



Reactive oxygen species levels and DNA fragmentation on astrocytes in primary culture after acute exposure to low intensity microwave electromagnetic field

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ABSTRACT

The exposure of primary rat neocortical astroglial cell cultures to acute electromagnetic fields (EMF) in the microwave range was studied. Differentiated astroglial cell cultures at 14 days *in vitro* were exposed for 5, 10, or 20 min to either 900 MHz continuous waves or 900 MHz waves modulated in amplitude at 50 Hz using a sinusoidal waveform and 100% modulation index. The strength of the electric field (rms value) at the sample position was 10 V/m. No change in cellular viability evaluated by MTT test and lactate dehydrogenase release was observed. A significant increase in ROS levels and DNA fragmentation was found only after exposure of the astrocytes to modulated EMF for 20 min. No evident effects were detected when shorter time intervals or continuous waves were used. The irradiation conditions allowed the exclusion of any possible thermal effect. Our data demonstrate, for the first time, that even acute exposure to low intensity EMF induces ROS production and DNA fragmentation in astrocytes in primary cultures, which also represent the principal target of modulated EMF. Our findings also suggest the hypothesis that the effects could be due to hyperstimulation of the glutamate receptors, which play a crucial role in acute and chronic brain damage. Furthermore, the results show the importance of the amplitude modulation in the interaction between EMF and neocortical astrocytes.

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The effects of electromagnetic fields (EMF) on human health have been widely studied using a broad range of the electromagnetic spectrum and several biological systems. In particular, the extremely low frequencies used in electrical power lines (50–60 Hz) [6,8,12,17], and the frequency range of radiofrequency (RF: from 3 kHz up to 300 MHz) and microwaves (MW: from 300 MHz up to 3 GHz), typical of cell phone transmissions [36] have been investigated. The thermal effects of EMF due to temperature increase for direct energy transfer are well established, while on the other hand there is a huge debate about the possible non-thermal effects [6,8–12]. In any case, due to the increasing spread in the use of EMF in telecommunications affecting everyday life, the possible existence of non-thermal effects caused by exposure to low intensity EMF in the RF and MW frequency range becomes an interesting research issue.

The remarkable electrical activity of the neurotransmitters in the Central Nervous System suggests that they should be especially sensitive to exogenous EMF. Several reports show experiments on chronic exposure of brain neuroglial cells to RF and MW-

EMF, covering a wide range of frequency, intensity and exposure time [8,10–12,16,17,20,22,25,28,33,34,37,38]. Instead only very few studies have been performed to investigate the acute exposure to EMF [3,19,24]. In these cases, the presented results are very controversial and have failed to point out an unequivocal correlation between EMF exposure and neurotoxicity. Moreover, the observed effects are strongly related to the time, frequency and intensity of EMF. It has been reported that exposure of rat primary neuronal cultures to 900 MHz EMF for 24 h, with a specific absorption rate (SAR) of 2 W/kg, may induce neuronal apoptosis through the caspase independent mitochondrial pathway activation [19]. In contrast, no significant increase in the apoptosis rate was found in the same cell cultures exposed to 900 MHz GSM modulated EMF for 24 h, with a SAR of 0.25 W/kg [20]. Any effect on damage-related factors in primary glial cell cultures has also been shown for exposure to 900 MHz GSM modulated EMF for 4, 8 and 24 h with SAR of 3 W/kg or continuous wave at SAR of 27 W/kg [34]. *In vivo* studies have demonstrated the potential effects of MW on neurotransmitters, blood–brain barrier permeability and behaviour changes [3,24]. It has been shown that a 15 min exposure to 900 MHz GSM-type radiation at a SAR of 6 W/kg induced an increase in the striatum, hippocampus and cerebral cortex of the Glial Fibrillary Acid Protein (GFAP) which is a specific marker of growth, maturation, and

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differentiation in astrocytes [24]. However, this effect is temporary and disappears at between 6 and 10 days after exposure [3].

Herein, since astrocytes respond to various stimuli and represent a valuable tool to study the biochemical mechanisms involved in several physiological and pathological brain conditions [5], we assessed the effects of acute exposure to low intensity MW-EMF of primary rat neocortical astroglial cell cultures.

