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Validation of an original incubator set-up for the exposure of human astrocyte cells to X-band microwaves in a GTEM-chamber

R.N. Pérez-Bruzón[1], A. del Moral[2], C. Pérez-Castejón[1], M. Llorente[1], A. Vera[1] and M.J. Azanza[1]

[1]Magnetobiology Laboratory, Department of Human Anatomy and Histology, School of Medicine, University of Zaragoza, Zaragoza and [2]Magnetism Laboratory, Department of Condensed Matter Physics and Material Science Institute (ICMA), University of Zaragoza and CSIC, Zaragoza, Spain

Summary. A current concern about the biological effects of electromagnetic fields (EMF) is increasing with the wide spread use of X-band microwaves (MW, 8-10 GHz range). Gigahertz transverse electromagnetic (GTEM) field flat transmission lines are currently being used for experimental exposure of biological samples to high frequency EMF. Experiments carried out on human cells in culture require optimal growing temperature conditions, i.e. 37°C, 5% CO_2 in a humidified atmosphere. The aim of our work has been: i) to built up an original incubator set-up, the so called *GTEM-incubator*, for exposure of human cells in culture to MW inside a GTEM-chamber, under optimal growing physical conditions; ii) to make the validation of the *GTEM-incubato*r by growing cell samples inside the non-energized GTEM-chamber (test sample) comparing the results with the ones obtained from cell samples grown inside a standard incubator (control samples). The features for comparison were: cell morphology, expression and distribution of cytoskeleton proteins, genotoxicity, viability and cell cycle progression. Any variation in any of the studied parameters would allow for detecting any possible failure or misconception in our *GTEM-incubator* working test. The results obtained in control and test incubators showed non-significant differences in the development of both cell populations for any of the studied parameters. Thereby our *GTEM-incubator* is considered valid for our purposes of human cell exposures to X-band MW.

Offprint requests to: Prof. M.J. Azanza, Departamento de Anatomía e Histología Humanas, Facultad de Medicina, Universidad de Zaragoza, C/Domingo Miral s/n, 50009 Zaragoza, Spain. e-mail: mjazanza@unizar.es

Key words: *GTEM-incubator* set-up, *GTEM-incubator* validation, Human normal astrocytes, Human astrocitoma cells

Introduction

The aim of our current research is to culture mammalian cells for exposure to X-band (9, 6 GHz) MW within a GTEM-cell. GTEM cells are exposure systems that combine characteristics such as generation of uniform field, i.e. a uniform electromagnetic (EM) plane wave, shielding from EM fields (EMF) from outside, which operates over a wide frequency band, allowing for well-defined and characterized exposure conditions, and which provides a volume inside to place the biological test samples. Well defined exposure conditions are essential and it is a condition to obtain reproducible results, exposure systems being widely used for *in vitro* and *in vivo* biological studies of EMF effects on biological systems (Salovarda Lozo and Malaric, 2010). Experiments inside a GTEM-cell, or other kind of EMF irradiation devices, for application of MW to mammal cells growing in culture, face the crucial problem of maintaining growing cells at the suitable physical conditions. Temperature (37°C) and 5% CO_2 in a humidified atmosphere are rather restrictive parameters for optimal mammalian cell growth. In some laboratories small TEM-cells were introduced inside standard incubators (Stagg et al., 1997; Kwee and Raskmark, 1998). Frequently, GTEM-cells are settled inside rooms maintained at 37°C. To obtain a uniform distribution of temperature, 5% CO_2 and a humidified atmosphere in a large room is by no means easy. In several experiments the regulation of temperature was undetermined (Ji et al., 2006; Pavicic and Trosic, 2008).

More frequently, once the cells were cultured inside a standard-incubator, cell exposures to RF-EMF were performed at room temperature. In these conditions the growing temperature dropped from the optimum 37°C, down to values of 34.0±0.1°C, 27.0±0.9°C and 26.2±0.6°C (French et al., 1997); 27°C (Donnellan et al., 1997) or 23°C (Dorsey et al., 2005), too low to be considered as optimal exposure experimental conditions. To solve this problem, a small incubator, made of plastic material to enable specimen irradiation without wave interferences and attenuation, was constructed by the Olympus firm *to our specifications*. Incubator temperature and CO_2 in a humidified atmosphere were controlled and monitored at 37.0±0.1°C and 5% respectively.

Validation studies were done by growing human astrocyte cell samples in Falcon flasks in a standard-incubator. After reaching 70-80% confluence half of the flask cell samples were transferred to the *GTEM-incubator* and placed inside the GTEM-cell where, following the manufacturers specifications, the volume of EMF is highly uniform (EUT, equipment under test, 20x20x15cm) when the GTEM cell is energized. The other half of the flasks was maintained inside the standard incubator for comparison of results. Two series of experiments for the *GTEM-incubator* validation were performed by growing cells for 6 and 24 h. The following parameters were studied as subjects of comparison: cell morphology, expression and distribution of cytoskeleton proteins, genotoxicity, cell viability and cell cycle progression. Any variation in any of the studied parameters would allow for detecting differences with control samples and thereby for correcting any working faults for the *GTEM-incubator*. The results showed non-significant differences in the development of both incubator cell populations, test and control, for any of the studied parameters. As a consequence the *GTEM-incubator* was considered validated for our purposes of cell exposure to X-band MW range.

Taking into account that the aim of our research is to culture mammalian cells for exposure to X-band range MW in a GTEM-cell once the *GTEM-incubator* had been validated, the dosimetry of the experimental system was carried out. The dosimetry was made for two different exposure experimental conditions, to be applied in further series of experiments and, importantly, designed to maintain cells in *sub-thermal* conditions at low EM powers, to rule out other possible thermal effects on cells under EMF exposure.

