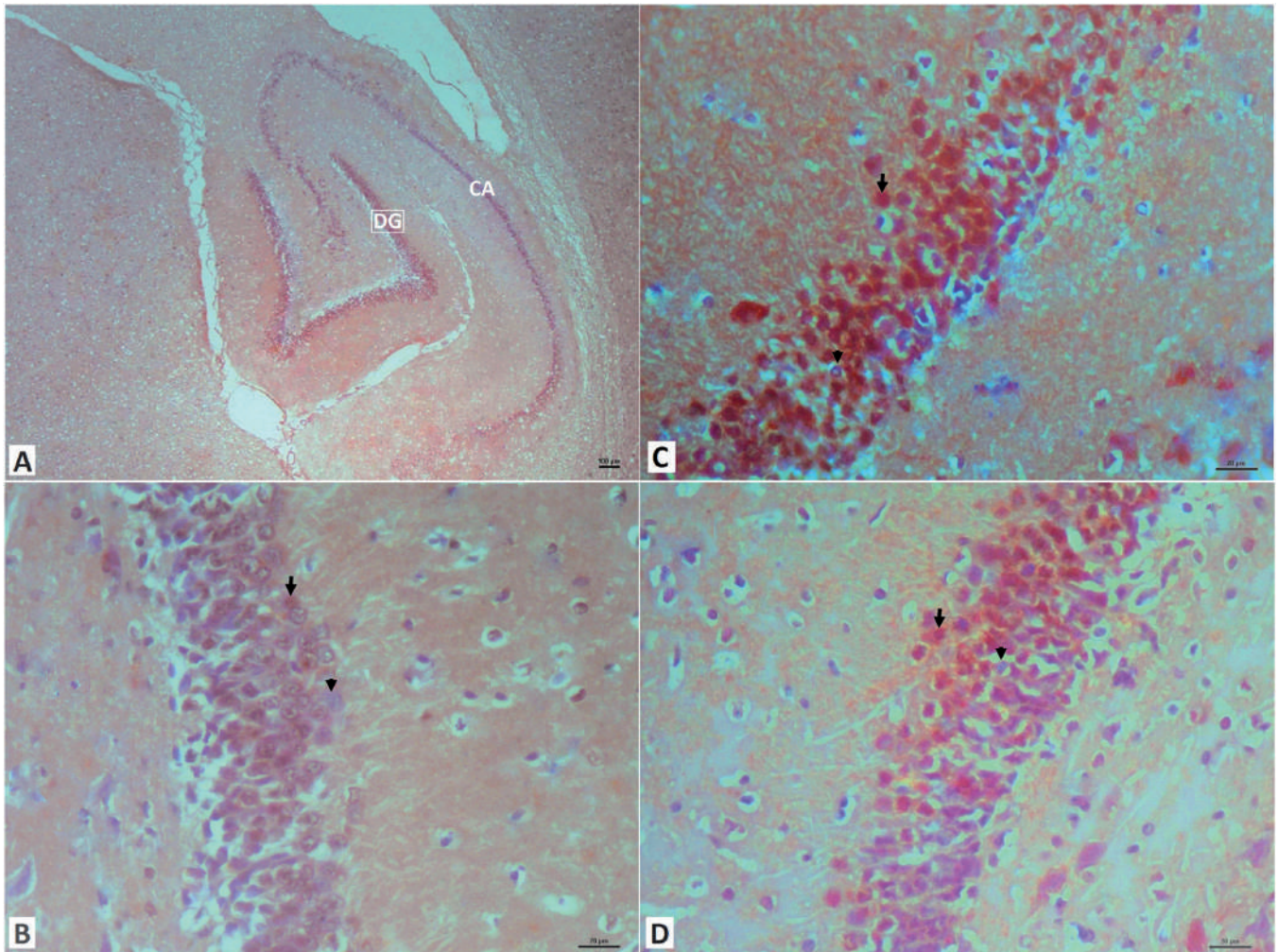


Fig. 2. Immune reactivity of the Bax protein is shown in DG region of hippocampus in all groups. The location of the DG images in the magnified pictures is the where that has been indicated with the rectangle in the panoramic picture. As stated in the result section EMR exposure caused a significant increase in Bax immunohistochemical expression of DG granule cells in comparison with the EMR+Egb761 or Control groups. **A.** Panoramic views of the hippocampus. **B.** Control. **C.** EMR. **D.** EMR+Egb761. Arrow and arrow head indicates one sample of immune stained and non-stained cells for Bax respectively. Immunohistochemical staining. Scale bars: A, 100 μ m; B-D, 20 μ m.

