Calcium channel blockade alleviates brain injury induced by long term exposure to an electromagnetic field

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Summary
This study aimed to examine the effects of short and long term exposures to 81 mG EMF intensity. It focused on the roles of ROS, Ca²⁺ and calcium channel blocker (CCB) on the rat brain. Rats were exposed to 81 mG EMF intensity at the mobile phone base station for one and four weeks (2 hr/day, EMF exposed group). Another group of rats was pretreated with CCB (amlodipine 20 mg/kg) for four weeks and similarly exposed to EMF (EMF + amlodipine group). Sham exposed and amlodipine control groups were used. At the end of the study, Ca²⁺ as well as pro-inflammatory and oxidative stress markers were measured. Immunohistochemical staining for Bax in brain samples was carried out. Short term exposure evoked a cellular adaptation response. This was evident by a transient increase in brain levels of Ca²⁺, glutathione (GSH) and serum tumor necrosis factor alpha (TNFα). Long term exposure to EMF was lethal; progressive oxidative damage, and a prolonged increase in the Ca²⁺ level accompanied by a marked pro-inflammatory reaction (TNFα and CRP) were demonstrated. These alterations were ameliorated by pre- and con-comitant treatment with amlodipine. Furthermore, it restored the EMF induced apoptosis in brain to near normal. In conclusion, EMF is a stressor agent that induces an imbalance between ROS generation and antioxidant defense response. Calcium ions may play a pivotal role in enhancing oxidative stress, pro-inflammatory reactions and apoptosis associated with EMF exposure. Therefore calcium channel blockade seems to play a role in brain protection.

Key words: electromagnetic field – calcium – oxidative stress – apoptosis – amlodipine – rats

INTRODUCTION
An electromagnetic field (EMF) is a force field associated with an electric current containing electromagnetic energy. Every electrical home appliance from kitchen stove to television is an EMF source which may affect human health (Yeniterzi et al. 2002, Qui et al. 2004), and mobile phones have become, within a short period of time, very commonly used throughout the world.
There is widespread public concern that there may be a health hazard specifically during long term exposure (Kaune et al. 2002, Yokous et al. 2005), although there is no clear evidence to show the harmful effect of EMF at the levels used by mobile phones or their base stations and the possible risk of EMF exposure for the human body is still an open question. Some publications have shown that exposure to mobile phone signals can influence cellular processes such as proliferation (Sun et al. 2006), cell morphology (Orendacova et al. 2007), heat shock protein expression (Kwee et al. 2001, Leszczynski et al. 2002) and intracellular calcium ion concentrations (Minelli et al. 2007). Concern has been expressed about the possible activation potential of EMF on human immune relevant cells. Activation of macrophages and other blood cells can be induced by external signals to produce reactive oxygen species (ROS) such as hydrogen peroxide and superoxide (Simko et al. 2006). These free radicals can directly induce oxidative damage of DNA (Lai and Singh 2004) leading to cytotoxicity, and implying influence on cancer development and ageing (Hwang and Kim 2007).

Ca2+ ion plays an important role in a wide variety of cellular functions, including superoxide anion generation through NADPH oxidase and nitric oxide production by constitutive nitric oxide synthetase activity (Oelze et al. 2006), cytoskeleton function (Quinn et al. 2007), secretion of proteins (Berridge et al. 1998) and activation of transcription factors (Zhou et al. 2006).

The relationship between intracellular Ca2+ and oxidative stress may be a complex process. Oxidative stress may initiate changes in cytosolic Ca2+ concentration in cells that subsequently activate further production of ROS (Ernack and Davies 2002). This would consequently enhance upstream events of pro-inflammatory cytokine production such as tumor necrosis factor α (TNFα) (Brown et al. 2004).

In this study, we investigated the effects of short and long term exposures to 81 mG EMF intensity. It focuses on the role of ROS, Ca2+ on the rat brain as well as the effect of the calcium channel blocker (CCB) in manipulating EMF hazards.

