Exposure to ELF-pulse modulated X band microwaves increases *in vitro* human astrocytoma cell proliferation

**C. Pérez-Castejón[1], R.N. Pérez-Bruzón[1], M. Llorente[1], N. Pes[1], C. Lacasa[1],
T. Figols[1], M. Lahoz[1], C. Maestú[2], A. Vera-Gil[1], A. del Moral[3] and M.J. Azanza[1]**

[1]Magnetobiology Laboratory, Department of Human Anatomy and Histology, School of Medicine, University of Zaragoza, Zaragoza,
[2]Biomedical Technology Center, ESIT Complutense University, Madrid and [3]Magnetism Laboratory, Department of Condensed
Matter Physics and Material Science Institute (ICMA), University of Zaragoza-CSIC, Zaragoza, Spain

Summary. Common concern about the biological
effects of electromagnetic fields (EMF) is increasing
with the expansion of X-band microwaves (MW). The
purpose of our work was to determine whether exposure
to MW pulses in this range can induce toxic effects on
human astrocytoma cells. Cultured astrocytoma cells
(Clonetics line 1321N1) were submitted to 9.6 GHz
carrier, 90% amplitude modulated by extremely low
frequency (ELF)-EMF pulses inside a Gigahertz
Transversal Electromagnetic Mode cell (GTEM-cell).
Astrocytoma cultures were maintained inside a *GTEM-
incubator* in standard culture conditions at 37±0.1°C,
5% CO_2, in a humidified atmosphere. Two experimental
conditions were applied with field parameters
respectively of: PW 100-120 ns; PRF 100-800 Hz; PRI
10-1.25 ms; power 0.34-0.60 mW; electric field strength
1.25-1.64 V/m; magnetic field peak amplitude 41.4-54.6
μOe. SAR was calculated to be 4.0×10^{-4} W/Kg.
Astrocytoma samples were grown in a standard
incubator. Reaching 70-80% confluence, cells were
transferred to a *GTEM-incubator*. Experimental
procedure included exposed human astrocytoma cells to
MW for 15, 30, 60 min and 24 h and unexposed sham-
control samples. Double blind method was applied. Our
results showed that cytoskeleton proteins, cell
morphology and viability were not modified.
Statistically significant results showed increased cell
proliferation rate under 24h MW exposure. Hsp-70 and
Bcl-2 antiapoptotic proteins were observed in control
and treated samples, while an increased expression of
connexin 43 proteins was found in exposed samples. The
implication of these results on increased proliferation is
the subject of our current research.

Offprint requests to: C. Pérez Castejón, Departamento de Anatomía e
Histología Humanas, Facultad de Medicina, Universidad de Zaragoza,
C/Domingo Miral s/n, 50009 Zaragoza, Spain. e-mail: capercas@
unizar.es

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microwave effects, Astrocytoma cell proliferation

Introduction

Common concern about the biological effects of
MW in the range of 10 GHz is increasing with the
expansion of X-band EMF for meteorology,
governmental institution applications or, more widely
extended, speed and aerial traffic control radars.
Therefore, it is important that the biological effects of
EMF in this range of frequencies are understood at a
cellular level to reveal the molecular mechanisms and,
possible health implications. A number of
epidemiological studies have raised concerns about the
relationship between exposure to EMF and cancer
promotion (Ahlbom et al., 2001). The number of reports
on radiofrequencies (RF) induced effects on various
cellular processes for phone radiation range has
increased rapidly, but not for X-band MW range. There
is even a paucity of investigation into the cells of central
nervous system (CNS), i.e., neurones and glia cells,
although astrocytoma risk related to job exposure to
electric (EF) and magnetic fields (MF) has been reported
to be significant (Mack et al., 1991). Several, more
recent studies, point out the potential contribution of
occupational exposure to EMF to the pathogenesis of
neurodegenerative processes such as Alzheimer's or
Parkinson´s diseases (Feytching et al., 2003; Park et al.,
2005).

Morphological changes in mouse neuroblastoma
cells have been reported to occur after growing *in vitro*
and being exposed to MW (2.7 GHz, 1.7-3.9 kV/cm, 1μs
pulses at 330 pulses/s) (Webber et al., 1980).
Vacuolation of neurones, but not glia cells, was observed
in the hypothalamic region of Chinese hamsters' brain
exposed to 2.45 GHz (50 mW/cm^2, SAR of 7.5 W/kg)

(Albert and De Santis, 1975). A human astrocytoma cell line (U-87 MG) exposed to 835 MHz, power densities 40 ± 15 mW/cm^2 and 8.1 ± 3 mW/cm^2, showed at the lower power density a DNA synthesis rate decrease and modification of cell morphology. At 40 mW/cm^2 there were no effects on cell proliferation but there was some cell morphology alteration. No increased cell proliferation of normal or transformed cultures of glial origin were observed when exposed to 836.55 MHz modulated RFs (Stagg et al., 1997). Rat hippocampal slice exposure to 9.3 GHz (pulse width 0.5-2.0 μs; 2 μs (0.5 Hz repetition rate) or 0.5 μs (2 Hz repetition rate)) gave no indication of any specific biological action of a brief RF exposure, even at peak SAR as high as 330 kW/g for 2 min (Pakhomov et al., 2003). Chronic exposure (5 days a week for 24 weeks) of glia cells to 900 MHz signal for 15 min/day (SAR = 6W/kg) showed persistent astroglia activation in the rat brain, revealed by increased GFAP protein expression. Primary culture of neurones and astrocytes from mice brains submitted to 2 h direct exposure to a cell phone rated at frequency of 1.9 GHz showed up-regulation of caspase-2, caspase-6 and Bax genes in astrocytes (Zhao et al., 2007).

Numerous *in vitro* biological effects, on different cell lines, have been reported under exposure to ELF-EMF. A number of studies showed adverse effects on cell membrane structure, apoptosis pathways and cell morphology (Blumenthal et al., 1997; Lisi et al., 2000). The distribution and expression of growth associated protein-43 (GAP-43), in human malignant glioma cells MO54, was studied after exposure to a MF (60 Hz, 5 mT). GAP-43 protein was present in the cytoplasm, accumulated in the perinuclear area. After 12 h exposure to 5 mT MF it was observed a protein expression increase (Ding et al., 2002). However, a satisfactory explanation by what biophysical and biological mechanisms these fields exert their biological effects have not yet been offered. The above data are of interest when applying MW modulated by ELF-EMF to separate the effect of the carrier wave out from the modulating ELF field one. Non-modulated carrier waves did not affect cell membranes (Byus al., 1984), but a *demodulation* effect has been observed in experiments made on single unit neurones by recording the bioelectric activity in real time. The biological effect was produced by the modulating ELF-field, however the application of the carrier wave alone did not produced any significant effect (del Moral et al., 2008).

