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EXPOSURE TO 2.45 GHz MICROWAVE RADIATION PROVOKES CEREBRAL CHANGES IN INDUCTION OF HSP-90 α/β HEAT SHOCK PROTEIN IN RAT

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Abstract—Physical agents such as non-ionizing continuous-wave 2.45 GHz radiation may cause damage that alters cellular homeostasis and may trigger activation of the genes that encode heat shock proteins (HSP). We used Enzyme-Linked ImmunoSorbent Assay (ELISA) and immunohistochemistry to analyze the changes in levels of HSP-90 and its distribution in the brain of Sprague-Dawley rats, ninety minutes and twenty-four hours after acute (30min) continuous exposure to 2.45 GHz radiation in a the Gigahertz Transverse Electromagnetic (GTEM cell). In addition, we studied further indicators of neuronal insult: dark neurons, chromatin condensation and nucleus fragmentation, which were observed under optical conventional or fluorescence microscopy after DAPI staining. The cellular distribution of protein HSP-90 in the brain increased with each corresponding SAR (0.034 ± 3.10^{-3}, 0.069 ± 5.10^{-3}, 0.27 ± 21.10^{-3} W/kg), in hypothalamic nuclei, limbic cortex and somatosensorial cortex after exposure to the radiation. At twenty-four hours post-irradiation, levels of HSP-90 protein remained high in all hypothalamic nuclei for all SARs, and in the parietal cortex, except the limbic system, HSP-90 levels were lower than in non-irradiated rats, almost half the levels in rats exposed to the highest power radiation. Non-apoptotic cellular nuclei and a some dark neurons were found ninety minutes and twenty-four hours after maximal SAR exposure. The results suggest that acute exposure to electromagnetic fields triggered an imbalance in anatomical HSP-90 levels but the anti-apoptotic mechanism is probably sufficient to compensate the non-ionizing stimulus. Further studies are required to determine the regional effects of chronic electromagnetic pollution on heat shock proteins and their involvement in neurological processes and neuronal damage.

1. INTRODUCTION

In the past decade, there has been a considerable increase in the industrial and medically-related use of 2.45 GHz electromagnetic fields, with the development of Bluetooth wireless technologies [1] and rehabilitative treatments for various pathologies and types of pain [2]. As a consequence there has been increased interest in studies of electromagnetic contamination at this frequency and of related thermal [3, 4] and non thermal effects [5, 6] in biological systems.

Heat shock proteins (HSP), which act as intracellular chaperones for other proteins, are frequently used in toxicological studies as biomarkers of cell damage [7]. Studies involving experimental exposure to electromagnetic fields have revealed numerous modifications in the expression of heat shock proteins in vivo [8] and in vitro, in

cell lines [9]. HSP-90 is the most common type of heat shock protein [10] and occurs at higher levels in nerve tissues than in non nerve tissues [11] and is distributed in neurons in the limbic system, neocortex, striatus and thalamus [12, 13]. This protein acts to regulate the activity of other proteins such as steroid hormone receptors [14] kinase [15], calmodulin [16], actin [17], and tubulin [18]. Ischaemic or corticoid-induced stimuli cause an increase in HSP-90 in CA1 and CA3 as well as in the dentate gyrus and in the paraventricular nucleus of the rodent hypothalamus [19]. Other stress-related stimuli such as fever, convulsions [20], drugs (amphetamine or LSD) [21], injury and neurodegenerative diseases [22] cause modifications in the basal levels of HSP-90. An increase in HSP-90 in neuronal subpopulations [23] and/or its activation in new non neuronal glial and microglial cell populations [24] indicates participation by this protein in neuroprotective mechanisms [25], oxidative stress [26] and anti-apoptic activity [27, 28].

The present study was carried out in response to the great diversity of the results reported in previous studies as regards the levels of heat shock proteins in diverse cell populations following in vivo or in vitro exposure to electromagnetic fields, including in some cases no apparent changes [29], and in our own experience [30, 31] and that of other authors [9], important changes in the levels. We have focused the present study on the brain of rats exposed in vivo to electromagnetic fields, as the metabolic activity is more important in brain than in other tissues and the effect of radiation may be greater [32]. For this, we studied the quantitative and qualitative expression of the HSP-90 protein in different anatomical regions of the rat brain following acute, continuous exposure of Sprague-Dawley rats to 2450 MHz radiation, at different SAR, in an experimental GTEM cell. In addition, we examined some morphological lesions in the histopathological sections from these animals to look for dark neurons or DAPI-stained nuclei. The objective of the study was to determine the variability in expression of the HSP-90 protein as a biological marker in the brain, as well as regional differences and the associated relation with visible changes in cytoprotective effect of this molecular chaperone in response to non ionizing radiation.