**MATERIALS AND METHODS**

**Experimental animals**

Adult male albino rats aged about 4–5 months were supplied by the Egyptian Organization for Biological Products and Vaccine. Rats were subjected to controlled conditions of temperature (25±2 °C), relative humidity of approximately 50%, and illumination (12h light/dark). They were allowed free access to rodent chow diet (El-Naser Co, Egypt) and water. All experiments were carried out in accordance with protocols approved by the local experimental ethics committee.

**Measurement of the magnetic field intensity**

A Photon Gausses-Teslameter of 100 mG range accuracy ± 1% count and high resolution was used to measure the magnetic field intensity at different distances from the mobile phone base station (at El-Quamia Square, Zagazig). From table 1, we selected the magnetic field intensity which is very close to that emitted from GSM mobile phones (81 mG at distance 7 meters) and exposed the cages of animal according to the design listed below.

**Experimental Design**

**Part I: Short term study**

After a two weeks acclimatization period, animals were assigned to four groups (10 rats per group). Of these, two groups were exposed to EMF emitted from a mobile phone base station at an intensity of 81 mG for one week (2hr/day). One of these groups was pretreated with amlodipine (Global Napi Pharmaceuticals, Egypt) at a dose level of 20 mg/kg/daily/orally (Toba et al. 2005) for four weeks before and during the exposure to EMF. A sham exposed group and drug control rats (who received amlodipine 20 mg kg/daily/orally) were used in this study.

**Part 2: Long term study**

Four groups of male albino rats (10 rats per group) were used. The previously mentioned experimental design, classification, and treatment regimen were applied except that the duration of exposure to EMF was four weeks.

The animals were examined daily using simple observation for neurological signs. At the end of EMF exposure periods (one and four weeks), all animals from each group were anaesthetized with urethane (1.3 mg/kg) and blood samples were collected, subjected to serum and plasma separation and stored at −20 °C as aliquots for further biochemical analysis. Rats were then killed by decapitation; the brains were immediately excised and washed in 0.1 M sodium phosphate buffer (pH 7.4). The two cerebral hemispheres were isolated. One of them was frozen in liquid nitrogen for biochemical study and samples from the frontal lobe cerebral cortex were excised carefully,
processed for paraffin section preparation and directed for histological and immunohistochemical assessment.

**Methods**

Brain calcium was determined following acid digestion with nitric acid and perchloric acid (2/1) using an Atomic Absorbing Flame Emission Spectrometer (UNICAM 969, Unicam, UK) (Campillo et al. 2000).

Oxidative stress biomarkers were determined in brain tissues. Briefly, samples were placed in ice-cold 50 mmol/l Tris HCl buffer (pH 7.4) containing 1.15 % KCl, 1 mmol/l EDTA, 5 mmol/l D-glucose, 0.1 mmol/l DL-dithiothreitol and homogenized with a glass-glass homogenizer. The crude homogenate was centrifuged twice at 750 g for 5 minutes to remove the bigger fragments. The total protein in the brain tissue homogenate was assayed according to the method of Chromy and Fischer (1977) using a diagnostic kit (Biocon, Germany). Aliquots (50 µg total protein) were directed for spectrophotometric determination of superoxide anion (O$_2^-$) (Hassoun and Stohs 1996) and nitrite content using Griess reagent (Moshage et al. 1995). Reduced glutathione (GSH) (Ahmed et al. 1991) and lipid peroxide measured as malondialdehyde (MDA) were estimated as previously described (Buege and Aust 1978).

Serum TNFα (Kim et al 1994) and CRP (Highton and Hessian 1984) were evaluated by the ELISA technique using kits purchased from Biosource, USA. DNA was extracted from brain homogenate as previously described (Shibko et al. 1967) and quantified at 260 nm using UV spectrophotometer.