Image analysis

Analyses were performed with a microscope (Nikon Eclipse E200) focused on the CA and DG regions of the hippocampus, and pictures were taken by a digital camera system (Nikon DS-Fi1, Japan). An image program (NIS-Elements BR2.30 SP4, Nikon, Japan) was used to determine the apoptotic and control cell counts. The investigator performing these analyses was blinded to the identification of the groups. The boundaries of each CA region did not exist as clear lines. So, to avoid controversy in the results, the effect of cellphone EMR on the whole CA and GD tissues of the hippocampus were analyzed.

Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Windows (SPSS, IBM Co., Somers, NY, US). The results were expressed as means $\pm$ standard errors of the mean (SEMs). Differences among groups were statistically analyzed by one-way analyses of variance (ANOVA) followed by Tukey's honest significance difference (HSD) and least significant difference (LSD) multiple comparison tests. The differences were considered statistically significant if $P < 0.05$.

Results

Histopathological observations

Following cellphone exposure, the histological appearance of the hippocampi of all experimental groups was examined under light microscopy. Marked morphological changes were detected in the DG and CA regions of the hippocampus in the EMR-exposed rats compared with the controls, in which no changes were observed. These changes were moderate in the EMR+Egb761 group rats.

Granule and pyramidal cell numbers in the hippocampal DG and CA regions

The numbers of granule and pyramidal cells in the DG and CA regions, respectively, of the hippocampus were estimated for all study groups. Substantial cell loss was seen in the DG and CA sections of the EMR group. The total numbers of both granule and pyramidal cells in the EMR group were significantly lower than those of the control ($P = 0.029$, $P = 0.039$). In the EMR+Egb761 group, moderate cell loss was seen, although no significant differences were found in comparison to the other two groups ($P > 0.05$) (Table 1). When the two cell types were combined and evaluated together, similar statistical results were found (Fig. 1). The coefficient of error (CE) of the estimated numbers of granule and pyramidal cells in the control, EMR, and Egb761 groups are shown in Table 1. These values were within

acceptable ranges (Gundersen and Jensen, 1987; West et al., 1991).

Immunohistochemical analysis

The immunohistochemical analysis of apoptotic (Bax, Acas-3) and anti-apoptotic (Bcl-2) proteins showed that cellphone EMR induced the Bax apoptosis pathway. It was observed that apoptotic protein expression (Acas-3 and Bax) was upregulated, whereas anti-apoptotic protein expression (Bcl-2) was downregulated in pyramidal and granule cells of the CA and DG regions, respectively, of the hippocampal tissue of rats following cellphone exposure. However, a significant difference was only found in Bax immunohistochemical expression in DG granule cells versus the control (Fig. 2).

Egb761 administration generally caused a decrease in the expression of apoptotic proteins Bax and Acas-3 and an increase in the expression of anti-apoptotic protein Bcl-2, although these differences were not statistically significant (only Bax expression in the DG granule cells was significant). Compared to the control group, Bax immunohistochemical expression in granule cells did not differ, so the two groups were statistically similar ($P > 0.05$). Also, these results did significantly differ from the EMR group ($P = 0.035$) (Fig. 2, Table 2).

TUNEL assay

The apoptotic cell index markedly increased in both CA pyramidal and DG granule neurons of the hippocampus of rats in the EMR group following cellphone exposure, while this index was the lowest in the control group. The mean apoptotic index values for the Egb761 group were between those of the control and EMR groups (Fig. 3A-C). Compared to the control and the EMR+Egb761 groups, the DG granule neurons of the hippocampus of the EMR group were significantly

Table 2. Mean immunohistochemical H-Score values of apoptosis proteins expression and mean API in CA pyramidal and DG granule neuron of hippocampus in all groups.

		EMR G	EMR+Egb761	Control G	P
Bax	CA	68,12 $\pm$ 4,1	62,99 $\pm$ 0,9	51,95 $\pm$ 5,2	>0.05
	DG	94,00 $\pm$ 5,8*	72,43 $\pm$ 2,7	62,39 $\pm$ 4,4	*<0.05
Bcl-2	CA	60,24 $\pm$ 4,2	62,13 $\pm$ 3,5	65,91 $\pm$ 4,1	>0.05
	DG	75,83 $\pm$ 7,5	84,10 $\pm$ 7,5	87,23 $\pm$ 8,9	>0.05
Acas-3	CA	82,72 $\pm$ 13,5	76,24 $\pm$ 6,6	66,79 $\pm$ 9,7	>0.05
	DG	57,75 $\pm$ 2,8	55,28 $\pm$ 2,8	54,40 $\pm$ 5,4	>0.05
API	CA	0.93 $\pm$ 0,14	0.82 $\pm$ 0,15	0.48 $\pm$ 0,10	>0.05
	DG	0.87 $\pm$ 0,12#	0.75 $\pm$ 0,11	0.46 $\pm$ 0,07	#<0.05