2. MATERIAL AND METHODS

2.1. Animals

All experiments were carried out in accordance with European regulations on animal protection (Directive 86/609), the Declaration of Helsinki and/or the Guide for the Care and Use of Laboratory Animals,

as adopted and promulgated by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Santiago de Compostela.

Adult female Sprague-Dawley rats weighing 230–250 g, housed with free access to food and water in individual cages, and maintained at 22°C under a 12 : 12 h light/dark regime, were used in the study.

2.2. Experimental Design

A total of 72 female Sprague-Dawley rats were used and distributed equally between the following groups:

Group A: The rats were divided into 4 subgroups, which were each exposed to different levels of microwave radiation: 0, 1.5, 3.0 and 12 W (the first group was the non-irradiated or control group), for 30 minutes. The rats were maintained for 90 minutes then killed and perfused with fixative.

Group B: The rats were divided into 4 subgroups, which were each exposed to different levels of microwave radiation: 0, 1.5, 3.0 and 12 W (the first group was the non-irradiated or control group), for 30 minutes. The rats were maintained for 24 hours then killed and perfused with fixative.

2.3. Microwave Radiation in a GTEM Cell. Calculation of Specific Absorption Rates

2.3.1. A. Description of the Experimental Radiation System

The power delivered by such a signal is controlled during the radiating procedure. The output coming from amplifier (AMP) was connected to the directional coupler (DC) in order to feed the Gigahertz Transverse Electromagnetic (GTEM 250 model of Schaffner, 1.25 m × 0.65 m × 0.45 m) cell where the irradiated rat (R) was located, with the animal conveniently placed in a region within which the maximum field

Table 1. Distribution of groups in relation to irradiation power and time post irradiation. (Exposure time was 30 minutes for all irradiated groups).

Experimental Groups	POWER/ TIME POST-IRRADIATION			
Group A	0 W/90 min	1.5 W/90 min	3 W/90 min	12 W/90 min
Group B	0 W/24 h	1.5 W/24 h	3 W/24 h	12 W/24 h

uniformity is guaranteed [33]. The rat was immobilized by means of a methacrylate holder (MH). The DC allows measurement of the input power (PIN) and reflected power (PREF) values. The PIN was read in a power meter (PM), which also monitored the purity of the input signal. On the other hand, the PREF value was obtained directly by means of a spectrum analyzer (SA), (see Figure 1). All the measurement instrumentation are certified and calibrated by Agilent Technologies. With this exposure setup good field uniformity is obtained and the behavior of a traveler wave is simulated.

2.3.2. Description of the Numerical Simulations

The values of the specific absorption rate (SAR) were estimated with the aid of SEM-CAD X [34], an FDTD-based software tool. For this purpose, a Sprague-Dawley numerical (voxel) phantom (model R8 [34]), weighing 198.3 g and composed of 60 different tissues assembled by slices 1.15 mm thick (tissue morphologies obtained by magnetic resonance images) was used. The numerical phantom was radiated by a plane wave impinging over its left zone, with the magnetic field **H** parallel to its main axis (see Figure 1). The field value E was specified by considering Eq. (1):

$$E = \sqrt{Z_0 P/(h^2 \zeta)} \tag{1}$$

where, h is the septum height in the exposure zone (position of the MH), P_{TR} is the input power on the GTEM cell, $P_{TR} = P_{IN} - P_{REF}$, $Z_0 = 50\,\Omega$ the input impedance of the cell, and ζ is coefficient which depend of the ripple of the field in the position el MH, is considerated 2 [35].

The simulations were performed at 2.45 GHz (by use of a desktop PC with an Intel Core 2 Quad processor working at 2.40 GHz, and with 4 GB RAM available); the corresponding grid positions composed of 6.762.000 voxels (minimum grid step of 0.7 mm and maximum grid step of 3 mm), for computation time of 3.5 hours.