**Histological and immunohistochemical assessment**

Samples were fixed in Bouin's fixative for two days before processing. They were processed to obtain 4 µm thick paraffin sections. Sections were stained by hematoxylin and eosin (H&E) (Drury and Wallington 1980) and immunohistochemically for polyclonal antibodies (anti-Bax). Standard immunohistochemistry procedures were carried out as previously described (Bancroft and Steven1996). Briefly, sections were deparaffinized, dehydrated, rinsed in tap water, embedded in 3% H$_2$O$_2$ in ethanol and rinsed in phosphate buffer. 100 µl of primary antibody were applied to each slide. Secondary antibody was applied. Strept – Avidin – Biotin and 2 drops of diaminobenzidine (DAB) were added. Mayer's hematoxylin was used as a counter stain.

**Morphometric Study**

Using a Leica Qwin 500 LTD image analysis for the counting of apoptotic cells in 10 low power fields (LPF), the area of Bax (apoptotic cells) immunoexpression was carried out in 10 high power fields (HPF) using the interactive measurements menu. The optical density of Bax was measured in HPF. The morphometric study was done at the image analysis unit, Histology Department, Faculty of Medicine, Zagazig University, Egypt.

**Statistical analysis**

Statistical evaluation was carried out using one-way analysis of variance (ANOVA) according to Snedecor and Cochran 1981 followed by Duncan's Multiple Range Test (DMRT) using SPSS version 10.0 for Windows. The level of significance was $\alpha = 0.05$.

**RESULTS**

**Physical Examination**

The sham exposed rats and drug control group showed the same normal behavior and exhibited no signs of neurological abnormalities. Meanwhile, rats of the EMF exposed group showed neurological manifestations in the form of disturbed equilibrium, aggressiveness and hyperactivity. In addition, they showed redness of the eyes and altered visual functions. These signs started to appear two weeks after irradiation and progressively increased in severity. In contrast, these signs were not so apparent in the rats pre- and concomitantly treated with amlodipine.

**Effects of short term exposure to EMF**

Animals exposed to EMF (2hr/day) for one week showed a significant increase in brain Ca$^{2+}$, GSH, MDA, O$_2^-$ and serum TNFα concentrations in comparison with the sham exposed and drug control groups. No significant increment was observed in other inflammatory and oxidative stress biomarkers. Pre- and con-comitant treatment with amlodipine induced a remarkable decrease in Ca$^{2+}$ and TNFα levels (Table 2, Fig. 1).

**Effects of EMF long term exposure**

EMF exposure for four weeks induced a marked increase in brain Ca$^{2+}$ content (Fig. 1). An imbalance between liberation of ROS and antioxidant defense was elicited. This was
manifested by a marked increase in brain $O_2^-$, NO and MDA, whereas GSH exhibited a significant decrease in comparison with the sham exposed and drug control groups (Table 3). A marked increase in pro-inflammatory markers as demonstrated in the EMF exposed rats (serum TNF$\alpha$ and CRP), whereas, brain DNA content was significantly decreased (Table 4).

Morphometric measurement for brain apoptotic cells following immunohistochemical staining showed a marked increase of apoptotic index and strong optical density of Bax stain in the EMF exposed group (Table 5).

Histological (H &E) and immunohistochemical (Bax stain) findings of rat cerebral cortical sections of the test animals are illustrated in Fig.2A-H.

**Fig. 1. Changes in brain Ca$^{2+}$ contents following short and long term exposure to EMF.** Data are expressed as means ±SD of 8 rats from each group. Values not sharing a common superscript differ significantly (DMRT)

**Effects of amlodipine**

Pre- and con-comitant treatment of EMF exposed rats with amlodipine significantly reduced brain Ca$^{2+}$ content and attenuated the pro-inflammatory response as indexed by decrease in serum levels of TNF$\alpha$ and CRP. An increment of brain GSH, inhibition of $O_2^-$ liberation and MDA formation restored the balance between ROS and antioxidant defense in the amlodipine treated group (Table 3).

Amlodipine has also a remedial effect against apoptosis. This is evident by a significant increase in brain DNA contents, a 3 fold decrease in the apoptotic index and 50% reduction of optical density of Bax reaction. (Tables 4, 5).

The histological examination of the amlodipine + EMF exposed group showed few small sized and dark nerve cells and multiple normal size nerve cells (H&E, Fig. 2G). Bax stained brain sections revealed moderately positive immunooexpression compared to EMF control group (Figs. 2H and 2F).