$\pm$, SEM; CA, cornu ammonis; DG, dentatus gyrus; G, group; Bax, Bcl-2-associated X protein; Bcl-2, Beta cell lymphoma-2; Acas-3, Active caspase-3; API, Apoptotic index. * and #: $p < 0.05$ vs EMR+Egb761 and Control groups.

different ($P=0.29$). In the EMR+Egb761 group, the mean apoptotic index values of both the CA and DG regions decreased relative to the EMR group; nevertheless, no statistically significant differences were found ($P>0.05$). In the CA region, the apoptotic cell index increased in the EMR group compared with the control but decreased upon comparing the EMR group to the EMR+Egb761 group (Fig. 3A-C); however, these differences were not statistically significant ($P>0.05$). In table 2, the mean apoptotic cell index values of pyramidal and granule cells of the CA and DG, respectively, of the hippocampus for all groups were demonstrated.

Discussion

Wireless communication, especially cellphones, is rapidly gaining popularity, although little is known about its effects on health. For this, the expansion of mobile communication over the last 20 years has raised doubts about the potentially adverse effects of EMR emitted by cellphones on the human brain, particularly on the hippocampus. As a result of the close proximity of the brain region to the RF source of cellphones, the hippocampus is exposed to relatively high SAR levels (Mausset et al., 2001; Odaci et al., 2008). EMR may have several neurological effects, including headaches and changes in sleep patterns (Wagner et al., 1998; Borbely et al., 1999; Butterfield and Lauderback, 2002;

Jing et al., 2012).

In particular, the hippocampus is a limbic structure of the brain that controls behavioral and cognitive functions, including memory, learning, spatial learning, and regulation of the neuroendocrine axis (Ragbetli et al., 2009). The production and plasticity of new neurons in the DG may have an important role in hippocampal functions, as the DG is a major source of afferent input into the hippocampus (Snyder et al., 2001). Deleterious environmental conditions can profoundly affect neurogenesis in the brain and may also induce brain activities that manifest in altered behavioral and cognitive functions, especially following long-term exposure.

Salford et al. (2003) first reported evidence of neuronal damage caused by nonthermal microwave exposure. The cortex, hippocampus, and basal ganglia in the brains of exposed rats contained dark neurons. These authors identified a significant relationship between the EMR dose and the number of dark neurons. Also, they observed that Egb761 treatment prevented EMR-induced histopathological changes in the brains of rats. EMF has been used in a number of biological and experimental studies, wherein neuronal damage in the hippocampus, cortex, and basal ganglia has been reported in rats (Salford et al., 2003). The hippocampus, especially the granule cells of the DG (Odaci et al., 2008) and the CA areas (Bas et al., 2009), are selectively vulnerable to