The SARs were estimated by application of a correction factor to the values obtained from the numerical simulations, in proportion to the ratio between the weight of the model rat and the weights of the experimental rats, as specified by the following expression:

$$SAR_E = SAR_S \times W_S/W_E \tag{2}$$

where SAR_E is the estimated value of the experimental SAR, SAR_S the SAR obtained during the simulation, $W_S = 198.3$ [g] the weight of the model rat (see above), and W_E [g] the weight of the experimental rat.

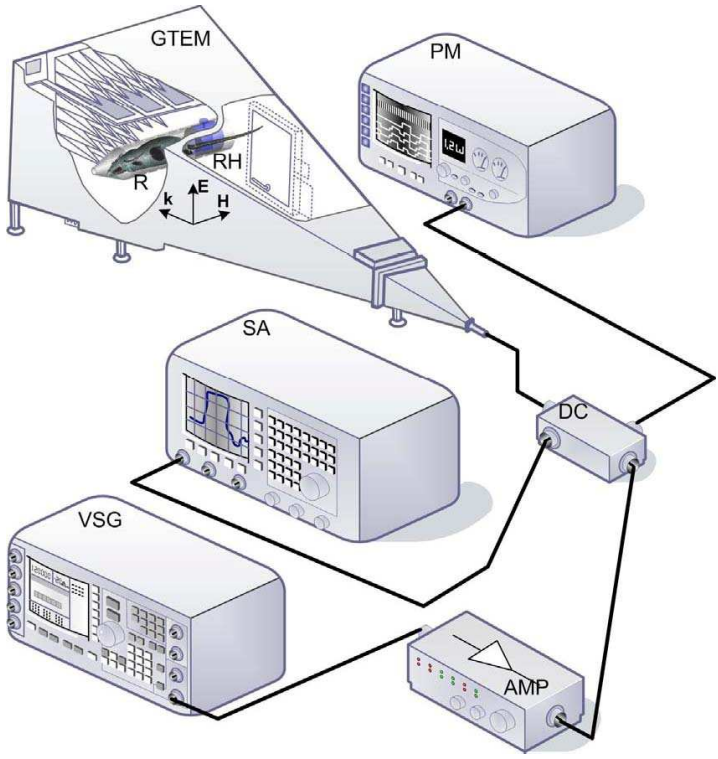


Figure 1. Illustration of the experimental system used. GTEM cell (Schaffner 250 irrad-iation chamber); VSG: Vector Signal Generator (Agilent E4438C: 250 kHz–4 GHz); AMP: Amplifier (Aethercomm 0.8-3.2-10); DC: Directional Coupler (NARDA 3282B-30: 800–4000 MHz); SA: Spectrum Analyzer (Agilent E4407B: 9 kHz–26.5 GHz); PM: Power Meter (Agilent E4418B); RH: Rat Holder.

2.4. Tissue Extraction and Preparation of Cell Extracts

After exposure to radiation, the rats were maintained for 90 minutes (Group A) or 24 hours (Group B). The animals were then killed by overdose of inhaled diethyl ether. Tissues from different areas of the brain: somatosensory cortex, limbic cortex, hippocampus and hypothalamus, were then dissected out under a stereomicroscope (Nikon Eclipse CFI60). The tissue samples were added to 500 µl of phosphate buffer (PBS; 0.015 M phosphate buffer, 0.15 M NaCl, pH 7.2) containing 0.1 mM pepstatin A, 0.02 mM *N*-(trans-epoxysuccinyl)-L-leucine 4 guanidinobutylamide (E-64), 1 mM phenyl-methanesulfonyl fluoride (PMSF) and 2 mM ethylenediaminetetraacetic acid (EDTA) as pro-

tease inhibitors (all from Sigma-Aldrich. The samples were disaggregated and homogenized in a Polytron tissue homogenizer (Kinematica AG, Littau, Luzern, Switzerland) at 35,000 rpm for 5 min on ice, and finally lysed ultrasonically in a Branson W-250 sonifier (Branson Ultrasonic Corporation, USA) with 5 cycles of sonication of 10 pulses each with a 50% duty cycle output. The whole process was performed on ice. The lysate obtained was centrifuged at 15,000 g for 10 minutes at 4°C. The supernatant was then aliquoted and frozen at −80°C until use.

2.5. Enzyme-Linked ImmunoSorbent Assay (ELISA)

2.5.1. Determination of Protein Concentration

Protein concentration in the tissue extracts was determined by the Bradford method with a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Germany), with BSA (Sigma-Aldrich, Spain) as standard.