Materials and methods

Description of the GTEM-incubator set-up

The *GTEM-incubator* (PECON), built by the Olympus firm (to our specifications) is a temperature incubator of 15 cm x 15 cm x 6 cm size made out of plastic material to enable specimen irradiation without wave interferences (Fig. 1). It is heated by warm water circulating inside the bottom and top walls and distributed from the heater. Water temperature range was regulated at the thermostat by means of an electronic feedback system (Julabo F25) (Fig. 2a,b). 5% CO_2 atmosphere was regulated by the CO_2 controller (PeCon GmbH, Germany) (Fig. 2c). The air coming from the controller was passed through sterile water to be humidified before entering in the *GTEM-incubator* (Fig. 2d). Cells were maintained in this way at a temperature of 37.0±0.1°C, accurately controlled at 5% CO_2

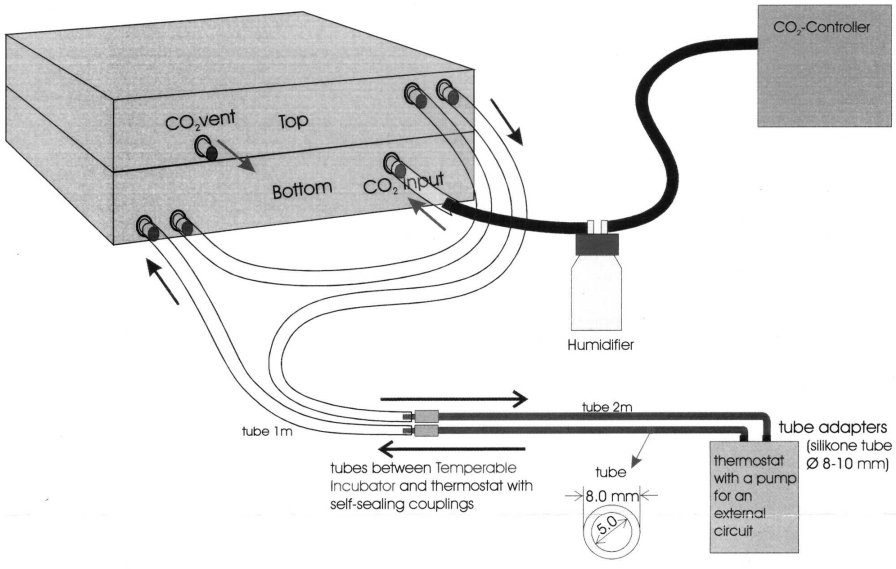

Fig. 1. The *GTEM-incubator* (PECON) is a temperature regulator for connection to an external bath water circulator, provided by a pump. It is made of a cage of 15 cm x 15 cm x 6 cm size fully made out of plastic material to enable specimen irradiation without wave interferences and eddy current production, with unfolded top and bottom parts, for introducing the Falcon flasks. Water circulation is through internal circuits within the two cage components (top, bottom). The incubator is heated up by the warm water in contact with the bottom and top walls. The water is warmed from the heater. Temperature range is regulated with a thermostat. 5% CO_2 atmosphere is regulated by the CO_2 controller. The air coming from the controller passes through a sterile water container, thus being humidified before entering in the *GTEM-incubator*.

atmosphere and within a humidified atmosphere. The incubator temperature was steadily monitored and accurately controlled by a Pt100 temperature sensor (Labfacility Ltd.) placed inside the incubator (Fig. 3b).

Validation methodology

Growing cell samples were introduced in the *GTEM-incubator*, and this was placed in turn inside the non-energized GTEM-cell (Fig. 3a). The results from *GTEM-incubator* (test sample) were compared with the ones obtained from samples of cells growing in a standard incubator (control sample). As a matter of comparison the considered validation parameters were: cell morphology, expression and distribution of cytoskeleton proteins to confirm morphology results, genotoxicity test to distinguish apoptosis from necrosis, cell viability, which gives quantitative information about cell survival

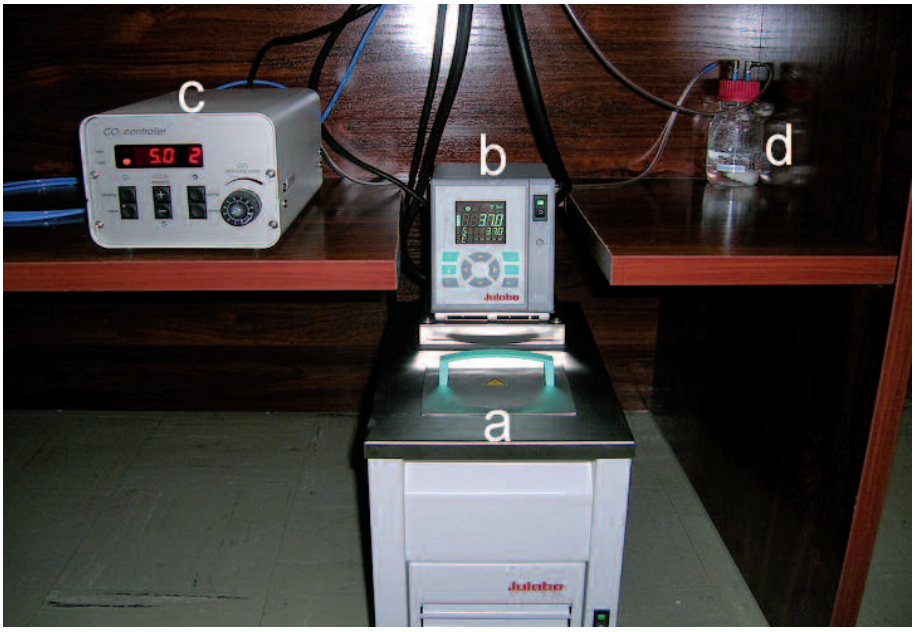

Fig. 2. Water temperature was regulated with the thermostat, controlling it by means of an electronic feedback system (Julabo F25). The thermostat is formed by the water reservoir and pump **(a)**, the electronic feedback and temperature measurement system **(b)**. **c.** CO_2 controller (PeCon). Cells were maintained in this way at a stable temperature of 37.0±0.1°C, within an accurately controlled 5% CO_2 atmosphere, humidified after passing the circulating water through a sterile water flask **(d)**.