**DISCUSSION**

Short term exposure to EMF seems to play a role in the activation of innate and acquired immunity. This is evidenced by a significant increase in the serum level of TNF$\alpha$ and brain GSH content, whereas no significant elevation in MDA and were observed. These results are consistent with Rollwitz et al. (2004), who demonstrated that 50 Hz EMF induces activation of immune relevant cells and enhances the phagocytes capacity of macrophages.
Fig. 2. Photomicrographs of adult rats cerebral cortex showing pyramidal (P), fusiform (F), granular (G) nerve cells, neuroglia cells (arrows) and blood capillary (double arrows) (A), weak positive immunoexpression for Bax (arrows) (B) in sham exposed group. Similar histological structure to sham exposed control (C), weak positive immunoexpression for Bax (arrows) (D) in amlodipine control group. Small sized and dark pyramidal (P), granular (G) nerve cells and multiple microglia cells (arrows) (E), strong positive Bax immunoexpression (arrows) (F) in EMF exposed group. Few small sized and dark nerve cells (arrows) and multiple normal sized nerve cells (double arrows) (G), moderately positive Bax immunoexpression (arrows) (H) in amlodipine + EMF group.

A, C, E, G – using H&EX 400
B, D, F, H – using Bax X 400
Table 1. Magnetic field intensity (mG) at different distances (meters) from mobile phone base station

<table>
<thead>
<tr>
<th>Distance (meters)</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnetic field intensities (mG),</td>
<td>99</td>
<td>95</td>
<td>91</td>
<td>88</td>
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<td>81</td>
<td>76</td>
<td>65</td>
<td>57</td>
<td>51</td>
<td>46</td>
<td>38</td>
<td>28</td>
<td>22</td>
</tr>
</tbody>
</table>

Table 2. Effects of short term exposure to EMF on some biochemical markers in serum and brain tissues

<table>
<thead>
<tr>
<th></th>
<th>Sham exposed</th>
<th>Amlodipine</th>
<th>EMF exposed</th>
<th>Amlodipine +EMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF α (pg/ ml)</td>
<td>15.6±2.8b</td>
<td>15.2± 2.1b</td>
<td>33.1±5a</td>
<td>17.4± 3.1b</td>
</tr>
<tr>
<td>CRP (µg/ml)</td>
<td>1.25 ±0.21b</td>
<td>1.15 ± 0.19b</td>
<td>1.32 ±0.25a</td>
<td>1.17±0.21b</td>
</tr>
<tr>
<td>MDA (nmol/g)</td>
<td>1± 6c</td>
<td>53 ± 6a</td>
<td>60±8a</td>
<td>57 ± 9b</td>
</tr>
<tr>
<td>GSH (µ mol/g)</td>
<td>269± 23b</td>
<td>281± 26b</td>
<td>357 ± 28a</td>
<td>324± 31a</td>
</tr>
<tr>
<td>O₂⁻ (µmol cytochrome C reduced/min/g protein)</td>
<td>9.6 ± 0.88c</td>
<td>8.5 ± 0.96c</td>
<td>11.8 ± 1.3a</td>
<td>10.6 ± 1b</td>
</tr>
<tr>
<td>NO (µmol/g protein)</td>
<td>17± 1.5bc</td>
<td>18 ± 1.3bc</td>
<td>20 ± 1.7b</td>
<td>22 ± 2.3a</td>
</tr>
</tbody>
</table>

Values are mean ± SD of 8 rats from each group
Values not sharing a common superscript differ significantly (DMRT)

Table 3. Imbalance between ROS formation and limited antioxidant defense following long term exposure to EMF