Primary cultures of astrocytes were prepared from the neocortex of newborn albino rat brains (1–2-day-old Wistar strain) as reported [3]. Isolated cells were suspended in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Milan, Italy) supplemented with 20% (v/v) heat-inactivated Fetal Bovine Serum (FBS, Invitrogen), 2 mM glutamine (Invitrogen), streptomycin (50 µg/ml, Invitrogen) and penicillin (50 U/ml Sigma, Milano, Italy), and plated at 3×10^6 cells per 6 multiwell Petri dish, 1.5×10^6 cells per 12 multiwell Petri dish, 0.5×10^5 cells per 96 multiwell dish, and 0.5×10^5 cells/chamber in Lab-Tek multichambered slides. Cells were maintained at $37.0 \pm 0.1^\circ\text{C}$ in a 5% CO_2 /95% air humidified atmosphere for 2 weeks and the medium (Sigma) changed every 3 days. Astroglial cells were characterized at 14 days *in vitro* (DIV) by immunofluorescent staining for GFAP (Chemicon, Prodotti Gianni, Milan, Italy) [5]. All efforts were made to minimize both the suffering and number of animals used. All experiments conformed to the guidelines of the Ethical Committee of the University of Catania, Italy.

The EMF was emitted by a double horn antenna (ETS-Emco-3115) plugged to an Agilent-8648D signal generator. The amplitude modulation of sinusoidal waveform was obtained by an external oscillator (Philips-PM5127). Two different irradiation conditions at the same power density of 0.26 W/m^2 were used: 900 MHz carrier wave (900 MHz CW) and 900 MHz CW modulated in amplitude with a frequency of 50 Hz and 100% modulation index (50 Hz AM) for 5, 10, and 20 min of exposure time. The irradiation was performed from the top and the distance between the antenna and the sample was 60 cm. The produced electric field was measured by a Holaday HI-6005 electric field probe, and its rms value at the sample position was 10 V/m both for modulated and not modulated EMF. The measured uniformity of the electrical field in a region of $15 \text{ cm} \times 20 \text{ cm}$ around the centre of the samples holder is 90%. The intensity of the used EMF is too low to induce a thermal change of the cell cultures. Indeed, for 20 min of exposure time, the EMF produces at maximum an increase of temperature of about 0.03°C . To check the occurrence of extra exogenous EMF, the environmental fields were also evaluated. The background value of 0.4 V/m was measured.

The astroglial cell cultures at 14 DIV were divided into three groups (*control*, *sham* and *test*). During each exposure, *control* cells were maintained into the incubator, while the *sham* and *test* samples were placed into two identical covered water thermal baths ($37.0 \pm 0.1^\circ\text{C}$). The *test* sample was exposed to EMF while the *sham* sample was not exposed.

Astroglial cell viability was assessed by MTT test, which evaluated mitochondrial dehydrogenase activity [4]. To verify the occurrence of necrotic cellular death, lactate dehydrogenase (LDH release) was evaluated in the culture medium and in the cellular lysates at 340 nm by spectrophotometric analysis (Hitachi-U-2000, Tokyo, Japan) of NADH reduction during pyruvate–lactate transformation [27]. The intracellular reactive oxygen species (ROS) levels were also tested [4]. Furthermore, to evaluate whether EMF exposure can lead to DNA fragmentation, Comet assay on single cell was performed according to Singh et al. [32]. DNA fragmentation was quantified by measuring the tail moment (TMOM) expressed as the product of tail head distance and percentage of the fragmented DNA, using Scion Image Software [1].