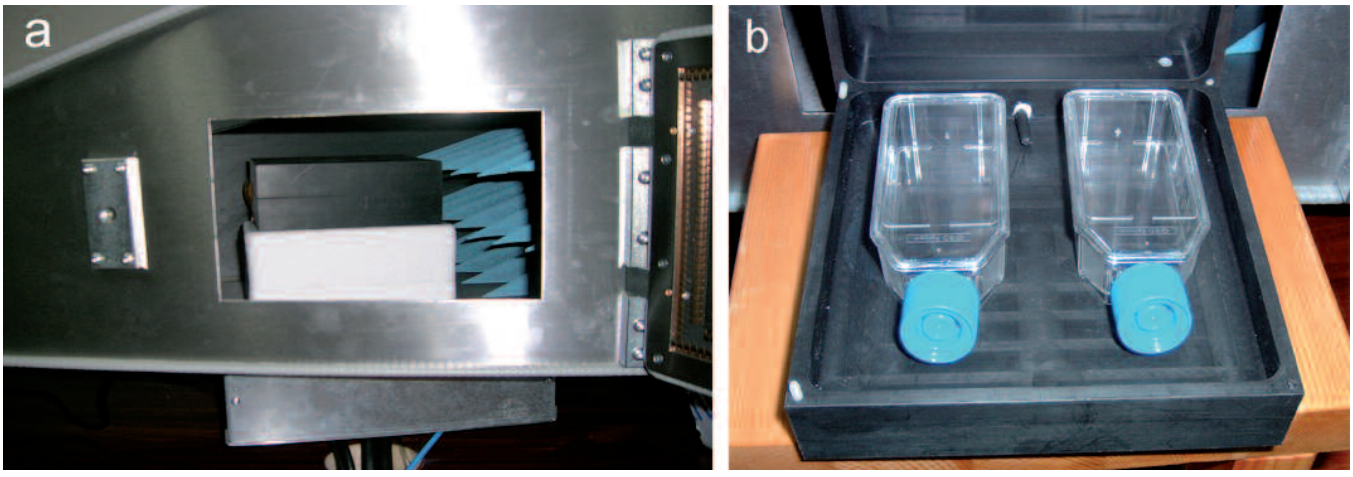

Fig. 3. a. The *GTEM-incubator* was placed inside the non energized GTEM-cell in the place where following the manufacturer specifications, the volume of EMF is highly uniform when the GTEM-cell is energized. The GTEM-cell chamber is terminated by a plastic foam EM anechoic wall (blue color pyramids) to reduce unwanted reflections. **b.** Two Falcon flasks with cells in culture were placed inside the *GTEM-incubator* test, with their long sides along the EMF incidence. Inside the *GTEM-incubator* the Pt100 temperature sensor (Pt filament covered by a plastic material) is placed inside the bottom cage and connected with the temperature regulator.

or death, and cell cycle progression which, allows localization of the course of the cell cycle. Experiments were replicated *five times* for all the validation tests. Double blinded method was applied for morphology, cell counting, and image capture processes.

Cell culture

Experiments for *GTEM-incubator* validation were performed on human normal astrocytes (Clonetics line CC-2565NHA) and cells from human astrocitoma (human astrocytes tumor) (Clonetics line 1321N1). Specific growth medium for normal astrocyte cells was: astrocyte basal medium supplemented with single quotes of 15 ml foetal bovine serum, 0.5 ml ascorbic acid, 0.5 ml epithelial growth factor (EGF), 0.5 ml gentamicin sulphate and amphotericin B, 1.25 ml insulin and 5.0 ml L-glutamine (LONZA). The specific growth medium for astrocytoma cells was: Dulbecco's Minimum Essential Medium (DMEM, SIGMA) supplemented with 10% fetal bovine serum (SIGMA), 0.4 mM L-Glutamine (SIGMA) and 100 U/ml penicillin and 0.1 mg/ml streptomycin (SIGMA).

According to proliferation rate cells were seeded at densities of $30x10^3$ and $300x10^3$ cells/ml for astrocitoma and normal astrocyte cells respectively into Falcon flasks (25 mm^2 basal surface), and maintained as an adherent monolayer in a humidified atmosphere, 5% CO_2 at 37°C. After reaching 70-80% confluence two Falcon flasks were maintained in the standard incubator (control sample) while another two Falcon flasks were transferred to the *GTEM-incubator* (test sample) and placed inside the non energized GTEM-cell (Fig. 3a,b). The experiments were performed maintaining the cells in culture for 6 h and 24 hours in two sets of experiments. Cell morphology was studied with an inverted Olympus microscope (Model IX 71).

Culture conditions inside the GTEM-cell

The *GTEM-incubator* with two Falcon flasks with growing cells was placed inside the non-energized GTEM-cell in the location where, according to the manufacturers specifications the volume of EMF, once energized, is highly uniform (Fig. 4a,b). Falcon flasks were placed with their longitudinal axis along the direction of electromagnetic wave incidence when the GTEM-cell was energized.

Apoptosis vs. necrosis

Apoptosis like cell death was identified on the basis of morphological criteria: condensed and fragmented nuclei (genotoxicity), blebbing of plasma membranes, a decrease in cell size and appearance of apoptotic bodies. To distinguish apoptosis from necrosis the Hoechst-propidium iodide protocol was applied. Normal astrocyte and astrocytoma cells were cultured on coverslips with poly-L-lysine (SIGMA) to reach 90% confluence in two series of experiments. 40 coverslips were plated per sample and experimental condition placed inside Petri dishes with growth medium. Cells were fixed for 5 min in ethanol, dried for 5 min and then washed with PBS for 10 min three times. Cells were stained for 30 min with Hoechst plus propidium iodide (0.05 μg/ml, H33258 SIGMA + 0.05 mg/ml, P4170, SIGMA), washed in PBS for 10 min twice and mounted with Dako solution (53023 Dako).

Immunofluorescence methods

The expression and distribution of proteins was studied by indirect immunofluorescence staining method for biomarker cytoskeleton proteins: glial-fibrillary-acidic-protein (GFAP, dilution 1/100, polyclonal rabbit-