Protoplasmic astrocytes are the main glia cells found in human brain grey matter. Astrocytes surround neurones and neuronal processes forming the structural matrix for the CNS. They provide neurones with structural support, maintain local conditions for neuronal function, feed neurones by supplying metabolites from blood and provide a neurotransmitter and ions buffer system to the brain. Astrocytes are more numerous than neurones and retain the capacity to proliferate, being responsible for more than 90% of human cellular brain tumours (benign or malignant gliomas). Any

modification in their activities will potentially produce negative effects on brain function and thereby on human health. Although so far, many reported biological effects under exposure to EMF are considered to be simply indeterminate with respect to their significance to health, we consider that astrocytes are cells of crucial interest for the search of potential health effects of radar EMF radiation.

The aim of this study was to evaluate whether 9.6 GHz MW amplitude modulated by ELF-pulsed EMF, at low power density to rule out thermal effects, induces alterations in the morphology, viability and proliferation of astrocytes from *in vitro* human astrocytoma cells.

Material and methods

Astrocytoma cell culture

Experiments were performed on astrocytes from human astrocytoma (Clonetics line 1321N1). Specific growth medium for astrocytoma cells was used: Dulbecco's Minimum Essential Medium (DMEM, SIGMA) supplemented with 10% foetal bovine serum (SIGMA), 0.4 mM L-Glutamine (SIGMA) and 100 U/ml penicillin and 0.1 mg/ml streptomicyn (SIGMA). Cells were maintained as an adherent monolayer in a humidified atmosphere at $37\pm0.1°C$, 5% CO_2, in a standard incubator subcultured once per week. Cells were seeded at a density of $3x10^4$ cells/ml into Falcon flasks and cultured under standard conditions, with a 70-80% confluence, and were transferred to a *GTEM-incubator* for exposure to MW-EMF.

To determine any cytotoxic effect under MW exposure the following parameters were studied: cell morphology, expression and distribution of cytoskeleton (GFAP, α-,ß- and tuc-tubulins) and transmembrane (connexin 43) proteins, viability, proliferation rate, cell cycle progression and apoptosis vs. necrosis. Experiment 1 and 2 parameters (Table 1) were applied sequentially. Exposure experiments were replicated five times keeping cells in culture for 15, 30, 60 min and 24 h. A series of experiments were made by exposing cells to experiment 2 parameters for 24 h, keeping 24 additional hours in culture before proceeding to cell counting and characterization for apoptosis related proteins. Double blinded method was applied for morphology, cell counting, and image capture processes. Sham-control cell samples were kept in *GTEM-incubator,* under the same experimental conditions, but were not exposed to radiation.

Immunofluorescence

The expression and distribution of proteins was studied by indirect immunofluorescence staining method for: glial-fibrillary-acidic-protein (GFAP, dilution 1/100, polyclonal rabbit-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); tubulin anti-tuc-4 (dilution 1/100, polyclonal AB5454 Chemicon; FITC-dilution 1:50, F0205 Dako); connexin 43 (Cx43) (anti GJB6, dilution 1/300, A014846 SIGMA; FITC- dilution 1:64, F2012 SIGMA); Bcl-2 (dilution 1/100, anti-Bcl-2, rabbit monoclonal 04-436 Millipore; FITC- dilution 1:50, F0205 Dako); Hsp-70 (dilution 1/100, rabbit-anti-Hsp AB3470 Millipore; FITC- dilution 1:50, F0205 Dako); and mouse-anti-Bax (dilution 4 μg/ml, mouse-anti-Bax, MAB4601 Millipore; FITC- dilution 1:64, F2012 SIGMA). The antibodies were diluted with Dako (S2022) and Chemicon (21544) diluents. Astrocytoma cells were cultured on coverslips impregnated with poly-L-lysine (SIGMA) reaching 90% confluence. 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 made 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).

Viability

The trypan blue exclusion method was applied on astrocytoma cells cultured on Falcon flasks, 70-80% confluence, after 15, 30, 60 min and 24 h exposure, checking for any toxicity effect. Cell monolayer was detached chemically, after removing the culture medium with 2 ml of trypsin-EDTA solution for 5-10 min. Cells were observed under inverted microscope to check out if 95% of cells had been detached from the bottom of the Falcon. For trypsin neutralization 1.0 ml of culture medium was added. Astrocytoma 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 staining dead cells in blue colour. Viability was calculated as the percentage of normal cells with respect to total number of cells.

Apoptosis vs. necrosis

Apoptosis like cell death was identified on the basis of morphological criteria: condensed and fragmented nuclei (genotoxicity), blebbing of plasma membranes, decrease in cell size and appearance of apoptotic bodies. To distinguish apoptosis from necrosis the Hoechst–propidium iodide protocol was applied. Astrocytoma cells were cultured on coverslips with poly-L-lysine (SIGMA) to raise 90% confluence. Cells were fixed 5 min in methanol, dried for 5 min and then washed with PBS for 10 min three times. Cells were stained 30 min with Hoechst plus propidium iodide (0.05 μg/ml H33258 SIGMA + 0.05 mg/ml, P4170, SIGMA) and washed in PBS for 10 min twice. Slides were mounted with Dako solution (53023 Dako).

Electromagnetic field exposure equipment

Experiments of cell exposure to X band MW of 9.6 GHz were done in a certified horn shape Gigahertz Transversal Electromagnetic Mode Cell (GTEM-cell), where the TEM radiated MW is from a flat strip line along a border (Pérez Bruzón et al., 2009). MW are produced with a solid state MW functions generator provided with a versatile modulator of different wave profiles (100 KHz-20 GHz range, GIGANTRONICS 2520A), followed by a high power (50W maximum output) MW travelling-wave tube amplifier (T186-50), a power meter (BOONTON 51013) and after a directional coupler (DMN 10-1-30), which inject the MW signal into the GTEM chamber through a 50 ohm coaxial cable. Direct and reflected powers are monitored via a diode bridge. GTEM chamber is provided with anechoic walls to reduce unwanted reflections. The EMF-MW mode is the TEM one, the same as usually used in wireless telecommunication.