All data were statistically analysed using one-way ANOVA, followed by Newman–Keuls *post hoc* test. All values are presented

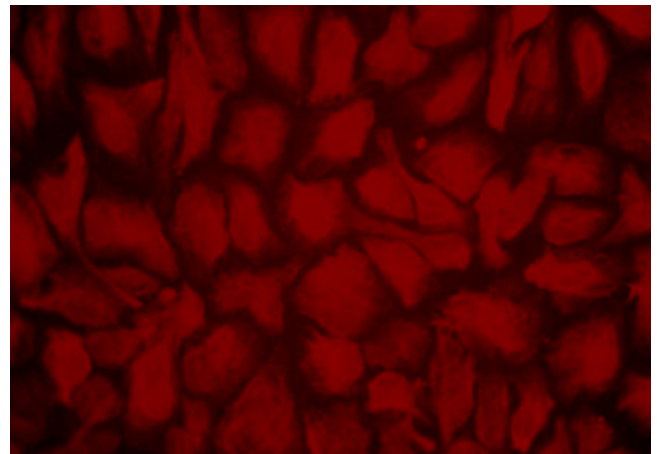


Fig. 1. Immunofluorescent staining for GFAP expression in primary rat neocortical astroglial cell cultures at 14 DIV. Rabbit monoclonal antibody against GFAP and polyclonal anti-goat IgG conjugated to tetramethylrhodamine-isothiocyanate (TRITC) were used. GFAP positive cells were fixed and analysed by a fluorescent microscopy (Leica, Germany). The photographs from the microscopic field showed that about 90–95% of cells were GFAP-positive, indicating that cultures were astrocyte enriched.

as means $\pm$ S.D. of 5 experiments. The Student *t*-test was used to compare the values of each assay; differences were considered statistically significant at $*p < 0.05$.

Primary rat neocortical astroglial cell cultures were characterized at 14 DIV by immunofluorescent staining of GFAP. About 90–95% of the cells were GFAP-positive, indicating that cultures were enriched in astrocytes (Fig. 1).

Preliminary assays by MTT test [4] and morphological observations have been made to establish the suitable exposure time intervals of the cultures to EMF. No differences between the *control* sample left in the incubator and the *sham* sample placed in the thermal baths until 20 min were observed (data not shown).

Consequently in the experiments 5, 10 and 20 min as exposure time intervals were chosen.

No significant changes, both for modulated and not modulated waves, in LDH release were found (data not shown). These results evidenced that EMF did not induce necrotic cellular death in our experimental conditions.

In parallel, to determine if the EMF was able to modify the oxidative status, the intracellular ROS production was evaluated. The results obtained are shown in Fig. 2. No significant difference is detected between *test* and *sham* cultures exposed to 900 MHz CW for all the used exposure times (Fig. 2a). Surprisingly, ROS levels appeared to have significantly increased only in *test* samples exposed for 20 min to modulated 900 MHz CW, and not these in *sham* samples (Fig. 2b).

To verify whether the altered redox status in astrocytes was able to induce DNA damage, DNA fragmentation was evaluated by COMET assay. This test highlights the presence of more or less extended cracks, variously produced in the nuclear DNA molecule [14,15,32]. Since it does not provide for DNA extractions with solvents, the cells are analysed in the same state, just disclosed, allowing the analysis on individual cells [14,15,32]. Images of the COMET assay analysis for *control*, *sham* and *test* samples are shown in Fig. 3. DNA damage is clearly visible in the *test* sample, when compared with the undamaged *sham* and *control* group. In Fig. 4 TMOM analysis is reported. In the neocortical astroglial cell cultures exposed to modulate EMF for 20 min a remarkable increase of TMOM amounting to $63 \pm 4\%$ was found.

These results demonstrate, for the first time, that even acute exposure to low intensity EMF induces ROS production and DNA