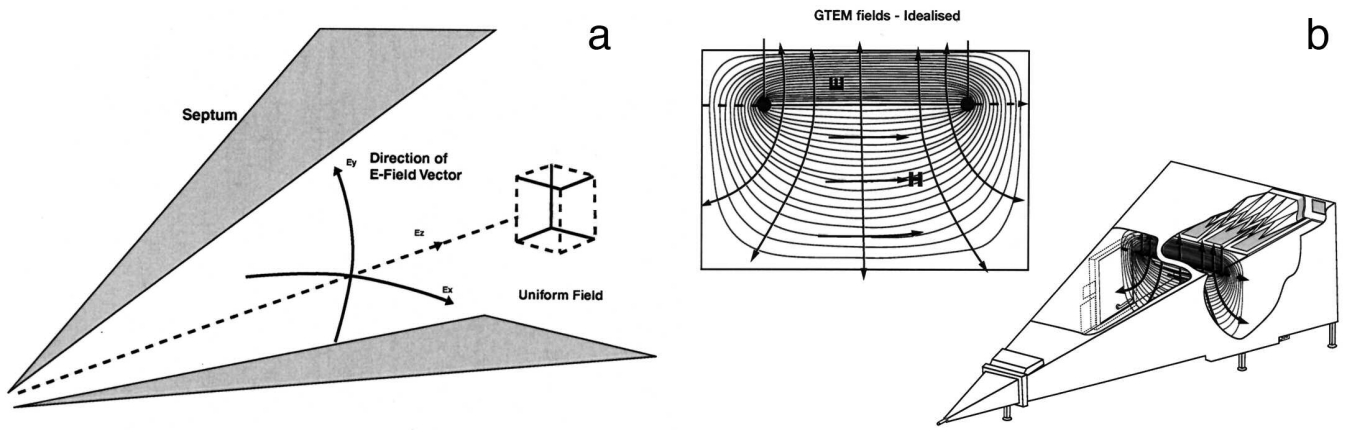

Fig. 4. Diagram of the angular aperture GTEM-cell chamber. **a.** The place where the incident EMF is more uniform and where the *GTEM-incubator* is placed for validation and MW exposure experiments. **b.** The schematic distribution lines of electric field, **E** and magnetic field, **H** are schematically drawn in a cross section of the chamber in front of the line termination.

antimouse Z0334 Dako; FITC-conjugate-anti-rabbit IgG dilution 1:50, F0205 Dako); tubulin α- (dilution 1/2000, monoclonal produced in mouse T5168 SIGMA; FITC-conjugated goat-anti mouse IgG dilution 1:64, F2012 SIGMA); tubulin-ß-III (dilution 1/100, produced in rabbit, T2200 SIGMA; FITC- dilution 1:50, F0205 Dako) and tubulin anti-tuc-4 (dilution 1/100, polyclonal AB5454 Chemicon; FITC- dilution 1:50, F0205 Dako). The antibodies were diluted with Dako (S2022) and Chemicon (21544) diluents. Astrocyte cells were cultured on coverslips impregnated with poly-L-lysine (SIGMA) inside Petri dishes reaching 90% confluence. 50 coverslips were studied per sample and biomarker. Cells were fixed for 5 min in methanol, dried for 5 min and then washed with PBS for 10 min three times. After treatment with Triton X100 plus 0.25 BSA (A4503 SIGMA) in PBS twice for 15 min, cells were incubated with the primary antibody solution for 20 h, followed by rinsing in PBS 10 min three times. The visualization was done by incubating cells with FITC-conjugate IgG for 90 min, washing in PBS 3 times for 10 min and mounted with solution for immunofluorescence (53023 Dako). Immunofluorescence studies were performed with an Olympus microscope (BX51).

Viability determination

Cell monolayer was detached chemically by removing the culture medium by adding 2 ml of trypsin-EDTA solution for 5-10 min. Cells were observed under inverted microscope to check if 95% of cells had been detached from the bottom of the Falcon. For trypsin neutralization 1.0 ml of culture medium was added. Cells were centrifuged at 1000 rpm for 5 min. The pellet was re-suspended in 0.25 ml of cultured medium adding the same volume of trypan blue. Trypan blue molecules are able to penetrate the plasma membrane of damaged cells. Dead cells are stained blue. Viability was calculated as the percentage of cells alive with respect to total number of cells.

Cell cycle progression

Cell cycle progression was determined by comparing the results obtained from astrocytoma cell growth in the two incubators: standard (control samples) and *GTEM-incubator* (test samples). Control cells were seeded at a concentration of $7x10^4$ cells/Falcon flask. Reaching a 95% confluence cells were counted on successive days (fourth to eighth) to determine the entrance in exponential phase and progression of the cell cycle up to the end of the growth phase. The results were compared with the ones obtained from test cells grown in the *GTEM-incubator* seeded at almost a double concentration of $15x10^4$ cells/Falcon flask. The protocol to follow up the cell cycle was the same as before.

Statistical analysis

Statistically significant differences between groups, control and test samples, were evaluated using independent sample t-test (SPSS Statistical Package, v.14.0). Data are expressed as mean ± SD. $p<0.05$ was considered to represent a statistically significant difference.

Experimental set-up for electromagnetic field exposure

Cell exposure to X-band (9.6 GHz) MW experiments is done in a certified horn shape Gigahertz Transversal Electromagnetic Mode (TEM) Cell (GTEM-cell) (Teseq GmbH) (Figs. 4b, 5a). The TEM MW is produced by a flat strip line, with the sample placed in front of the line end. The line of non-parallel plates is formed by a flat metallic sheet, the other part being the upper face of the GTEM-cell. Since line planes have an aperture angle the line can radiate in order to get an EMF outside and in front of the plate termination. MW are produced with a solid state function generator (51013-4E, S/N: 34341 of Z=50Ω output impedance) (Fig. 5b), provided with a versatile modulator of different wave profiles (Gigantronics, 100 KHz-20GHz range). The MW signal was amplified with a traveling wave tube (TWT) (Medium Power Wide Band Amplifier T186-50, 6.0-18GHz passband, of 50W nominal maximum output) (Fig. 5c). MW power was measured by a two port power meter (4232A, Boonton) (Fig. 5d), essentially consisting of a diode bridge power sensor.

Fig. 5. Experimental irradiation set-up. **a.** Gigahertz Transversal Electromagnetic Mode Cell (GTEM-cell). **b.** MW functions generator. **c.** Travelling wave tube power amplifier. **d.** Diode bridge power meter. **e.** Directional coupler. **f.** 50 Ω field probe for reflected EM wave power measurement.

The GTEM-cell chamber is terminated by a plastic foam EM anechoic wall to reduce unwanted reflections (Fig. 3a). The MW signal was injected into the GTEM cell using a ferrite directional coupler followed by a 50 ohm coaxial cable (COU-BD418G50W-35, 4-18GHz, 50W) (Fig. 5e). In this way direct and reflected powers were measured, the reflected signal being injected into the directional coupler through an adaptor of 50 ohm characteristic impedance (Fig. 5f). The EMF-MW mode is the sinusoidal TEM one (magnetic field, **H** on the strip plane and electric field **E** normal to it), the same as in normal wireless telecommunication (Fig. 4a). A cross section of the GTEM-cell with the scheme of the **E** and **H** field lines near to the terminals of the angular flat line is shown in Fig. 4b.