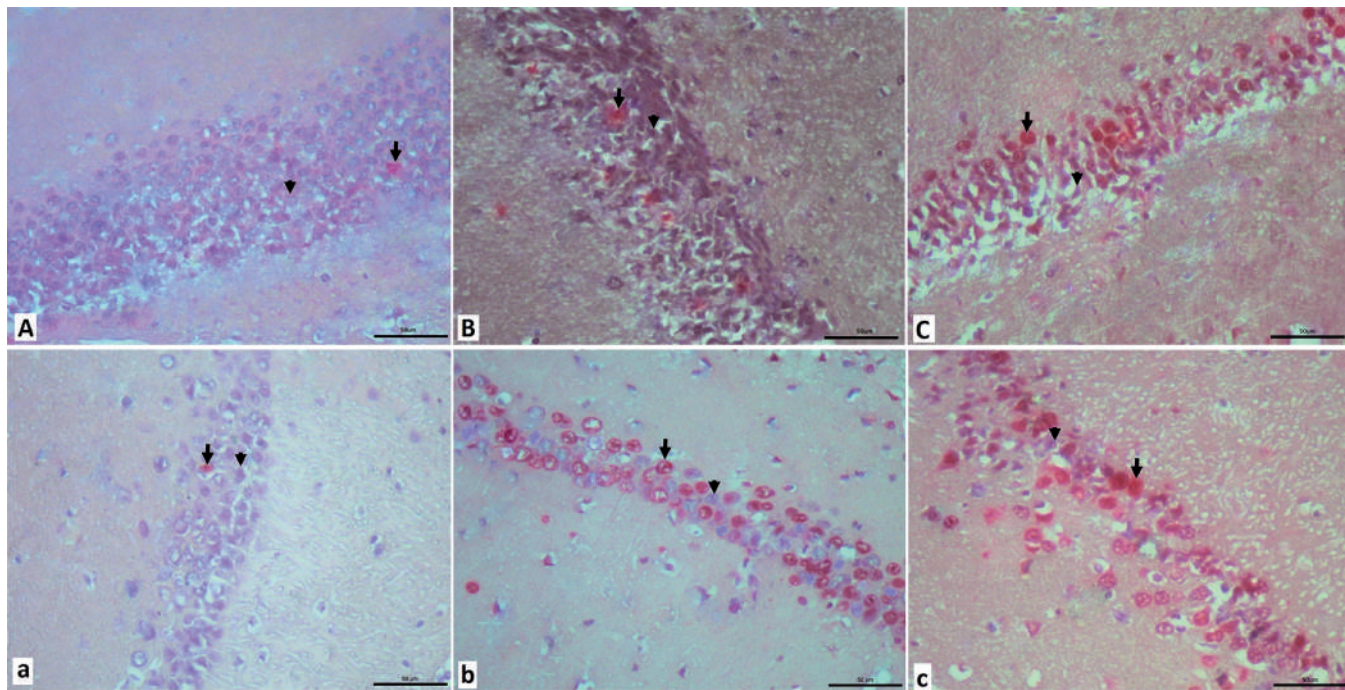


Fig. 3. Representative photomicrographs showing TUNEL labeling in the hippocampal CA (a-c) and GD (A-C) area in all groups. Apoptotic, TUNEL-positive cells is shown red staining. Arrow and arrow head indicates one sample of TUNEL stained apoptotic and non-stained normal cells respectively (TUNEL staining). Scale bars: 50 μ m.

EMR. The number of adult rat pyramidal cells in the CA decreased following four weeks of exposure at 900 MHz (Bas et al., 2009), and EMF exposure also caused granule cell loss in the DG of rats in another experimental study (Odaci et al., 2008). These findings, which are similar to those of this study, may indicate that RF exposure has a negative effect on hippocampal tissues. In another stereological study, Sonmez et al. (Sonmez et al., 2010) showed that the total number of Purkinje cells in the cerebellum of EMR-exposed rats was significantly lower than that of the controls. It was used an updated stereology technique to estimate the total viable neuron number and found similar results as these two previous studies. So, cellphone EMF can be postulated as hazardous for the hippocampus in the case of long-term exposure.

Furthermore, EMR emitted by cellphones may affect brain morphology as well as physiologic activities and neurons at the cellular level (Mausset et al., 2001;

Salford et al., 2003; Mausset-Bonnefont et al., 2004; Manikonda et al., 2007; Maskey et al., 2010). Maskey et al. (2010) reported that EMF exposure at 835 MHz for one month produced a loss of pyramidal cells in the CA area and disrupted the granular layer of the DG. Chronic RF exposure in rats lowered levels of calcium binding protein and resulted in an influx of calcium into cells. Subsequently, apoptosis and an increase of glial fibrillary acidic protein were evidenced as possible morphological parameters of hippocampus damage (Maskey et al., 2010). This study also revealed a loss of pyramidal cells in the CA area as well as a decrease in the granule cells of the DG area.

The present study demonstrated that prolonged exposure to cellphones in calling mode increased the levels of immunohistochemical expression of apoptosis biomarkers, induced DNA damage, and caused some histopathological changes and lowered cell numbers in the CA and DG regions of hippocampal tissues,