The two exposure conditions selected, named as experiments 1 and 2, are shown in Table 1. MW amplitude modulated by ELF-EMF -modulation was kept for all irradiations at 90%- parameters were: carrier frequency (CF), 9.6 GHz; pulse width (PW) 100-120 ns; pulse repetition frequency (PRF), 100-800 Hz; pulse repetition interval (PRI), 1.25-10 ms; power 0.34-0.60 mW; electric field (EF) strength 1.25-1.64 V/m; magnetic field strength 3.3×10^{-3} A/m (41.4 μOe) and 4.35×10^{-3} A/m (54.6 mOe) respectively.

Culture conditions inside GTEM cell

Cell exposure to MW was performed in the GTEM-cell inside a *GTEM-incubator* placed in the volume where the EMF is more homogeneous, on a horizontal plastic plate just in front of the radiating TEM-MW strip line (Pérez-Bruzón et al., 2009). Commercial standard incubators have a heating system based on a metallic plate calefactory, being prone to eddy current formation under MW excitation, with EMF distortion and Joule-heat production. To avoid this problem it was necessary to build an incubator made of a plastic material, and heated by circulating warm water, here called GTEM-incubator (built by Olympus firm under our specifications). Incubator temperature was steadily monitored and accurately controlled by an electronic feedback system within 0.1°C. Cells were maintained in this way at a temperature of 37±0.1°C, within a humidified atmosphere and 5% CO_2 (Pérez-Bruzón et al., 2009).

Previously to exposure experiments we made the validation of *GTEM-incubator* by studying morphology, cytoskeleton protein expression, cell viability and cell

cycle progression. Our results showed that culture conditions in *GTEM-incubator* were suitable for exposure of astrocytoma cells to MW (Pérez-Bruzón et al., 2009).

Dosimetry

The characterization of field patterns inside the GTEM-cell was made using analytical calculations and numerical simulations with the GTEM-incubator and two Falcon flasks inside the GTEM-cell (Pérez-Bruzón et al., 2009). Exposure conditions were design to maintain cells in non-thermal experimental conditions. Calculated SAR values were: 3.99×10^{-1} and 4.08×10^{-1} mW/Kg respectively for the two flasks. Calculated temperature increases due to MW power exposure were below 3×10^{-5} °C, the accuracy (0.01°C) of the calibrated Pt-resistor thermometer. Therefore we estimate negligible thermal effects in the cells.

Statistical analysis

Statistically significant differences between groups, treated and control 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 significant difference.

Results

Cell morphology has been studied in a number of experiments, in a wide diversity of conditions: control samples and samples under the two different sets of MW exposure (experiments 1 and 2, Table 1), plus exposure times of 15, 30, 60 min and 24 h. A group of samples were studied at the end of exposure time. Another group were left in culture an additional 24 h and then studied.

We have observed that astrocytoma cells show a natural polymorphism according to cell cycle phase and differentiation stage. Our conclusion was that no significant effects on cell morphology were induced for any of the experimental conditions and exposure times with respect to sham control samples (Fig. 1).

Lack of morphological effects was confirmed by studying the distribution and expression of biomarker cytoskeleton proteins. No significant differences between irradiated and sham-control samples were observed for any of experiment 1 and 2 parameters and, exposure times of 15, 30, 60 min and 24 h in the expression and distribution of α-, ß- (Fig. 2), and tuc-tubulins, and GFAP (Fig.3) proteins.

Since connexins are also considered biomarker proteins for astrocyte morphological and metabolic activities the expression of Cx43, specific for astrocytes, was studied under exposure to experiments 1 and 2 parameters for 15 min and 24 h respectively. Expression differences were observed in the results for Cx43 between treated and sham control samples for 24 h exposure (Fig. 4).

Table 1. Microwave EMF parameters selected for the two experiments carried out on human astrocytoma cells.

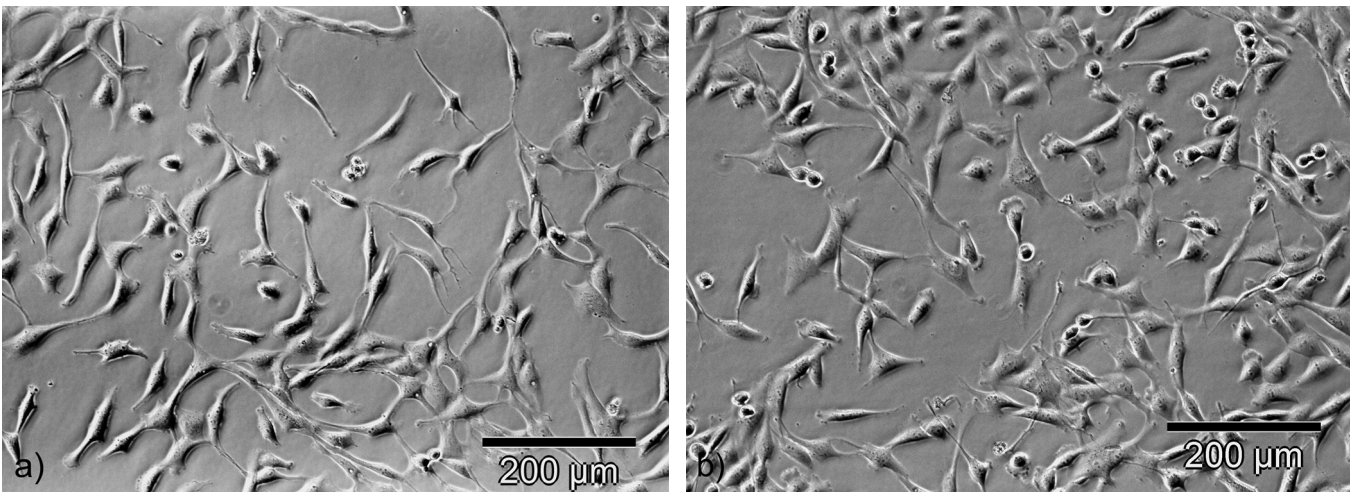| CF MW 9.6 GHz	PW	(PRF)	(PRI)	Power	MW electric field intensity (peak)	MW magnetic field intensity (peak) ELF modulation 90%
Exp.1	100 ns	100 Hz	10 ms	0.34 mW	1.25V/m	3.3×10^{-3} A/m (41.4 μOe)
Exp.2	120 ns	800 Hz	1.25 ms	0.60 mW	1.64V/m	4.35×10^{-3} A/m (54.6 μOe)

Fig. 1. Human astrocytoma cells show a natural polymorphism according to cell cycle phase and differentiation stage. No significant morphology differences were observed between sham-control and irradiated cell samples. **a.** Sham-control sample. **b.** Irradiated cell sample, 24 h exposure to experiment 2 parameters.