Outline of dosimetry, EM field analytical calculations and numerical simulations

For experiments of astrocyte human cell exposure to MW, two experimental exposure conditions were adopted: 9.6 GHz carrier frequency, modulated by short pulses with widths of 100 and 120 ns, with pulse repetition frequency of 100 and 800 Hz (pulse repetition interval of 10 and 1.25 ms). Experimental exposure conditions were designed to maintain cells in *sub-thermal* conditions at low powers of 0.34 and 0.60 mW to rule out other possible thermal effects on cells under EMF exposure (Pérez Castejón et al., 2009).

The characterization of field patterns inside the GTEM-cell was made by using analytical calculations

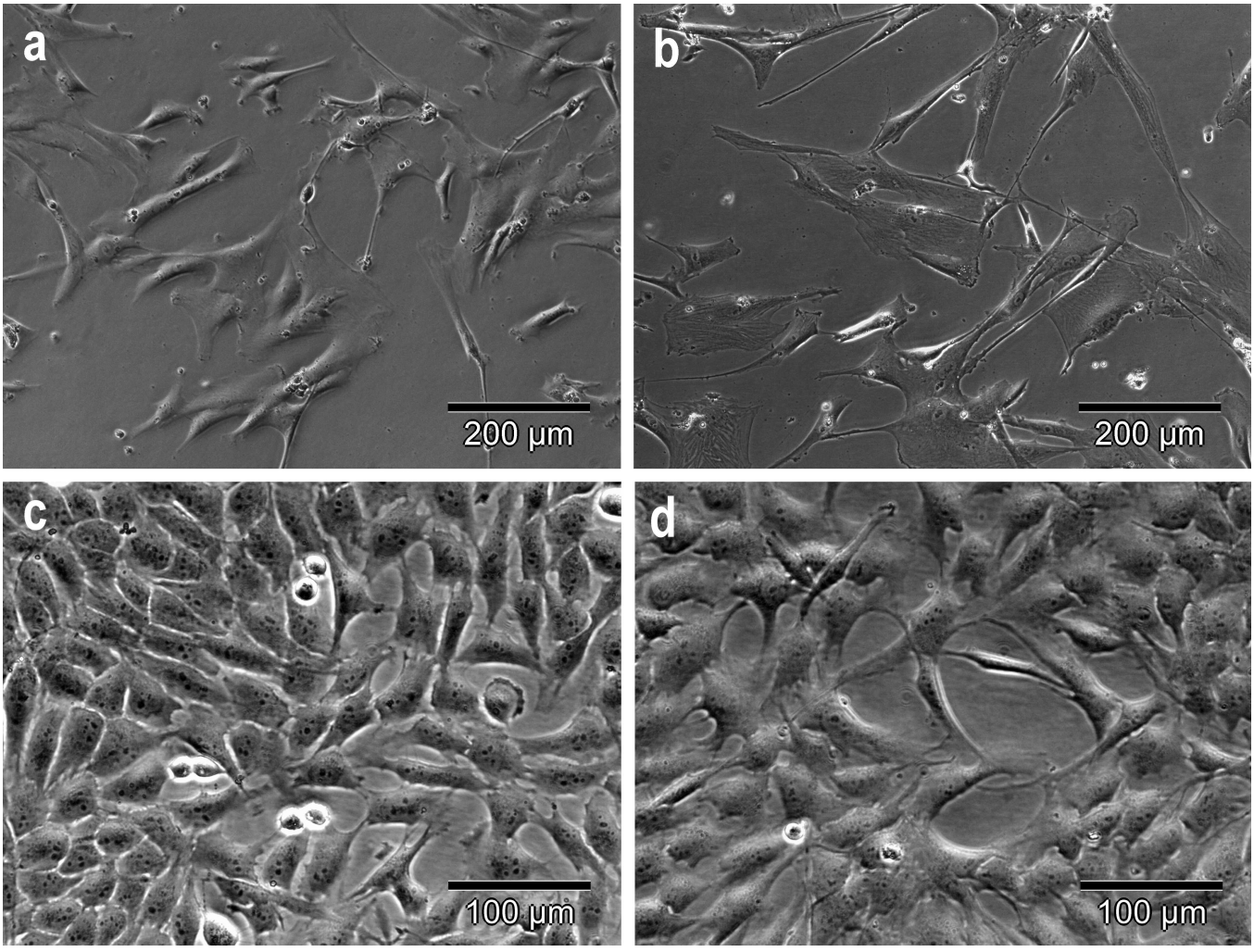

Fig. 6. No significant morphological differences after 24 h incubation time were observed between normal astrocyte cells growth in standard (**a**, control cells) and *GTEM-incubator* (**b**, test cells) or astrocitoma cells (**c**, control cells; **d**, test cells).

and numerical simulations for the *GTEM-incubator* with Falcon flasks inside the GTEM-cell. Also, different locations of flasks were tested. The simulations to calculate the **E** and **H** field distributions, its homogeneity at the sample positions, the specific absorption rate (SAR, EM frequency spectrum absorbed by a living tissue) and other EMF characteristics will be the subject of a further publication (Pérez-Bruzón et al., 2011).

After several preliminary test experiments of cell culture and dosimetry simulation in energized GTEM cell, was determined the optimal flask positions with EM wave incidence from the back of the flasks (+OX-direction). It was the position allowing the most uniform distribution of the incident field and was the Flask position inside the *GTEM-incubator* for the validation experiments (Fig. 3b).

Results

We have observed that under standard conditions normal astrocyte and astrocytoma cells show a natural polymorphism according to cell cycle phase and differentiation stage. Our conclusion is that no significant effects on cell morphology, either on astrocyte or astrocytoma cells were induced either after 6 h or 24 h incubation times (Fig. 6a-d).