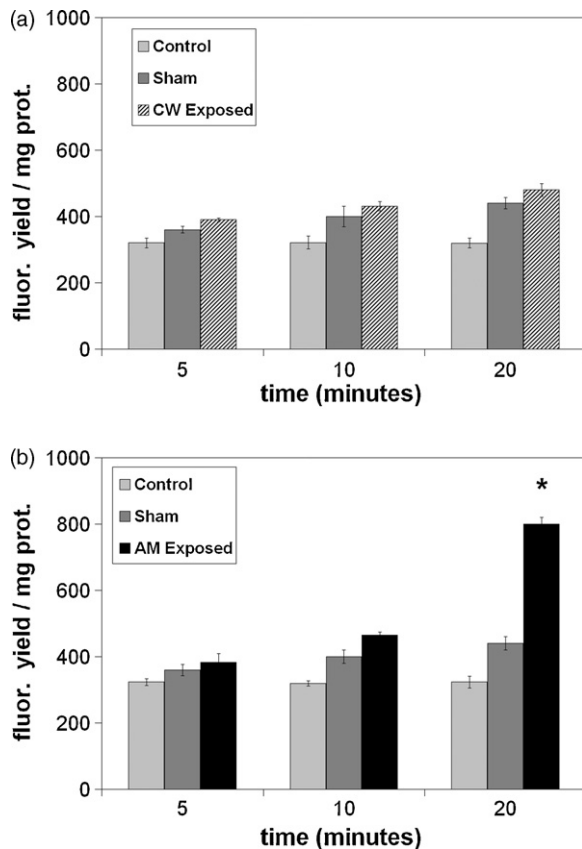


Fig. 2. ROS production levels in control, sham and test primary rat astroglial cell cultures: effect of non-modulated (a) and modulated (b) electromagnetic fields at different (5, 10, and 20 min) exposure times. Values are the mean $\pm$ S.D. of five separated experiments (* statistically significant vs control, $p < 0.001$).

fragmentation in astrocytes in primary cultures, which also represent the principal target of modulated EMF.

In contrast, other reports have shown that prolonged exposure of 70% enriched primary rat astroglial cell cultures to either CW or GSM modulated 900 MHz EMF was not able to modify cellular viability and morphology [34]. The discrepancy with our data can be explained by the different modulation used in our experimental conditions and the short exposure time which prevents the activation of the cellular repair mechanisms.

Our findings are consistent with other experiments on the effect of prolonged exposure of rat neuronal cell to cell phone radiation [38]. These experiments have reported up-regulation of specific intermediaries of apoptotic pathway [38], reduction of numbers of generated neurites [10], reduction of excitatory synaptic activity [37]. Our data are also in agreement with results achieved by *in vivo* experiments for acute exposure to EMF, which have shown a significant increase of GFAP expression 2 days after the exposure due to astrocytes activation in some brain structures [3,24]. Furthermore, the observed increase of ROS levels supports the hypothesis that the increased GFAP expression represents the response of astrocyte to oxidative stress [26]. However, the effects induced by EMF exposure in *in vivo* experiments are limited with respect to *in vitro* experiments, because of the activation of reparation mechanisms [3].

As already stressed, the observed increase of ROS production and DNA fragmentation induced by exposure to modulated 900 MHz EMF for 20 min cannot be attributed to thermal effects. The observed effects must be attributed to the amplitude modulation of EMF. Indeed, exposures to not modulated EMF for 5, 10 or 20 min did not produce any significant effect in the astroglial cell cultures, while the exposure to modulated EMF for 20 min of the same power density induced clearly measurable effects. These