2.5.2. Enzyme-Linked ImmunoSorbent ELISA Assay

An ELISA test was used to detect the rat heat shock protein HSP-90. One µg aliquots of protein extract in 100 µl of carbonate-bicarbonate buffer (pH 9.6) were added to 96-well ELISA plates (IWAKI) and incubated overnight at 4°C. The plates were then washed three times with TBS (50 mM Tris, 0.15 M NaCl, pH 7.4), blocked for 1 h with TBS containing 0.2% Tween 20 (TBS-T1), 5% non-fat dry milk, incubated for 2 h at 37°C with 100 µl of a 1 : 100 dilution (in TBS-T1 containing 1% non-fat dry milk) of a murine monoclonal antibody anti-HSP 90 (Santa Cruz Biotechnology, USA) at 1 : 100 dilution, and washed five times with TBS containing 0.05% Tween 20. For detection of mouse immunoglobulins (Ig), 100 µl of the polyclonal antibody peroxidase-conjugated rabbit anti-mouse Ig (Dako) was diluted 1 : 1000 in TBS-T1 and incubated for 1 h at 37°C. The wells were washed five times in TBS, then 100 µl of *o*-phenylendiamine (OPD; Sigma) at a concentration of 0.04% in phosphate-citrate buffer pH 5.0 containing 0.001% H_2O_2, was added to each well. The reaction was stopped after 20 min, with 3 N H_2SO_4, and the optical density (OD) was measured at 492 nm in an ELISA reader (Titertek Multiscan, Flow Laboratories).

2.6. Processing and Staining of Brain Tissue

2.6.1. Perfusion and Processing of Brain

Ninety minutes post irradiation, the rats were killed by overdose of pentobarbital and prefixed by transcardial perfusion with physiological saline followed by formaldehyde in phosphate buffer (pH 7.4). The brains were then immediately removed from the skull, placed in fresh fixative solution for 4 h at 4°C, and transferred to phosphate-buffered saline (PBS) for 12 h at the same temperature, after which transverse 50 μm sections were cut with a vibrotome, collected in PBS and processed as follows.

2.6.2. Immunohistochemistry

The free-floating sections were pretreated for 1 h with normal rabbit serum and Triton X-100 (respectively 10% and 0.25% in 0.02% potassium-phosphate-buffered saline (KPBS), and then exposed overnight at room temperature to polyclonal rabbit anti- HSP-90 antibody (from Santa Cruz Biotecnology) (1 : 1000 in KPBS), rinsed with KPBS, incubated for 1 h with biotinylated goat anti-rabbit antibody (from Vector, Burlingame, CA, USA) (1 : 500 in KPBS), rinsed three times with KPBS, incubated for 30 min with an avidin-biotin-peroxidase complex prepared according to the manufacturer's instructions (DAKO, Glostrup, Denmark), and labelled with 3,3-diaminobenzidine (DAB) in imidazole-HCl-buffered H_2O_2 solution (DAKO).

2.6.3. Histopathological Examination: Toluidine Blue

In order to study dark neurons, some of the 50 μm sections were stained for RNA/DNA with toluidine blue. The toluidine blue-O Toluidine blue-O (Panreac) stain was prepared by additing 1 g dye to 100 ml distilled water and filtering this solution, and then adding 100 mg/100 ml sodium borate to the toluidine blue-O solution. The staining procedure was as follows. The sections were rehydrated through a series of alcohol to distilled water, then washed with tap water for 3 min. The sections were then incubated in toluidine blue-O solution for 2 min, and washed with tap water for 5 min. The slides were incubated in a 3 : 1 solution of 100% ethanol and glacial acetic acid for 10 min. Finally the slides were incubated in ammonium molybdate (4% in water), then immediately washed in tap water for 5 min, rapidly dehydrated in alcohol, cleared with xylene, and mounted with Entellan (Panreac).

The occurrence of dark neurons was judged semiquantitatively as 0 (no or occasional dark neurons) 1, (moderate occurrence of dark neurons), or 2 (abundant occurrence). The sections were examined by optical microscopy with the analyst blind to the treatments.

2.6.4. DAPI Staining of Nuclei

Sections were washed with PBS, fixed in methanol for 5 min, washed again with PBS and incubated with 0.8 mg/ml of 40, 6-diamidine-20-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) in PBS for 15 min at room temperature.