Cell cycle progression showed that cells enter into exponential phase after four days in culture, reaching maximum growth on the 8th day.

Viability results for experiment 1, exposure times of 15, 30 and 60 min (Fig. 5) and, for experiments 1 and 2, exposure times of 24h, (Fig. 5) showed that the difference of the mean-population was not significant.

Cell proliferation rate was studied by considering the number of cells alive for all experimental studied conditions. Results showed that the differences between sham control and irradiation conditions of experiments 1 and 2 parameters, 24h exposure times were statistically significant. Proliferation increase was of 42% and 44%

for experiments 1 and 2 exposure conditions respectively (Fig. 6). After observing proliferation increase rate, the experiments were replicated another five additional times by applying experiment 2 parameters, exposure for 24 h. In this set of experiments, cells were maintained in culture 24 additional hours before proceeding to cell count. The results we have show that the differences between sham control and exposed experiments were also statistically significant.

The method of Hoechst and propidium iodide, which specifically distinguishes processes of apoptosis from necrosis ones, showed no genotoxic effects (Fig. 7).
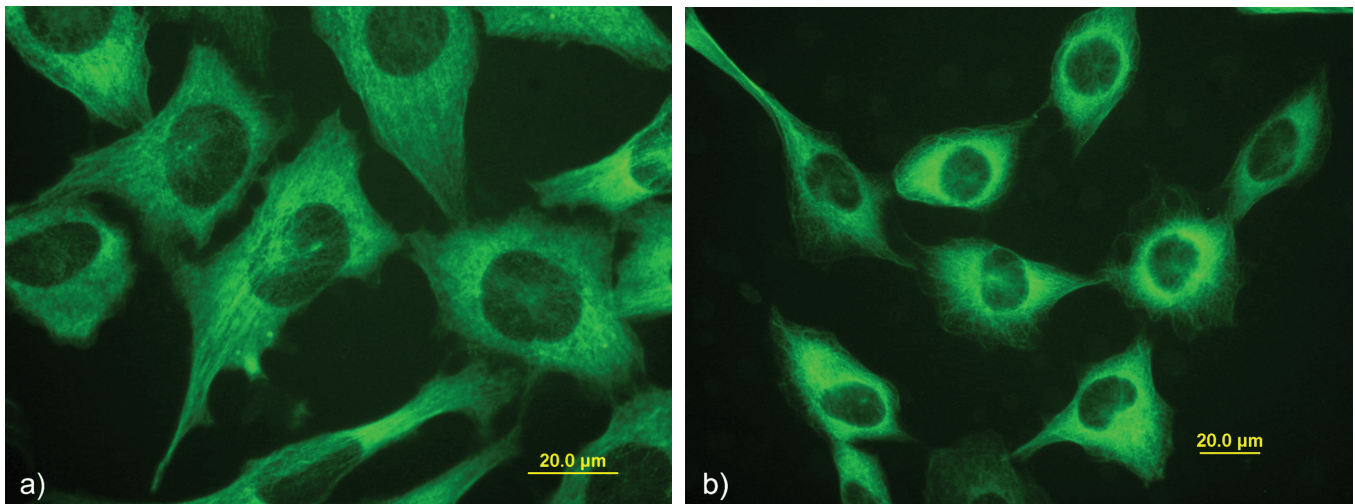
Additional experiments were undertaken to try to

Fig. 2. Immunofluorescence reaction for ß-tubulin. No significant differences between sham-control and irradiated cell samples were observed in expression of ß-tubulin proteins for any of the experimental or exposure time conditions. **a.** Sham-control samples. **b.** Irradiated cell sample, 24 h exposure to experiment 2 parameters.
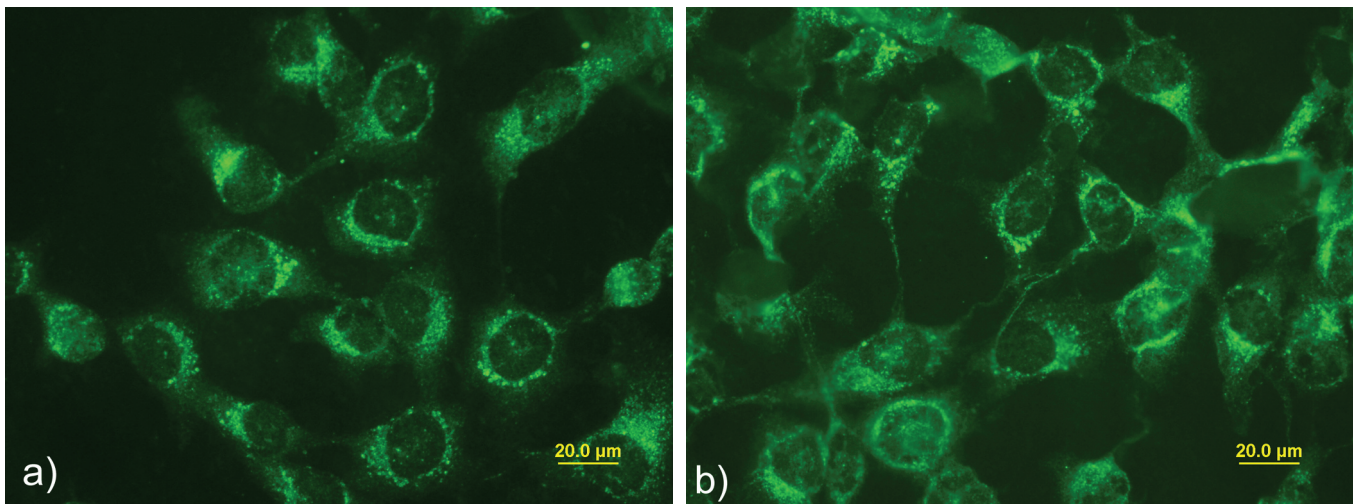

Fig. 3. Immunofluorescence reaction for GFAP proteins. No significant differences between sham-control and irradiated cell samples were observed in expression of GFAP proteins for any of the experimental or exposure time conditions. **a.** Sham-control sample. **b.** Irradiated cell sample, 60 min exposure to experiment 1 parameters.

find out molecular data to explain the observed increased proliferation. We have centred our attention on expression and localization of hsp-70, antiapoptotic oncogen Bcl-2 protein and pro-apoptotic Bax protein. It is well known that hsp proteins show an extremely short life time, but we have been able to find a positive reaction for hsp-70 protein expression in control and at the end of exposure of 24 h at experiment 2 parameters (Fig. 8). The expression of Bcl-2 protein in astrocytoma cells was observed in 100% of control and exposed cells for exposure of 24 h at experiment 2 parameters (Fig. 9).