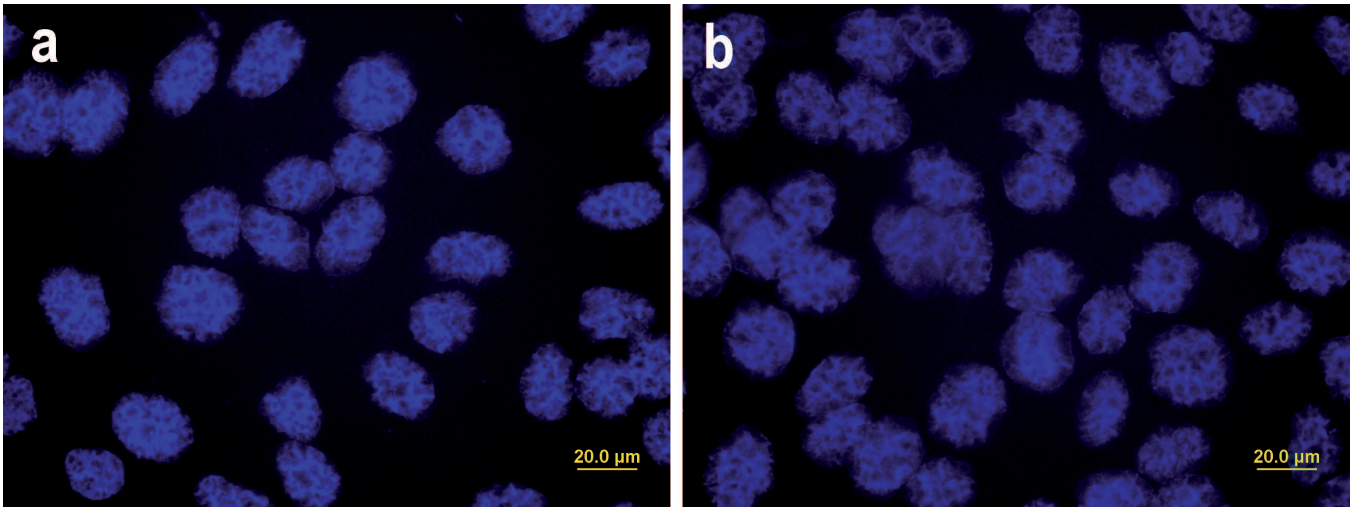

Fig. 7. Hoescht and propidium iodide reaction showed any significant morphological difference between normal astrocyte cells growth in standard (**a**, control cells) and *GTEM-incubator* (**b**, test cells) after 24 h incubation time.

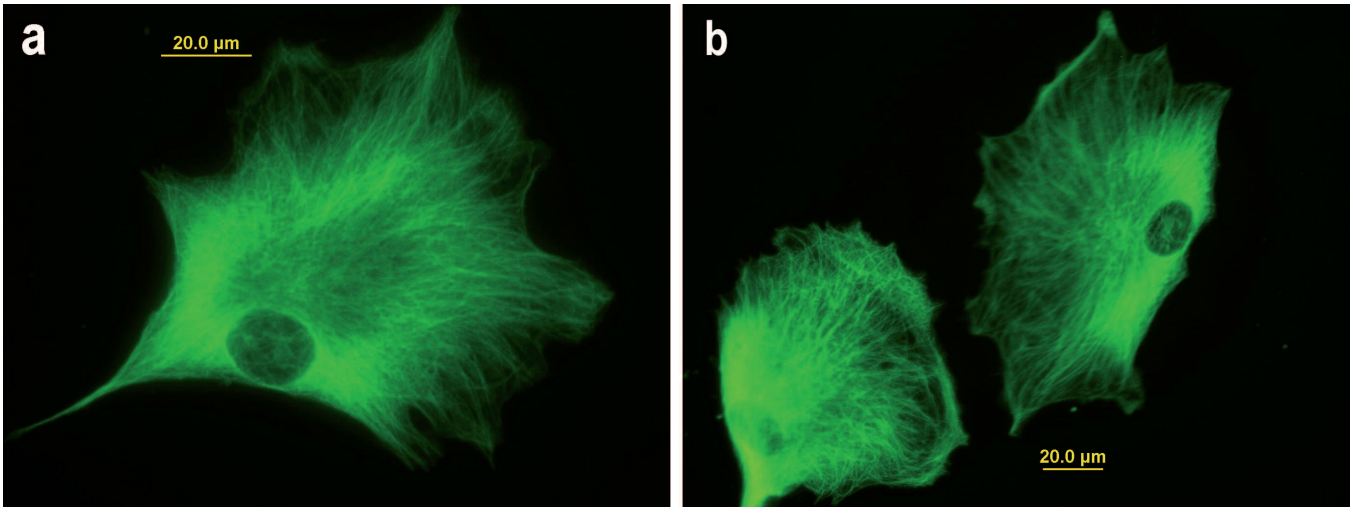

Fig. 8. No significant differences were observed in expression and distribution of ß-tubulins for normal astrocyte cells growth in standard (**a**, control cells) and *GTEM-incubator* (**b**, test cells) after 24 h incubation time.