<table>
<thead>
<tr>
<th></th>
<th>Sham exposed</th>
<th>Amlodipine</th>
<th>EMF exposed</th>
<th>Amlodipine + EMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/g)</td>
<td>55± 8c</td>
<td>61 ± 9c</td>
<td>124±13a</td>
<td>78 ± 8b</td>
</tr>
<tr>
<td>GSH (µ mol / g)</td>
<td>281± 25a</td>
<td>288± 31a</td>
<td>134 ± 16b</td>
<td>246± 21b</td>
</tr>
<tr>
<td>O₂⁻ (µ mol cytochrome C reduced/ min/g protein)</td>
<td>9.11 ±0.75c</td>
<td>10 ± 1.1c</td>
<td>21 ± 1.8a</td>
<td>13.7 ± 1.9b</td>
</tr>
<tr>
<td>NO (µ mol/ g protein)</td>
<td>15.8± 1.3c</td>
<td>15.3 ± 1.5c</td>
<td>27.4 ± 3.1a</td>
<td>18 ± 2.2b</td>
</tr>
</tbody>
</table>
Moreover, monocytes are a component of the immune system that perform a variety of functions including not only regulation of the immune response, phagocytosis, wound healing, repair and detoxification, but also generation of free radicals that are killing the invading microorganisms (Kriukov et al. 2006). This low level of free radicals enhances important physiological processes such as signal transduction of various membrane receptors and further immunological functions.

During long term exposure to EMF, we noticed redness and altered visual functions of the eyes as well as aggressiveness and hyperactivity in the rats. These signs were less evident in the amlodipine pretreated group and not detected in the control groups. These neurological signs may be attributed to an increase in the brain benzodiazepine receptors which are responsible for anxiety and stress responses in animals (Roy-Byrne 2005). The increase in the redness of the eyes and altered visual function may be related to damage of the corneal endothelial cells, degenerative changes in cells of the iris and retina after repeated exposure to EMF (Ozquner et al. 2006).

Increased free radical production here (NO) enhances lipid peroxidation (MDA) and changes the antioxidant status (GSH) following long term exposure to EMF. These findings are in agreement with others (Mostafa et al. 2001, Bediz et al. 2006). Subsequent to increased peroxidation, there is an increase in Ca²⁺ leakage from internal storage sites in the cells. This will trigger an increase in NO synthesis through activation of calmodulin dependent NO synthase. Accordingly, NO is quite involved in the biologic effect of EMF (Yoshikawa et al. 2000). Furthermore, NO can amplify free radical formation joined with depletion of cellular antioxidant and repair processes. Such an effect may lead to necrosis and apoptosis of brain cells. Necrosis is probably a consequence of progressive lipid peroxidative damage in the cell membranes, whereas apoptosis is mainly triggered by DNA

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Table 4. Effects of amlodipine on serum proinflammatory cytokine and brain DNA contents

<table>
<thead>
<tr>
<th></th>
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<th>EMF exposed</th>
<th>Amlodipine + EMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF α (pg/ml)</td>
<td>18.4±3.1c</td>
<td>16.7±2.3c</td>
<td>141±27a</td>
<td>66±11b</td>
</tr>
<tr>
<td>CRP (µ g/ml)</td>
<td>1.15 ± 0.19c</td>
<td>1.16 ± 0.17c</td>
<td>2.5±0.33a</td>
<td>1.38±0.23b</td>
</tr>
<tr>
<td>DNA (µ g/g)</td>
<td>8.89±1.31a</td>
<td>8.40±1.42a</td>
<td>5.81±0.46c</td>
<td>7.35±1.03b</td>
</tr>
</tbody>
</table>

Significance of data in Tables 3, 4 as per Table 2

Table 5. Effects of EMF long term exposure on some morphometric parameters using Bax stain

<table>
<thead>
<tr>
<th></th>
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<th>Amlodipine</th>
<th>EMF exposed</th>
<th>Amlodipine + EMF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptotic index (ratio of apoptotic cells to normal cells)</td>
<td>0.088±0.015c</td>
<td>0.09±0.013c</td>
<td>1.1±0.12a</td>
<td>0.36±0.05b</td>
</tr>
<tr>
<td>Optical density of Bax reaction</td>
<td>62±8c</td>
<td>58±6.5c</td>
<td>137±16a</td>
<td>69±7.6b</td>
</tr>
</tbody>
</table>

Significance as per Table 2
damage. This study demonstrated a significant decrease in the total DNA content of brain tissue and increase of apoptotic index following repeated exposure to EMF. However, there are other controversial negative findings (Frumkin et al. 2001, Simko et al. 2006).