We observed negative results for expression of Bax, proapoptotic-protein under the same experimental conditions.

Discussion

In considering the biological effects of exposure to high frequency EMF the misconception still persists that RF and MW effects are solely the result of heat generation, contrary to the fact that a number of reported studies showed significant effects, on various cellular
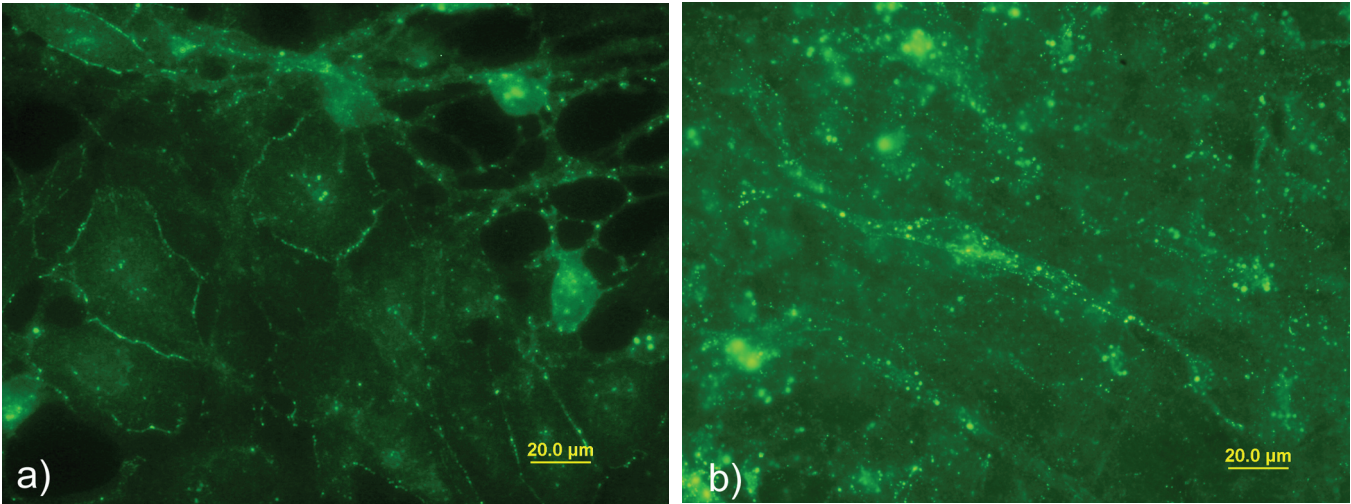

Fig. 4. Immunofluorescence reaction for Cx43 proteins. Differences between sham-control and irradiated cell samples were observed in expression of Cx43 proteins for 24 h exposure to experiments 1 and 2 parameters. **a.** Sham-control sample. **b.** Irradiated cell sample, 24 h exposure to experiment 2 parameters.
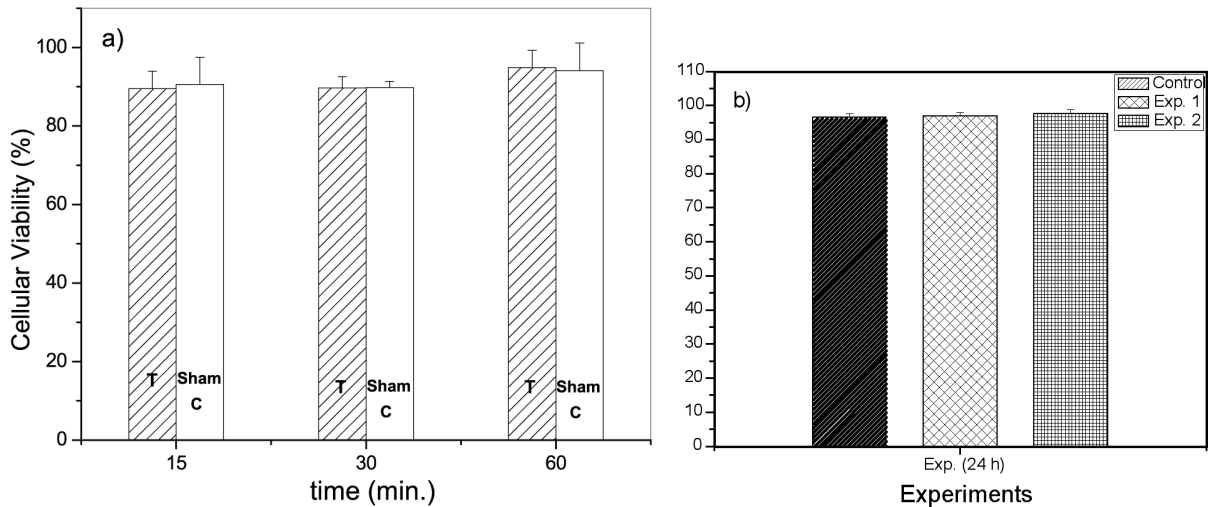

Fig. 5. Viability results show no significant differences between sham-control and cells exposure samples to any of experimental parameters (Exp.1) or experiment 2 (Exp.2) and exposure times. Viability results for 15, 30 and 60 min exposure **(a)** and for experiments 1 and 2 parameters, 24 h exposure **(b)**. T: treated samples; sham c: control samples. Experimental values represent means ± standard deviation for 15 measurements. The two sample paired t-test statistic method applied to calculate viability results showed that at the 0.05 level, the difference of the mean-population was no-significant.

activities, in experimental systems under well controlled isothermal conditions (Cleary et al., 1996; Velizarov et al., 1999). Temperature control for experiments under applied MW becomes a strategic end point. In our experiments it was afforded by applying a very low power EMF (0.34-0.60 mW) in non-thermal exposure conditions -increases due to MW power exposure were below 3×10^{-5} °C- and by maintaining astrocytoma cells growing in culture inside the so called *GTEM-incubator* (Pérez-Bruzón et al., 2009). SAR values in our

experimental conditions (4.0×10^{-4} W/Kg) were of the same order of magnitude of well tested non-thermal RF conditions inducing increased expression of hsp molecules (De Pomerai et al., 2000). Incubator validation, made before starting exposure experiments, showed that incubator physical conditions (37°C, 5% CO_2, humidified atmosphere) were suitable for proceeding with astrocyte exposure to pulsed MW (Pérez Bruzón et al., 2009).