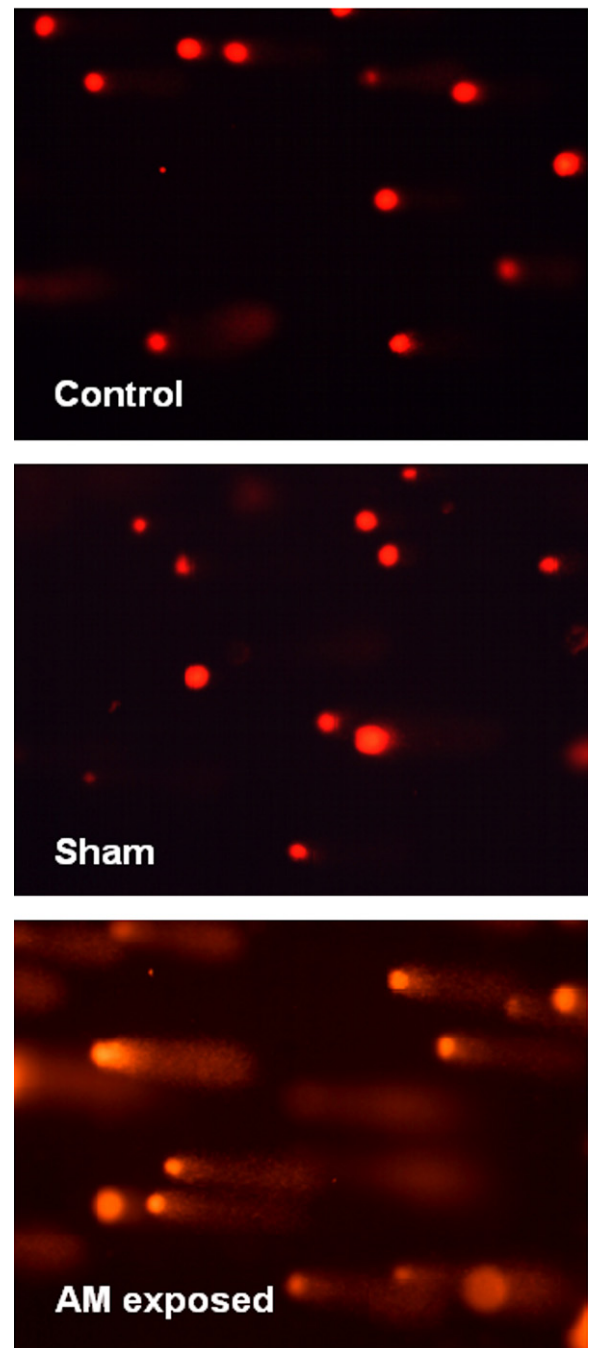


Fig. 3. COMET assay of genomic DNA of primary rat astroglial cell cultures was performed on single cell gel electrophoresis. DNA ethidium bromure stained minigels were analysed by using a fluorescent microscope (Leica, Germany). Control, sham and test samples are respectively shown in the middle and lower panel. The image analysis measurements of the overall intensity of fluorescence in the control (upper panel) and sham (middle panel) samples showed a circular head corresponding to the undamaged DNA. Single strand breaks damage was found in 20 min modulated EMF-exposed astroglial cell cultures (lower panel).

observations are in agreement with several theories used to explain the interaction of EMF with several biological systems [2,13,30,31], which invoke resonance phenomena to explain why modulated EMF can be more biologically active than continuous ones. The observed effects were found at an intensity of the irradiating field about four times lower than the limit for public exposure established by the International Commission on Non-Ionizing Radiation Protection (ICNIRP) [18]. For these reasons, it is important to investigate more deeply the non-thermal effects of EMF and the currently

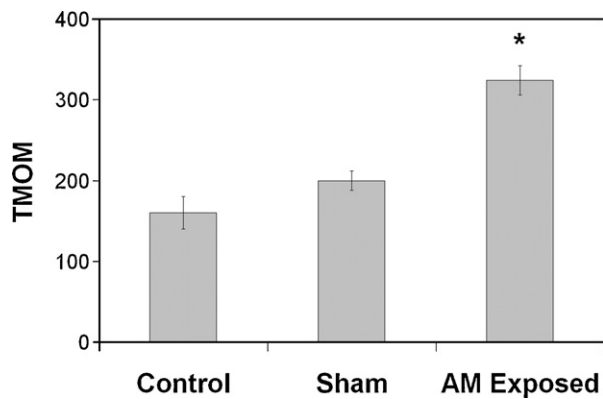


Fig. 4. TMOM analysis of genomic DNA fragmentation in *control*, *sham* and 20 min 50 Hz AM exposed primary rat astroglial cell cultures. Values are the mean $\pm$ S.D. of five separated experiments (* statistically significant vs *control*, $p < 0.001$).

existing safety standards, which refer only to the thermal effects in the MW frequency range.

In conclusion, the observed results have shown, for the first time and with high statistical reliability, that acute exposure to low intensity 50 Hz AM 900 MHz CW EMF for 20 min interferes with the normal mechanisms of development and control of primary rat neocortical astroglial cell cultures. Nevertheless, it cannot be excluded that the observed effects could be modified in the *in vivo* brain, where several mechanisms of neural repair are promoted. Furthermore, our data suggest that the increase of ROS levels and DNA fragmentation induced by the EMF exposure could be related to the increase of intracellular calcium ions levels, probably due to the hyperstimulation of the glutamate receptors [3,23,29], which play a crucial role in the acute and chronic brain damage [7,21,35]. This aspect is of crucial interest, because it involves the possibility of EMF influence on neurotransmitters, and this will be the subject of our future research.

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