After several washes in PBS, the slides were mounted in PBS/glycerine. DAPI staining was visualized by fluorescence microscopy.

2.7. Quantification and Statistical Analysis

- The results of ELISA shown in the text and figures are expressed as means ± SEM; significant differences ($P < 0.05$) were determined by one-way analysis of variance (ANOVA) (followed by the Tukey-Kramer test for multiple comparisons).

- The results of SAR shown in Table 3 are expressed as means ± DEM; significant differences ($P < 0.05$) were determined by one-way analysis of variance (ANOVA) (followed by the Holm-Sidak test for multiple comparisons).

- The numbers of HSP-90 positive cells were counted by investigators who were blind to exposure conditions. For each rat, three or four sections were taken from each of the following areas for examination of HSP-90 expression in localized regions: a) cortical areas: somatosensorial or parietal cortex, limbic or entorhinal cortex; b) hippocampal structures, dentate gyrus (DG) and CA1, and c) hypothalamus nuclei, paraventricular nuclei (Par), arcuate nucleus (Ar) and the Lateral Hypothalamic (LH) area. The transverse sections 50 µm through the rat brain were measured at the following interaural levels: 9.2 mm in parietal cortex, 6.88 mm in hypothalamic nuclei (Par,Ar,LH), 5.7 mm in hippocampal areas (GD and CA1) and 4.2 mm in entorhinal cortex [36]. In each area, HSP-90 positive cells were counted in a 0.32×0.24 mm field magnified 20× in a Nikon Eclipse E200 microscope connected to a computer running morphometric software (from Kappa, Monrovia, CA, USA). Counts of cells per field in each of the areas were expressed as means for individual animals or experiments ± SEM per group. The significance of between-group differences in HSP-90 positive cell counts was assessed by two way ANOVA, considering separately for each area: Power of

radiation (0, 1.5, 3,12 W) x Time post-irradiation (90 minutes or 24 hours); differences between groups were considered significant at $P < 0.05$ after Bonferroni correction. The histopathological results for dark neurons and DAPI nuclei were analysed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test for multiple comparisons.

3. RESULTS

3.1. SAR

The mean power absorbed by rats in the four groups, estimated by equation 2, as well as the average weight, mean SAR ± DEM in brain and body, and peak SAR ± DEM averaged for 1 g of brain or body and 2.45 GHz frequency in a Shaffner GTEM cell. One way ANOVA by different levels of radiation by mean SAR in brain or body and peak SAR averaged for 1gr of brain or body, showed significant differences in all SAR values ($p < 0.001$) are shown in Figure 2 and Table 2. Note, that the increases of SAR values (mean and peak) are directly proportional to the input power for each subgroup.

3.2. Levels of Protein HSP-90 Determined by ELISA

- Group A (rats killed and fixed 90 minutes after completion of irradiation): The amount of HSP-90 expressed at the level of the **somatosensorial cortex** in rats exposed to SAR radiation at 0.034 ± 3.10^{-3} W/kg and 0.069 ± 5.10^{-3} W/kg was not significantly different from that in non irradiated rats. The levels of the protein in rats irradiated at maximum SAR 0.27 ± 21.10^{-3} W/kg were

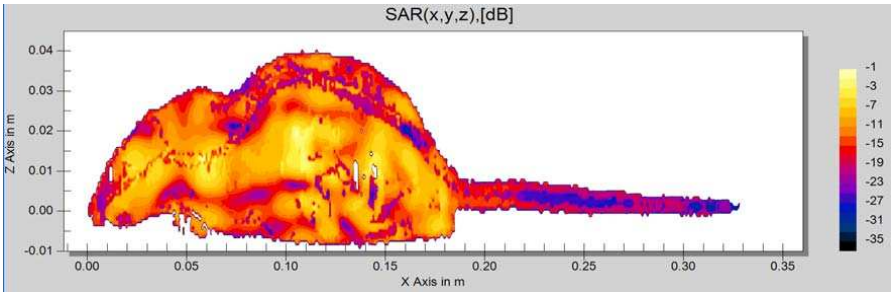


Figure 2. Distibution of local SARs in the phantom rat "exposed" to 12 W of 2.45 GHz, in the plane $X = 0.33$ m.