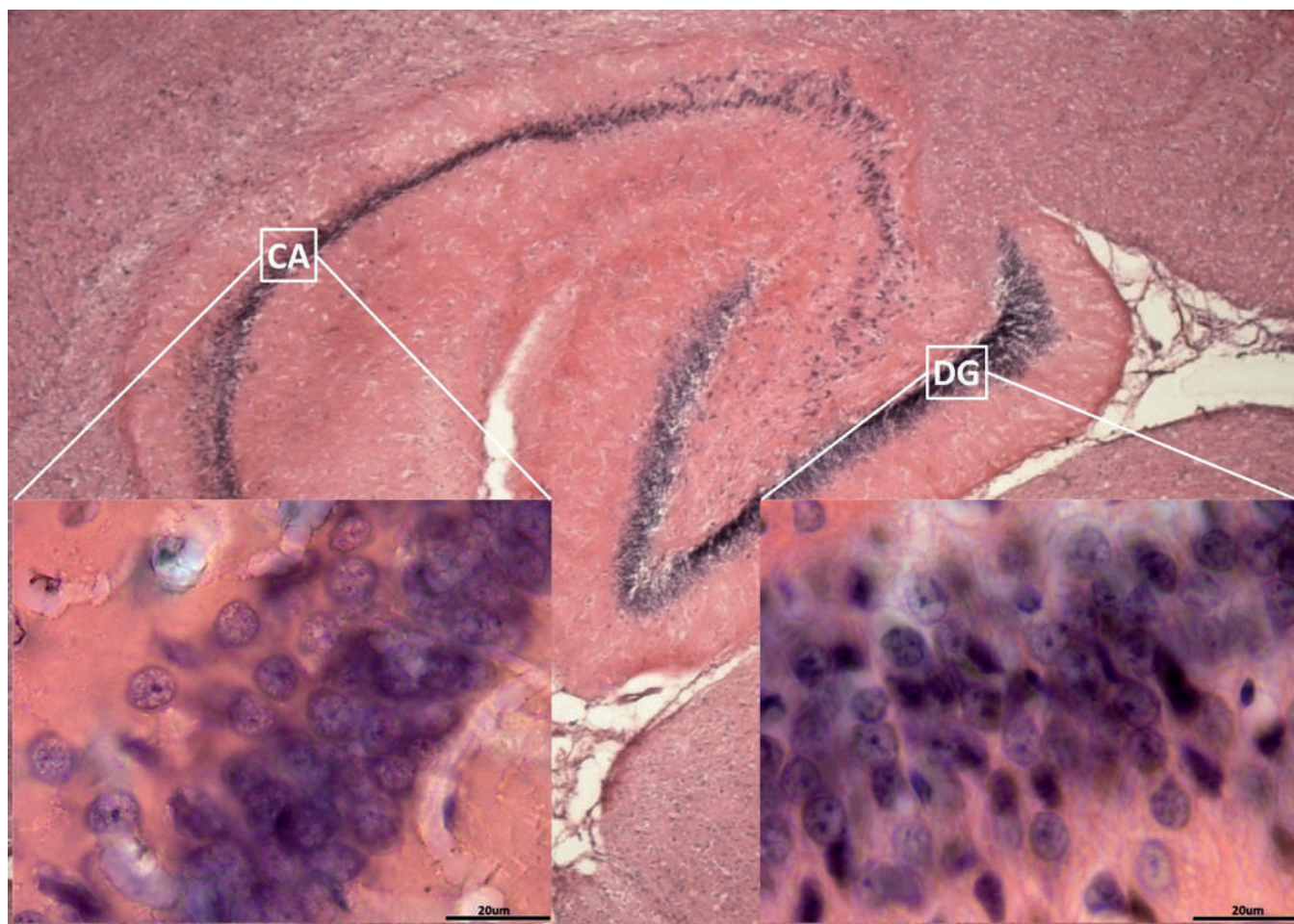


Fig. 4. Representative micrograph of the rat hippocampus from a stereological cross section. The inset in panel shows the pyramidal and granular cells with the widest profile of nucleus. The cells its nucleus and nucleolus are seen obviously were counted. CA: cornu ammonis; DG: dentatus gyrus. H&E staining. Scale bars: 20 μ m.

indicating the possibility of neuronal damage. Since the nervous system is particularly vulnerable to EMR because of its high metabolic rate, deficient oxidant defense mechanisms, and diminished cellular turnover, OS may contribute to the initiation and/or the progression of neuronal damage following exposure to cellphone-emitted radiation (Motawi et al., 2014).

Many studies have indicated that EMR can produce a stress response and affect apoptosis, yet additional negative results have also been reported depending on cell type and exposure characteristics (Hook et al., 2004; Maskey et al., 2010). Apoptosis is programmed cell death characterized by a variety of morphological, biochemical, and genetic markers (Tian et al., 2002). Studies have indicated that two distinct apoptotic pathways exist in mammalian systems: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The former involves the binding of death ligands to their cognate cell surface receptors, resulting in the activation of initiator caspase-8 and effector caspase-3. However, in the intrinsic pathway, cells respond to various stimuli by triggering apoptosis through the Bax/Bcl-2/caspase-9 cascade, wherein caspase-9 cleaves and activates apical effector caspases like caspase-3. Therefore, caspase-3 is one of the key executioners of apoptosis and is either partially or totally responsible for the proteolytic cleavage of many key proteins (Thornberry and Lazebnik, 1998; Nicholson, 1999).

In this present investigation of apoptosis, the detection of Acas-3 along with the up-regulation of Bax and the down-regulation of Bcl-2 indicated that exposure to cellphones for 30 days (SAR: 0.96 W/kg, 4 h/day) may disturb the intrinsic apoptotic machinery in rat hippocampal tissues and disrupt the neurogenesis of DG granule and CA pyramidal cells in the hippocampus, leading to the impairment of neuronal oxidant balance and probable adverse effects.