Tubulins and GFAP are considered biomarker proteins for morphological and metabolic activities for astrocytes. Microtubule proteins are extremely dynamic structures; their functions are based on dynamic instability: polimerization of α- and ß-tubulins and their degradation to dimmer protein subunits. 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 rearrangements. Modification of cytoskeleton proteins and consequently alteration of cell proliferation has been described under a wide range of experimental conditions by exposure to ELF-EMF (Blumenthal et al., 1997). Also, RF exposure produced modifications that affected microtubule structure and morphology, inducing a significant decrease in growth of astrocytoma cells (Ortner et al., 1983; French et al., 1997; Lisi et al., 2000; Pacini et al., 2002), mast cell line RBL-2H3 (Donnellan et al., 1997) and lung fibroblast line V79 (Pavicic and Trosic, 2008). GFAP is an intermediate filament protein highly specific for cells of astroglial lineage. This protein is involved in many cellular functioning processes, such as cell structure, movement and cell communication. Reactive astrocytoma cells may also contribute to increased expression of GFAP characterized by cell hypertrophy and hyperplasia.
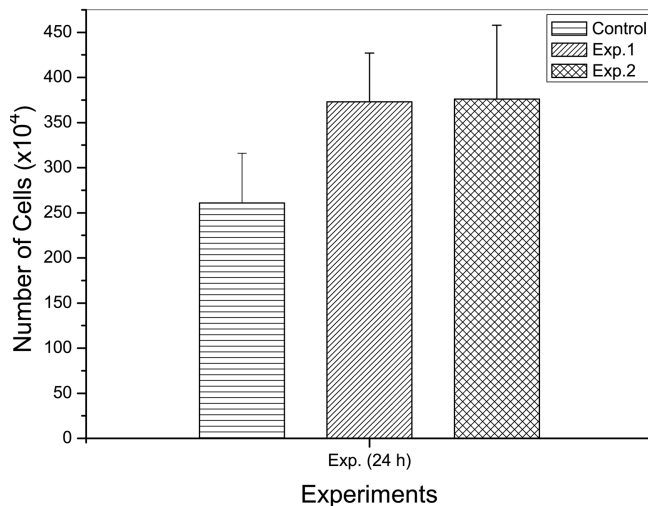

Fig. 6. Proliferation rate has been calculated by considering the number of cells alive for all experimental conditions. The differences between sham-control and exposure to experiments 1 (Exp.1) and 2 (Exp.2) parameters, 24 h exposure showed statistically significant differences. Experimental values represent means ± standard deviation for 15 measurements.
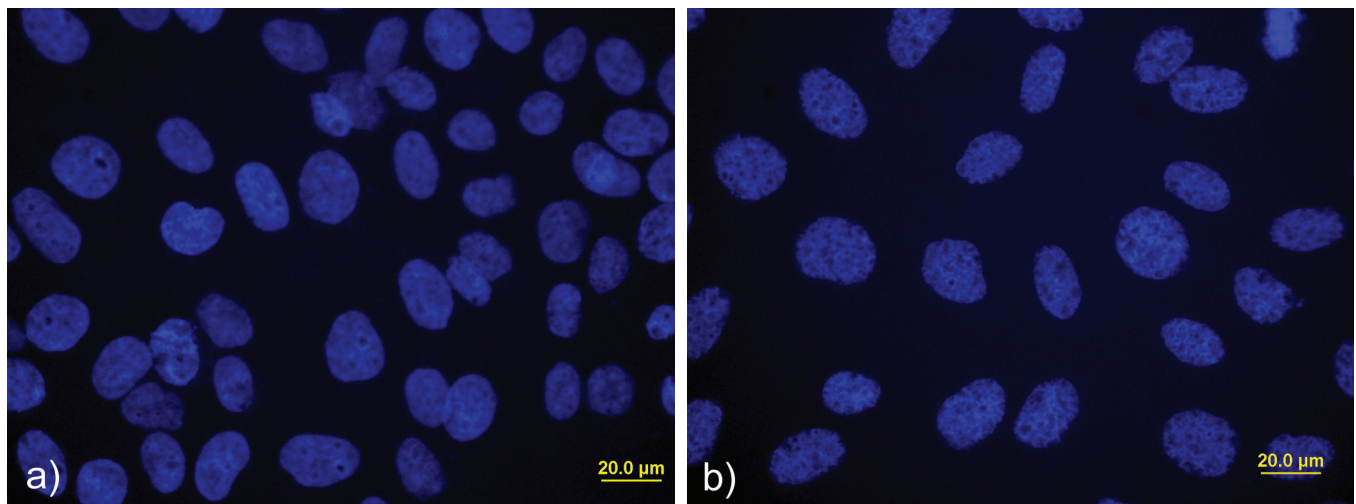

Fig. 7. Hoescht and propidium iodide reaction specifically reveals processes of apoptosis vs. necrosis. The reactions showed no genotoxic induced effects for any of the experimental conditions. **a.** Sham-control. **b.** Irradiated samples to experiment 1 parameters, 60 min exposure.

Chronic exposure to GSM signal of 900 MHz at a low dose (SAR = 1.5 W/kg) failed to induce evidence of damage in the brain, but for a SAR of 6 W/kg cellular gliosis was induced in different areas of the rat brain quantified by image analysis of immunoreactive astroglia staining for GFAP (Ammari et al., 2008).

Our experimental observations were not coincident with the above reported data. Astrocytes from human astrocytoma showed a highly variable morphology and size, depending on several variables, like number of cells in culture or cell cycle and differentiation stage. Modifications either in morphology or in the expression and distribution of microtubule proteins α-, ß- and tuc-4 tubulins (tuc-4 tubulin is expressed along the most distal regions of lamellipodia and filopodia) were not

observed. Our results on the expression of GFAP also showed no alterations in the expression and cytosolic distribution of these proteins. Hence, astrocytoma polymorphism cannot be correlated with alterations of cytoskeleton proteins under exposure to MW of 9.6 GHz according to experiment 1 and 2 parameters.