Table 2. SAR values in brain and bodies of the experimental rats calculated from the power (P) and electrical field (E).

	EXPERIMENTAL MEASUREMENT OF RADIATION			
	Mean SAR in brain	**Peak SAR in brain**	**Mean SAR in body**	**Peak SAR in body**
Subgroup B $P = 1.5\,\mathrm{W}$ $E = 28.48\,\mathrm{V/m}$	0.034 ± 3.10^{-3}	0.042 ± 4.10^{-3}	0.020 ± 20.10^{-4}	0.099 ± 10.10^{-3}
Subgroup C $P = 3\,\mathrm{W}$ $E = 40.28\,\mathrm{V/m}$	0.069 ± 5.10^{-3}	0.086 ± 7.10^{-3}	0.041 ± 34.10^{-4}	0.203 ± 16.10^{-3}
Subgroup D $P = 12\,\mathrm{W}$ $E = 80.56\,\mathrm{V/m}$	0.27 ± 21.10^{-3}	0.337 ± 26.10^{-3}	0.160 ± 120.10^{-4}	0.791 ± 61.10^{-3}

significantly different from those in the non irradiated group ($p < 0.05$) (Figure 3(a)).

At the level of the **limbic cortex** (only with SAR $0.27 \pm 21.10^{-3}\,\mathrm{W/kg}$) resulted in a significant increase in protein levels with respect to the non irradiated animals ($p < 0.01$), and for the other levels of radiation ($0.034 \pm 3.10^{-3}\,\mathrm{W/kg}$ and $0.069 \pm 5.10^{-3}\,\mathrm{W/kg}$) there were no significant differences (Figure 3(b)).

In the **hippocampus** the levels of HSP-90 positive cells in irradiated and non irradiated rats were not significantly different (Figure 3(c)).

At the level of the **hypothalamus**, the levels of the protein in rats exposed to radiation at SAR $0.034 \pm 3.10^{-3}\,\mathrm{W/kg}$ and $0.069 \pm 5.10^{-3}\,\mathrm{W/kg}$ were significantly higher than in non irradiated rats ($p < 0.05$), and in rats exposed to radiation at SAR 0.27 ± 21.10^{-3} the differences were even greater ($p < 0.01$) (Figure 3(d)).

- Group B (rats killed 24 h after completion of irradiation): At the level of the **somatosensorial cortex** the levels of HSP-90 in the subgroups of rats exposed to radiation at SAR values 0.069 ± 5.10^{-3} and 0.27 ± 21.10^{-3} were significantly higher than in non irradiated rats ($p < 0.05$) (Figure 3(a)).

In the **limbic cortex**, the levels of the protein were significantly lower in rats exposed to all levels of radiation than in non irradiated rats ($p < 0.01$) (Figure 3(b)).

In the **hippocampus**, the levels of HSP-90 were lower in rats exposed radiation at $0.069 \pm 5.10^{-3}\,\mathrm{W/kg}$ and 0.27 ± 21.10^{-3} than in