Stereological and gross histopathological findings also revealed that cellphone radiation led to severe damage in the neuronal cells of the CA and DG regions. Hippocampal neuronal cells were more vulnerable to disruption, even at low-energy radiation, than other types of cells. Neuronal damage was moderate in the Egb761-exposed group but severe in the EMR-exposure group. Therefore, the detrimental effect of cellphone radiation on rat hippocampi was alleviated through the administration of Egb761 (100 mg/kg/day, IP).

Notably, the mechanisms leading to the observed neuronal damage can be either direct or indirect. For instance, EMR can directly cause cell death as a consequence of oxidative stress, which induces apoptosis, for example, yet can indirectly cause damage via other toxic agents that might be taken up into neurons or released from neuronal stores. These toxic agents could be albumin or other substances conjugated to albumin that are transported across the BBB (Eberhardt et al., 2008). Nittby et al. (2009) also observed a significant increase in the BBB permeability

of mammalian brains after seven days of exposure to radiation from a GSM-900 cellphone.

Conclusion

In summary, cellphone radiation has harmful effects on hippocampal tissues. The effects of radiation on cell apoptosis were more obvious in cellphone EMR-exposed rats than in the EMR+Egb761 and control group rats. This study provided three important findings in regard to oxidative damage following experimental exposure to EMR. First, cellphones in calling mode could cause oxidative damage-induced cell death in hippocampal tissues (De Iuliis et al., 2009). Second, EMR exposure produced histopathological changes in the CA and the DG regions of the hippocampus. Third, Egb761 (*ginkgo biloba* extract) contains compounds with potent free-radical scavenger and antioxidant properties that may prevent some of the effects of cellphone EMR on hippocampal tissues. However, it is difficult to extrapolate these effects from rodents to humans; also, the entire body of the rat was exposed to EMR, while a person only receives direct exposure through his or her skull, affecting brain tissue near the cellphone. Long-term EMR exposure from cellphones has a destructive effect on hippocampal tissues that can lead to the disruption of tissue-related functions, such as learning and memory. Therefore, cellphones should not be kept in calling mode for long periods of time and should be held away from the body. More advanced technologies with fewer or no biological effects should be developed in the future.

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References

- Ameisen J.C. and Capron A. (1991). Cell dysfunction and depletion in AIDS: The programmed cell death hypothesis. *Immunol. Today* 12, 102-105.
- Andersen J.B., Mogensen P.E. and Pedersen G.F. (2002). Possible exposures from future mobile communications systems. In: *Review of radio science 1999-2002*. Wiley.
- Anderson V. (2003). Comparisons of peak SAR levels in concentric sphere head models of children and adults for irradiation by a dipole at 900 MHz. *Phys. Med. Biol.* 48, 3263-3275.
- Barnett J., Timotijevic L., Shepherd R. and Senior V. (2007). Public responses to precautionary information from the department of