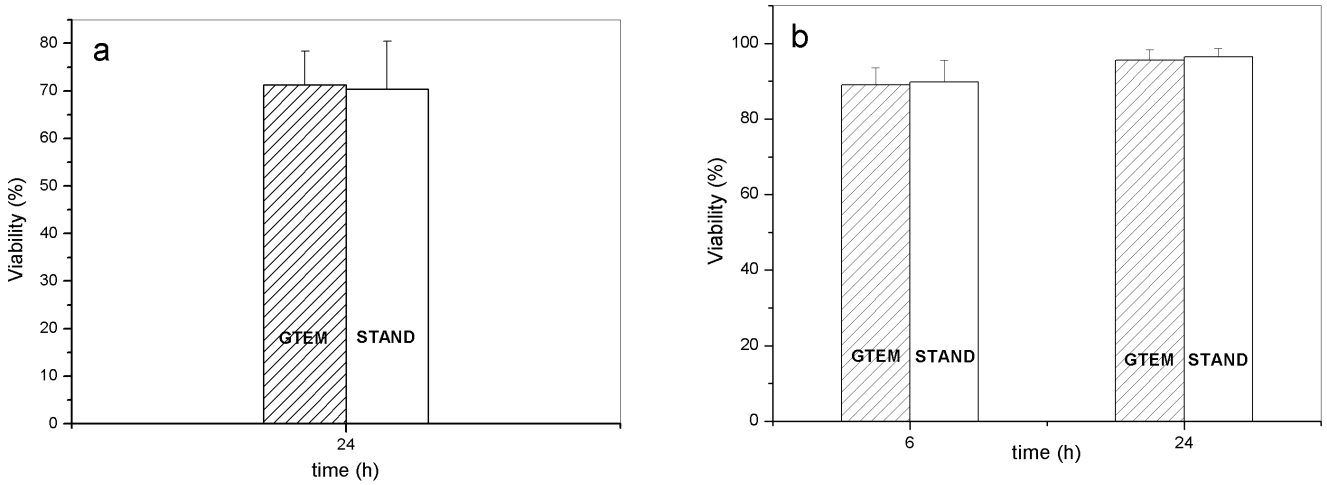

Fig. 9. Cell viability of normal astrocyte and astrocytoma cells in standard (control, STAND) and *GTEM-incubator* inside the GTEM-cell (test, GTEM) were compared after 6 and 24h in culture. No significant differences were observed. Experimental values represent means ± standard deviation (bar) for 15 measurements.

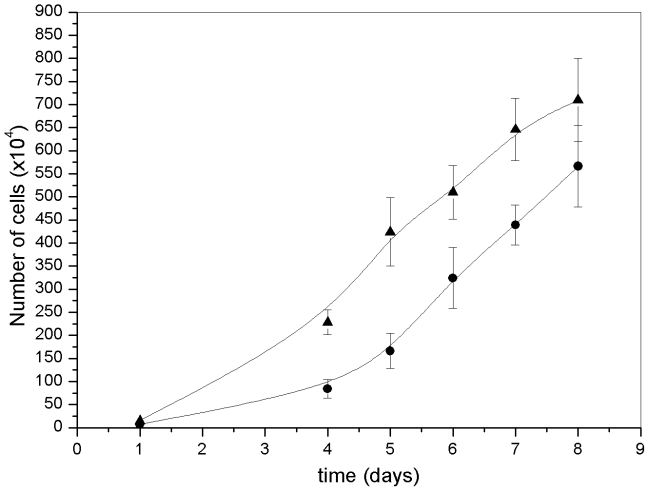

Fig. 10. Cell cycle progression was determined by comparing the results obtained from astrocytoma cells grown in standard ($7x10^4$ seeding cells, control sample) (circle) and *GTEM- incubator* ($15x10^4$ seeding cells, test sample) (triangle). Although the concentration of cells in *GTEM-incubator* was almost double with respect to the standard one, results were similar for both culturing conditions: cells enter in exponential phase after four days in culture, reaching maximum growth on 8th day. Experimental plotted points represent means ± standard deviation (bar) from 15 measurements.

A lack of morphological effects was confirmed by Hoechst and propidium iodide results, which showed no genotoxic effects i.e., normal nuclei, neither necrosis nor apoptosis alterations (Fig. 7a,b), and by studying the distribution and expression of biomarker cytoskeleton proteins. No significant differences were observed between the standard-incubator (control-samples) and the *GTEM-incubator* (test-samples) in the expression and distribution of GFAP, tuc-4, α- or ß-tubulin (Fig. 8a,b) proteins either after 6 or 24 h incubation times.

Viability results showed that the difference of the cells mean population was not significant for incubation times of 6 and 24h either in standard (control samples) or in the *GTEM-incubator* (test samples) for normal astrocyte or astrocitoma cells (Fig. 9a,b).

Astrocytoma cell cycle progression results were similar for both culturing conditions, standard incubator (control samples) and *GTEM-incubator* (test samples). It must be emphasized that although the number of cells in the *GTEM-incubator* were almost double those seeded in the standard one, cell cycle progression was in parallel for both experiments. Cells enter into exponential phase after four days in culture reaching a maximum growth on the 8^{th} day (Fig. 10).

As a conclusion we have observed no significant effects on cell morphology, expression and distribution of biomarker cytoskeleton proteins, cell viability, induction of genotoxic effects and cell cycle progression between both standard incubator (control samples) and *GTEM-incubator* (test samples) experimental growing conditions.

Our conclusions are that: i) our originally designed *GTEM-incubator* set-up allows the growth in culture of human normal astrocyte and astrocytoma cells in physical conditions comparable with standard incubator ones; ii) the preliminary calculated dosimetry confirms that the *GTEM-incubator* set up gives optimal conditions for studying the potential effects on human normal astrocyte and astrocytoma cells in culture, under exposure to X-band (9.6 GHz) MW inside a GTEM cell at very low power densities as described below.

Discussion

In considering the biological effects of exposure to MW a misconception still remains, i.e. that RF and MW effects are solely the result of heat generation, in contrast to the fact that a good number of reported studies showed significant effects on various cellular activities in experimental systems under well controlled isothermal conditions (Cleary et al., 1992; Velizarov et al., 1999). However it is a matter of concern that exposure to external agents like EMF could induce cell division alterations which would convey proliferation increase or decrease, and thereby produce a biological effect (Pérez-Castejón et al., 2009). Temperature control for experiments under applied MW becomes a strategic end point for proceeding with such kinds of experiments. Gigahertz transverse electromagnetic (GTEM) transmission lines are currently being used for experimental exposure of biological samples to high frequency electromagnetic fields. However biophysical conditions, like temperature and CO_2 for optimal cell growth inside the GTEM-cell are parameters by no means easy to control as mentioned before (French et al., 1997; Donnellan et al., 1997; Seaman et al., 2002; Dorsey et al., 2005). Our purpose has been to develop a *GTEM-incubator* device for maintaining optimal mammal cell growth conditions for in vitro exposure inside a GTEM-cell.

We have chosen for our study human astrocyte cells, which are responsible for more than 90% of human brain tumours, astrocitoma. Experiments on human normal astrocyte and cells from human astrocytoma would allow the comparison between two kinds of close cells, although normal and neoplasic ones show differential susceptibilities for genotoxic effects and proliferative behavior. The parameters studied were addressed to show the possible activation of any pathway related to a genotoxic effect and cell division which could be detected, in a first attempt, as changes in: cell and nuclei morphology, which could give information about necrosis or apoptosis processes, and expression and distribution of cytoskeleton proteins, viability and cell cycle progression, which would indicate modifications in the standard cell cycle.