The significance of our observations lies in the possibility that cells of different lineages respond in different ways to applied EMFs. In previous experiments we studied the distribution of Cx26, tuc-4 tubulins, MAP2 and TAU proteins in *Helix aspersa* neurones under exposure to various EMF parameters: i) sinusoidal ELF-MF (0.2 μT-15 mT, 0.1-217 Hz); ii) 1.8 GHz-RF (520 mW, SAR 4-10 W/kg) and iii) 13.6 GHz-MW amplitude modulated by square ELF-MF of 4 to 16 Hz
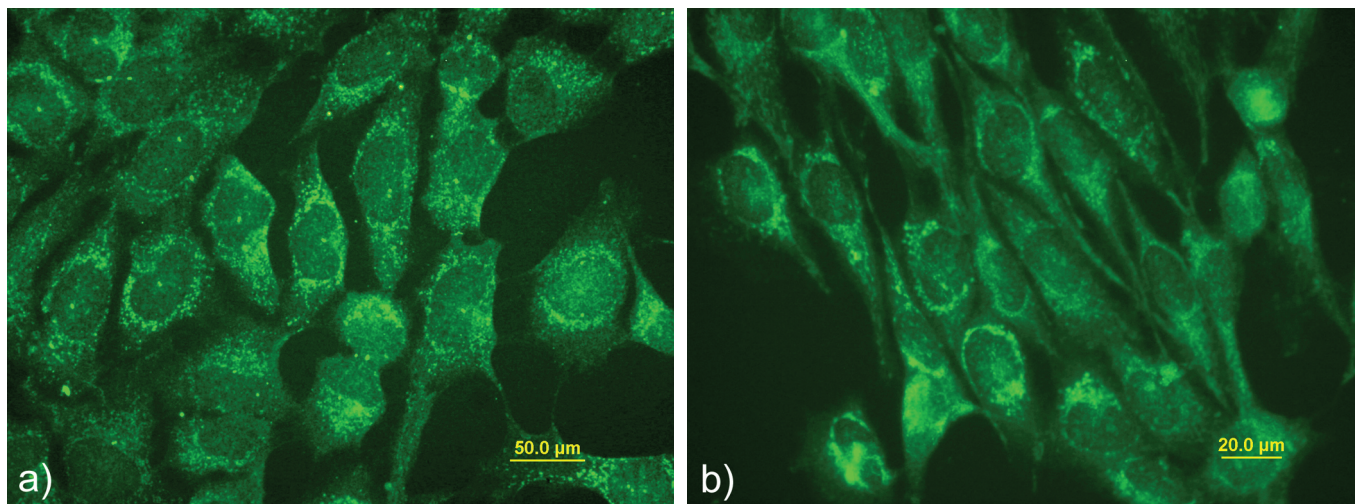

Fig. 8. Staining of Hsp-70 antiapoptotic protein expression by immunofluorescence. A positive reaction was observed either perinuclear or inside the nuclei in sham-control **(a)** and in cells exposed to experiment 2 parameters during 24 h **(b)**. The reaction was made immediately after the exposure.
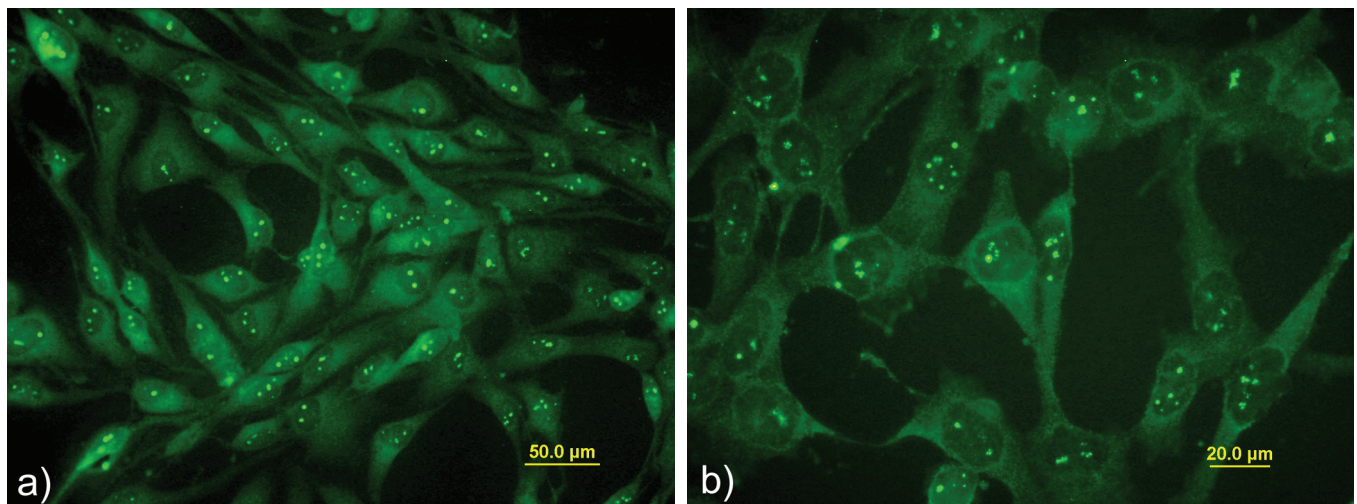

Fig. 9. Staining of Bcl-2 antiapoptotic protein expression by immunofluorescence. A positive reaction was observed in the nuclei of cells in sham-control **(a)** and cells exposed to experiment 2 parameters during 24 h **(b)**. The reaction was made immediately after the exposure.

($H_0 \approx 0.1$ Am^{-1} = 1.25 mOe, MW power 5 mW) (Azanza et al., 2007; Perez-Castejón et al., 2007). The exposure times were of 15, 30 and 60 min, according to effects observed on neurones electrophysiology for these time intervals. Immunoreaction of tuc-4 tubulin decreased after 60 min exposure. Cx26 expression decreased after 30 min exposure (Raso, 2005).