- health (uk) about possible health risks from mobile phones. Health Policy 82, 240-250.
- Bas O., Odaci E., Kaplan S., Acer N., Uco K. and Colakoglu S. (2009). 900 mhz electromagnetic field exposure affects qualitative and quantitative features of hippocampal pyramidal cells in the adult female rat. Brain Res. 1265, 178-185.
- Behari J. (2010). Biological responses of mobile phone frequency exposure. Indian J. Exp. Biol. 48, 959-981.
- Borbely A.A., Huber R., Graf T., Fuchs B., Gallmann E. and Achermann P. (1999). Pulsed high-frequency electromagnetic field affects human sleep and sleep electroencephalogram. Neurosci. Lett. 275, 207-210.
- Bridi R., Crossetti F.P., Steffen V.M. and Henriques A.T. (2001). The antioxidant activity of standardized extract of *ginkgo biloba* (egb 761) in rats. Phytother. Res. 15, 449-451.
- Butterfield D.A. and Lauderback C.M. (2002). Lipid peroxidation and protein oxidation in alzheimer's disease brain: Potential causes and consequences involving amyloid beta-peptide-associated free radical oxidative stress. Free Radic. Biol. Med. 32, 1050-1060.
- Cheon K.W., Lee H.S., Parhar I.S. and Kang I.S. (2001). Expression of the second isoform of gonadotrophin-releasing hormone (GNRH-II) in human endometrium throughout the menstrual cycle. Mol. Hum. Reprod. 7, 447-452.
- Daniels W.M., Pitout I.L., Afullo T.J. and Mabandla M.V. (2009). The effect of electromagnetic radiation in the mobile phone range on the behaviour of the rat. Metab. Brain Dis. 24, 629-641.
- De Iuliis G.N., Newey R.J., King B.V. and Aitken R.J. (2009). Mobile phone radiation induces reactive oxygen species production and DNA damage in human spermatozoa *in vitro*. PLoS One 4, e6446.
- Dogan M., Turtay M.G., Oguzturk H., Samdanci E., Turkoz Y., Tasdemir S., Alkan A. and Bakir S. (2012). Effects of electromagnetic radiation produced by 3G mobile phones on rat brains: Magnetic resonance spectroscopy, biochemical, and histopathological evaluation. Hum. Exp. Toxicol. 31, 557-564.
- Eberhardt J.L., Persson B.R., Brun A.E., Salford L.G. and Malmgren L.O. (2008). Blood-brain barrier permeability and nerve cell damage in rat brain 14 and 28 days after exposure to microwaves from gsm mobile phones. Electromagn. Biol. Med. 27, 215-229.
- Faruque M.R.I., Islam M.T. and Misran N. (2011). Analysis of electromagnetic absorption in mobile phones using metamaterials. Electromagnetics 31, 215-232.
- Ferreira A.R., Knakievicz T., Pasquali M.A., Gelain D.P., Dal-Pizzol F., Fernandez C.E., de Salles A.A., Ferreira H.B. and Moreira J.C. (2006). Ultra high frequency-electromagnetic field irradiation during pregnancy leads to an increase in erythrocytes micronuclei incidence in rat offspring. Life Sci. 80, 43-50.
- Godbole G.B., Modi D.N. and Puri C.P. (2007). Regulation of homeobox a10 expression in the primate endometrium by progesterone and embryonic stimuli. Reproduction 134, 513-523.
- Gundersen H.J. (1986). Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of william R. Thompson. J. Microsc. 143, 3-45.
- Gundersen H.J. and Jensen E.B. (1987). The efficiency of systematic sampling in stereology and its prediction. J. Microsc. 147, 229-263.
- Gupta N., Goyal D., Sharma R. and Arora K.S. (2015). Effect of prolonged use of mobile phone on brainstem auditory evoked potentials. J. Clin. Diagn. Res. 9, CC07.
- Hook G.J., Zhang P., Lagroye I., Li L., Higashikubo R., Moros E.G., Straube W.L., Pickard W.F., Baty J.D. and Roti Roti J.L. (2004). Measurement of DNA damage and apoptosis in molt-4 cells after *in vitro* exposure to radiofrequency radiation. Radiat. Res. 161, 193-200.
- Hossain M.I., Faruque M.R.I. and Islam M.T. (2015). Analysis on the effect of the distances and inclination angles between human head and mobile phone on sar. Prog. Biophys. Mol. Bio. 119, 103-110.
- Ilhan A., Gurel A., Armutcu F., Kamisli S., Iraz M., Akyol O. and Ozen S. (2004). *Ginkgo biloba* prevents mobile phone-induced oxidative stress in rat brain. Clin. Chim. Acta 340, 153-162.
- Jing J., Yuhua Z., Xiao-qian Y., Rongping J., Dong-mei G. and Xi C. (2012). The influence of microwave radiation from cellular phone on fetal rat brain. Electromagn. Biol. Med. 31, 57-66.
- Khullar S., Sood A. and Sood S. (2013). Auditory brainstem responses and emfs generated by mobile phones. Indian J. Otolaryngol. Head Neck Surg. 65, 645-649.
- Kundi M., Mild K., Hardell L. and Mattsson M.O. (2004). Mobile telephones and cancer--a review of epidemiological evidence. J. Toxicol. Environ. Health B. Crit. Rev. 7, 351-384.
- Manikonda P.K., Rajendra P., Devendranath D., Gunasekaran B., Aradhya R., Sashidhar R. and Subramanyam C. (2007). Influence of extremely low frequency magnetic fields on ca 2+ signaling and nmda receptor functions in rat hippocampus. Neurosci. Lett. 413, 145-149.
- Maskey D., Pradhan J., Aryal B., Lee C.-M., Choi I.-Y., Park K.-S., Kim S.B., Kim H.G. and Kim M.J. (2010). Chronic 835-mhz radiofrequency exposure to mice hippocampus alters the distribution of calbindin and GFAP immunoreactivity. Brain Res. 1346, 237-246.
- Mausset-Bonnefont A.L., Hirbec H., Bonnefont X., Privat A., Vignon J. and de Seze R. (2004). Acute exposure to gsm 900-Mhz electromagnetic fields induces glial reactivity and biochemical modifications in the rat brain. Neurobiol. Dis. 17, 445-454.
- Mausset A.L., de Seze R., Montpeyroux F. and Privat A. (2001). Effects of radiofrequency exposure on the gabaergic system in the rat cerebellum: Clues from semi-quantitative immunohistochemistry. Brain Res. 912, 33-46.
- Motawi T.K., Darwish H.A., Moustafa Y.M. and Labib M.M. (2014). Biochemical modifications and neuronal damage in brain of young and adult rats after long-term exposure to mobile phone radiations. Cell Biochem. Biophys. 70, 845-855.
- Narayanan S.N., Kumar R.S., Potu B.K., Nayak S., Bhat P.G. and Mailankot M. (2010). Effect of radio-frequency electromagnetic radiations (RF-EMR) on passive avoidance behaviour and hippocampal morphology in wistar rats. Ups. J. Med. Sci. 115, 91-96.
- Nicholson D.W. (1999). Caspase structure, proteolytic substrates, and function during apoptotic cell death. Cell Death Differ. 6, 1028-1042.
- Nittby H., Brun A., Eberhardt J., Malmgren L., Persson B.R. and Salford L.G. (2009). Increased blood-brain barrier permeability in mammalian brain 7 days after exposure to the radiation from a GSM-900 mobile phone. Pathophysiology 16, 103-112.
- Odaci E., Bas O. and Kaplan S. (2008). Effects of prenatal exposure to a 900 mhz electromagnetic field on the dentate gyrus of rats: A stereological and histopathological study. Brain Res. 1238, 224-229.
- Odaci E., Kaplan S., Sahin B., Bas O., Gevrek F., Aygün D., Ünal B., Sönmez O., Colakoglu S. and Bilgic S. (2004). Effects of low-dose oxcarbazepine administration on developing cerebellum in newborn rat: A stereological study. Neurosci. Res. Commun. 34, 28-36.
- Ozben T. (2007). Oxidative stress and apoptosis: Impact on cancer therapy. J. Pharm. Sci. 96, 2181-2196.