Such an effect may be mediated through the Ca\textsuperscript{2+}/ROS process. Ca\textsuperscript{2+} induced oxidant formation is known to cause DNA break and DNA protein cross links, to activate protein kinase C, increase the production of heat shock protein and to alter calcium homeostasis (Uccelletti et al. 2005).

In the current study, Bax immunostained brain sections showed strong expression in the EMF exposed group. These results may be related to the effect of heat shock developed after exposure to EMF and subsequent production of superoxide radical anions (Lantow et al. 2006). Therefore strong expression of Bax may refer to apoptosis or programmed cell death. It is also documented morphometrically by a marked increase in the count of apoptotic cells in the EMF group compared to the control groups.

Amlodipine is a dihydropyridine calcium channel blocker that is widely used for treatment of hypertension (Julius et al. 2004). It has anti-atherosclerotic, anti-inflammatory and anti-oxidant effects in animals and humans (Yoshii et al. 2006).

In particular, the brain is an organ that is strongly affected by progressive oxidative stress. Oral treatment with amlodipine for four weeks before and during EMF exposure reduced oxidative stress and protected the brain function. These data clearly showed a significant reduction of brain, NO, MDA and restored GSH levels near normal values. The lipophilic properties of dihydropyridines such as amlodipine and nifedipine not only enable this class to cross the blood brain barrier (Huang and Leenen 2003) and reduce the generation of ROS in the brain (Fukuo et al. 2002) but also may be associated with their sympathe-inhibitory effect (Yoshitaka et al. 2006).

Several lines of evidence suggest that amlodipine possesses antioxidant activity, primarily due to its chain breaking antioxidative action, and direct scavenging of peroxyl radical mediated oxidation (Mak et al. 2002).

A transient increase in Ca\textsuperscript{2+} may initiate a signal transduction pathway contributing to cell survival. Ronit et al. (2003) added that such transient increases in Ca\textsuperscript{2+} enable the accumulation of sub lethal and induced cellular adaptation responses in the cells, the transcription and translation of the oxidative stress related enzyme and heme oxygenase, and heat shock proteins and the stimulation of cell growth.

However, this study demonstrates that prolonged increase in Ca\textsuperscript{2+} can be lethal following long term exposure to EMF.

Changes in Ca\textsuperscript{2+} homeostasis and ROS may be implicated in progression of apoptosis (Annuzziato et al. 2003). Elevation of the cytosolic Ca\textsuperscript{2+} level may lead to Ca\textsuperscript{2+} dependent proteases calpains and caspase-3 activation and subsequent initiation of apoptotic events (Sasaki et al. 2007). This study showed the protective effect of CCB against apoptosis induced by EMF long term exposure.

Ca\textsuperscript{2+} is greatly implicated in TNF\alpha mediated cell damage through activation of proteases (Sasaki et al. 2007). and the levels of Ca\textsuperscript{2+}, TNF\alpha and CRP were markedly elevated following repeated EMF exposure. Amlodipine treatment suppresses the change in the mitochondrial membrane permeability leading to decrease in intracellular Ca\textsuperscript{2+} and subsequent inflammatory response (Kataoka et al. 2004). The anti-inflammatory effect of amlodipine is represented by a significant reduction of both serum TNF\alpha and CRP leading to protection of the brain tissues.

In conclusion, this study speculates that long term EMF exposure is a stressor agent especially on the oxidative system, whereas in the short term, it evokes a cellular adaptation response. Calcium ions may be implicated as a second messenger in activating pro-inflammatory transcription factors (TNF\alpha), amplifying free radical formation through the NO/Ca\textsuperscript{2+} calmodulin mechanism and reinforcing apoptosis during long term exposure. These results showed a remedial effect of Ca\textsuperscript{2+} channel blockade in alleviating cellular injury challenged by EMF long term exposure.

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