From our validation test experiments we can come to the conclusion that our *GTEM-incubator* attains optimal physical conditions for keeping human normal astrocyte and astrocytoma cells growing inside a GTEM-cell. Tubulins and GFAP are considered biomarker proteins for studying astrocyte morphological and metabolic activities. Microtubule proteins are extremely dynamic structures. Their functions are based on dynamic instability: polymerization of α- and ß-tubulins and their degradation to dimer protein. These processes are continuously occurring in the proliferating cells, and mitotic apparatus depends on correct microtubule arrangements. Consequently, cell proliferation depends on proper dynamic instability rate of microtubule rearrangement. Our studies on the expression of α- and ß, tuc-4 tubulins (tuc-4 tubulin is expressed along the most distal regions of lamellipodia and filopodia), have shown no alterations in the expression and distribution of microtubule proteins. Hence, observed normal astrocyte and astrocytoma cell polymorphism cannot be related to alterations of cytoskeleton proteins but to a quite normal morphology according to cell cycle and differentiation stages. Together with Hoestch method, which gives information about cell death through necrosis or apoptosis pathways, cell cycle progression allows us to determine the cycle phase where the alteration could have been produced. Our results on cell viability and cell cycle determined by comparing control and test astrocytoma samples constitute the strongest support for the validation of the so called *GTEM-incubator*, which maintains optimal physical growing conditions in order to submit mammal cells to exposure to MW-EMF.

GTEM-cells are considered to allow controlled experimental conditions wherein either *in vitro* cell cultures or *in vivo* biological samples are placed in a well-defined field environment (Lu et al., 1999; Yurekli et al., 2006; Li et al., 2007; Seaman 2007). However a precise knowledge of the electromagnetic dose received by each specimen is critical in accurately establishing a biological response-electromagnetic dose relationship. After our *GTEM-incubator* validation, which ensures growth temperature control in a humidified atmosphere with 5% CO_2, we proceeded with dosimetry simulation characterization of the GTEM-cell equipment. Dosimetry was done by using analytical calculations and numerical simulations with the *GTEM-incubator* and two Falcon flasks inside the GTEM-cell. Dosimetry studies ensure that for the two experimental parameters selected the estimated maximum temperature increases were negligible, so that any experimentally observed unusual cell function could be associated with non-thermal effects.

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References

Cleary S.F., Liu LM. and Cao G. (1992). Effects of RF power absorption in mammalian cells. Ann. N. Y. Sci. 649, 166-170.

Donnellan M., McKenzie D.R. and French P.W. (1997). Effects of exposure to electromagnetic radiation at 835 MHz on growth, morphology and secretory characteristics of a mast cell analogue, RBL-2H3. Cell Biol. Int. 21, 427-439.

Dorsey W.C., Ford B.D., Roane L., Haynie D.T. and Tchounwou P.B. (2005). Induced mitogenic activity in AML-12 mouse hepatocytes exposed to low-dose ultra-wideband electromagnetic radiation. Int. J. Environ. Res. Public Health 2, 24-30.

French P.W., Donnellan M. and McKenzie D.R. (1997). Electromagnetic

radiation at 835 MHz changes the morphology and inhibits proliferation of human astrocytoma cell line. Bioelectrochem. Bioenergetics 43, 13-18.

Ji Z., Hagness S.C., Booske J.H., Mathur S. and Meltz M.L. (2006). FDTD analysis of a Gigahertz TEM cell for ultra-wideband pulse exposure studies of biological specimens. IEEE Trans. Biomed. Eng. 53, 780-789.

Kwee S. and Raskmark P. (1998). Changes in cell proliferation due to environmental non-ionizing radiation 2. Microwave radiation. Bioelectrochem. Bioenerg. 44, 251-255.

Li B.F., Guo G.Z., Ren D.Q., Jing L. and Zhang R.B. (2007). Electromagnetic pulses induce fluctuations in blood pressure in rats. Int. J. Radiat. Biol. 83, 421-429.

Lu S.T., Mathur S.P., Akyel Y. and Lee J.C. (1999). Ultrawide-band electromagnetic pulses induced hypotension in rats. Physiol. Behav. 65, 753-761.

Pavicic I. and Trosic I. (2008). In vitro testing of cellular response to ultra high frequency electromagnetic field radiation. Toxicology in vitro 22, 1344-1348.

Pérez Bruzón R.N., Gómez Martín R., González García S. Azanza M.J. and del Moral A. (2011). GTEM-chamber dosimetry for human cells in culture exposure to pulsed MW. (in preparation).

Pérez Castejón C., Pérez Bruzón R.N., Llorente M., Pes N., Lacasa C., Figols T., Lahoz M., Maestú C., Vera-Gil A., del Moral A. and Azanza M.J. (2009). Exposure to ELF-pulse modulated X band microwaves increases in vitro human astrocitoma cell proliferation. Histol. Histopathol. 24, 1551-1561.

Salovarda Lozo M. and Malaric K. (2010). Use of GTEM-cell and wire Patch Cell in calculating thermal and non-thermal biological effects of electromagnetic fields. In: Advanced microwave and millimetre wave technologies: semiconductor devices, circuits and systems. Moumita Mukherjee (ed.). In-Tech, intechweb-org. Vukovar, Croacia. pp 573-588.

Stagg R.B., Thomas W.J., Jones R.A. and Adey W.R. (1997). DNA synthesis and cell proliferation in C6 glioma cells and primary glial cells exposed to a 836.55 MHz modulated radiofrequency field. Bioelectromagnetics 18, 230-236.

Seaman R.L., Parker J.E., Kiel J.L., Mathur S.P., Grubbs T.R. and Prol H.K. (2002). Ultra-wideband pulses increase nitric oxide production by RAW 264.7 macrophages incubated in nitrate. Bioelectromagnetics 23, 83-87.

Seaman R.L. (2007). Effects of exposure of animals to ultra-wideband pulses. Health Phys. 92, 629-634.

Velizarov S., Raskmark P. and Kwee S. (1999). The effects of radiofrequency fields on cell proliferation are non-thermal. Bioelectrochem. Bioenergetics 48, 177-180.

Yurekli A.I., Ozkan M., Kalkan T., Saybasili H., Tuncel H., Atukeren P., Gumustas K. and Seker S. (2006). GSM base station electromagnetic radiation and oxidative stress in rats. Electromagn. Biol. Med. 25, 177-188.

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