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- Pitchumoni S.S. and Doraiswamy P.M. (1998). Current status of antioxidant therapy for alzheimer's disease. *J. Am. Geriatr. Soc.* 46, 1566-1572.
- Ragbetli M.C., Aydinlioglu A., Koyun N., Ragbetli C. and Karayel M. (2009). Effect of prenatal exposure to mobile phone on pyramidal cell numbers in the mouse hippocampus: A stereological study. *Int. J. Neurosci.* 119, 1031-1041.
- Ragbetli M.C., Aydinlioglu A., Koyun N., Ragbetli C., Bektas S. and Ozdemir S. (2010). The effect of mobile phone on the number of purkinje cells: A stereological study. *Int. J. Radiat. Biol.* 86, 548-554.
- Repacholi M.H. and Greenebaum B. (1999). Interaction of static and extremely low frequency electric and magnetic fields with living systems: Health effects and research needs. *Bioelectromagnetics* 20, 133-160.
- Sakatani S. and Hirose A. (2002). A quantitative evaluation of the magnetic field generated by a ca3 pyramidal cell at epsp and action potential stages. *Ieee. T. Bio-Med. Eng.* 49, 310-319.
- Salford L.G., Brun A.E., Eberhardt J.L., Malmgren L. and Persson B.R. (2003). Nerve cell damage in mammalian brain after exposure to microwaves from gsm mobile phones. *Environ. Health Perspect.* 111, 881-883.
- Snyder J.S., Kee N. and Wojtowicz J.M. (2001). Effects of adult neurogenesis on synaptic plasticity in the rat dentate gyrus. *J. Neurophysiol.* 85, 2423-2431.
- Sonmez O.F., Odaci E., Bas O. and Kaplan S. (2010). Purkinje cell number decreases in the adult female rat cerebellum following exposure to 900mhz electromagnetic field. *Brain Res.* 1356, 95-101.
- Thornberry N.A. and Lazebnik Y. (1998). Caspases: Enemies within. *Science* 281, 1312-1316.
- Tian F., Nakahara T., Yoshida M., Honda N., Hirose H. and Miyakoshi J. (2002). Exposure to power frequency magnetic fields suppresses x-ray-induced apoptosis transiently in ku80-deficient xrs5 cells. *Biochem. Biophys. Res. Commun.* 292, 355-361.
- Tredici P.D. (1991). Ginkgos and people-a thousand years of interaction. *Arnoldia (Boston)* 51, 2-15.
- Wagner P., Roschke J., Mann K., Hiller W. and Frank C. (1998). Human sleep under the influence of pulsed radiofrequency electromagnetic fields: A polysomnographic study using standardized conditions. *Bioelectromagnetics* 19, 199-202.
- West M.J. and Gundersen H. (1990). Unbiased stereological estimation of the number of neurons in the human hippocampus. *J. Comp. Neurol.* 296, 1-22.
- West M.J., Slomianka L. and Gundersen H.J. (1991). Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *Anat. Rec.* 231, 482-497.

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