Genotoxic effects, not observed in our experimental conditions, can also induce proliferation modification but, there exist several other more indirect cellular pathways which can lead to increased proliferation effects. Gap junctions are regions of contact between adjacent astrocyte membranes that couple cells electrically and chemically by means of connexin proteins. Intercellular gap junction communication plays an essential role in regulating astrocyte growth, proliferation and differentiation. In our experiments we have observed an increased distribution and expression of Cx43, which needs to be confirmed by quantitative methods, in astrocytoma cells after 24h exposure to pulsed MW. Astrocyte DNA and connexins have been the subject of research under applied ELF-EMF and RF. Several authors have suggested that certain intensities and frequencies of ELF-MF, by inhibiting cell gap junctions, might act as cancer promoters (Li et al., 1999; Hu et al., 2001; Teng et al., 2002). Isothermal exposure of gliomas to 2.45 GHz (SAR > 5W/kg^{-1}) caused DNA synthesis increase in cells (Cleary et al., 1992) whereas no significant differences that could be attributed to altered DNA synthesis rates were found in rat glioma cells under exposure to 836.55 MHz at three power densities: 0.09, 0.9, 9 m/W/cm^{-2} (Stagg et al., 1997). ELF-MF (50 Hz, 0.8 mT) induced the inhibition of gap junctions by a mechanism of hyperphosphorylation of Cx43 in fibroblast cells (NIH3T3 line) (Hu et al., 2001). A decrease of Cx43 was also obtained in Chinese hamster lung cells, by a mechanism of protein internalization, under exposure to 50 Hz sinusoidal MF at 0.8 mT for 24 h (Zeng et al., 2003). Opposite results were found in a mouse fibroblast cell line (SV40-Swiss-3T3) under applied fields of 50 Hz, 2 mT. The results showed that the field exposure stimulated the gap junctions to 160% of the control level (Schimmelpfeng et al., 1995). In the human malignant glioma cells MO54 after exposure to an ELF-MF (60 Hz, 5 mT), an increased of GAP-43 protein in the perinuclear area was observed after 12 h exposure to 5 mT MF (Ding et al., 2002).

According to our negative results on cytoskeleton proteins expression modification and lack of genotoxic effects, additional experiments were carried out. Tumour production arises through a multistep, mutagenic process whereby tumour cells acquire common features, such as unlimited proliferation potentials and resistance to antiproliferative and apoptotic signals. Proliferation could be induced through expression increase of specific proteins involved in cell cycle regulation with anti-apoptotic effects. We centred our attention on hsp-70, Bcl-2- and Bax- expression proteins.

With respect to the influence of EMF on apoptosis, results are variable depending on the kind of cell and EMF exposure. It has been described that either an increase of (Blumenthal et al., 1997) or a decrease of proliferation responses are induced (Tofani et al., 2001). The exposure of cell to pulsed MW could induce cell stress and thereby a hsp proteins increase expression. Hsps proteins not only function as chaperon proteins to refold or eliminate misfolded or denatured proteins, but they also play a critical role in the regulation of the cell cycle, proliferation, differentiation and apoptosis. Up-regulation of heat shock proteins is a normal defence response to cellular stress. The synthesis of hsp can be produced under several stress conditions: alcohol, heavy metals, oxidative stress, osmotic pressure change, toxic chemicals, pore formations, virus infection and exposure to low-frequency EMF, which has been shown to produce increase of hsp-27, hsp-70 and hsp-90 protein expression. Hsp proteins are short lasting and the differences between sham and exposed cells are no longer detected 1 h and 4 h after the end of RF irradiation period (Pipkin et al., 1999; Leszczynski et al., 2002). EMF exposure has been observed to cause only transient effects, and long exposure times (hours, days) could result in a kind of adaptation, so that small changes may not be detected. However, chronic expression of hsps is known to induce or promote oncogenesis, metastasis and/or resistance to anticancer drugs. We have got increased hsp protein expression in control samples and also in samples under exposure to pulsed MW for 24 h. It could be considered that those astrocytoma cells are able to continuously express hsp-70 protein with antiapoptotic effect. Of interest for new research will be to quantify (Western blot method) the amount of hsp protein in control vs. treated samples to check if exposure to pulsed MW could induce increased anti-apoptotic effect in our experimental conditions.

It is worthwhile to consider the positive reaction for Bcl-2 protein also observed in 100% of control and exposed astrocytoma cell samples. The Bcl-2 protein family is involved in the pathway initiated by trophic factors, which operate through the activation of a Ca^{2+}-dependent-antioxidant metabolic pathway, acting in turn as anti-apoptotic protein by preventing the release of cytochrome *c* from mitochondria and blocking the caspase cascade. Through this pathway Bcl-2 protein could also be involved in the homeostasis of normal cells, and an over-expression has been described in epithelial, endocrine and nervous tissues. But in astrocytoma cells it could also be considered that these antiapoptotic proteins were constitutively expressed. Bcl-2 proteins expression observed after 24 h exposed astrocytoma samples, together with negative results obtained for expression of Bax, proapoptotic-protein, is in favour of the up-regulation of antiapoptotic protein in our samples. To decide whether pulsed MW increases the expression of this antiapoptotic protein requires the quantification (Western blot method) of Bcl-2 in control vs. exposed astrocytoma cells.

Considering the interaction mechanisms we have shown that neuron electrophysiological activity modification by static and alternating MF is promoted by a calcium dependent mechanism (Azanza and del Moral, 1994; Azanza et al., 2002). The proposed model of anisotropic superdiamagnetism and calcium liberation by a mechanism of coulomb explosion (del Moral and Azanza, 1992) explains the interaction of MF with plasma membrane and the free liberation of calcium ions bound to membranes to the cytosol. Increased intracellular calcium concentration induces stimulatory and inhibitory neurones elicited responses, this process being mimicked by caffeine, which interacts with the phosphatidylinositol-3,4,5-triphosphate pathway. It is well known that signal cascade of astrocyte apoptosis may enclose modifications of intracellular calcium concentration ($[Ca^{2+}]_i$), an interesting confluence of molecular pathways to explain the effects under exposure to EMF in two kinds of nervous system cells: neurones and astrocytes. An increase in $[Ca^{2+}]_i$, mediated by the reverse mode of Na^+-Ca^{2+} exchanger, was followed by delayed astrocyte cell death. Our future research will be devoted to studying the possible dependence of increased astrocytoma cell proliferation through an increased $[Ca^{2+}]_i$ concentration process and the implication of phosphoinositide-3-kinase (PI 3K) and activation of protein-kinase B (PKB), which induces the phosphorylation of Bad protein, increasing in turn the anti-apoptotic role of Bcl-2 protein. Both pathways play an essential role in preventing activation of proapoptotic proteins, thus promoting cell survival (Takuma et al., 2004).

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