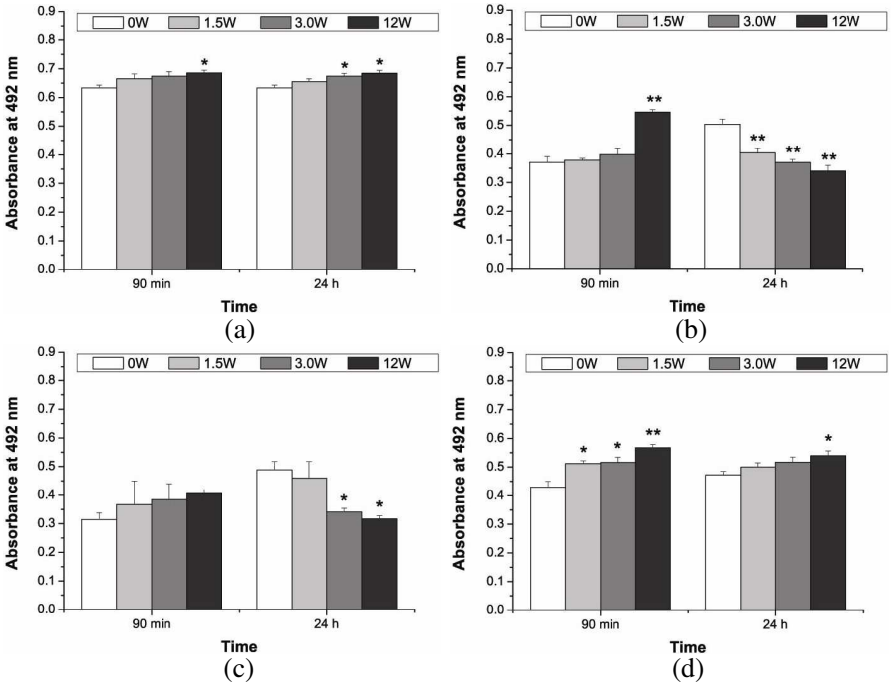


Figure 3. Histograms (a) and (b) show the amounts of HSP-90 detected by ELISA (expressed as absorbance) in the cerebral cortex: (a) In the somatosensorial cortex, (parietal cortex), (b) in the limbic cortex, (entorhinal cortex). Histograms (c) and (d) illustrate the amount of HSP-90 detected by ELISA in the subcortical regions: (c) In the hippocampus, (d) in the hypothalamus. Values are means ± S.E. * and ** indicate significant differences established by a one-way ANOVA and posterior Tukey-Kramer test for multiple comparisons.

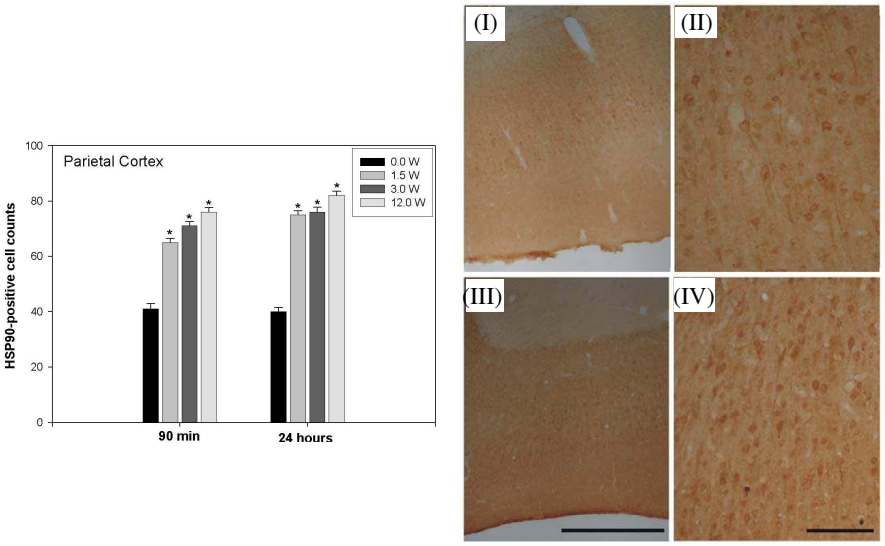
non irradiated rats ($p < 0.05$) (Figure 3(c)).

In the **hypothalamus** the levels of HSP-90 positive cells in rats exposed to radiation at 0.27 ± 21.10^{-3} W/kg were significantly higher than in non irradiated rats ($p < 0.05$) (see Figure 3(d)).

3.3. Distribution of HSP-90 Determined by Immunohistochemical Techniques

The distribution of HSP-90 expression as measured by counting the subpopulations of HSP-90 positive cells 90 minutes after the end of the exposure to radiation was different in rats subjected to maximum levels of SAR 0.27 ± 21.10^{-3} W/kg than in non irradiated rats. As regards the

groups of rats exposed to the other levels of radiation, the results were variable. The rats maintained for 24 h after being irradiated all showed differences with respect to non-irradiated ($p < 0.05$), although much of the variability in the cell counts depended on the type of brain tissue considered. The distribution, cell counts and the statistical analysis of the values obtained for the different cell populations studied in the different tissues are described as follows.



(a) (b)

Figure 4. (a) Histogram showing the number of HSP-90 positive cells in the parietal cortex. Significant differences (at $p < 0.05$) were established by two-way ANOVA and posterior Bonferroni test for multiple comparisons. Values are cells/mm^2 means ± S.E. * indicate significant differences respect non irradiated animals. (b) Photomicrograph showing more intense staining for HSP-90 in the cells of the parietal cortex of rats exposed to microwave radiation at 0.27 ± 21.10^{-3} W/kg ((III) and (IV)) compared with non-irradiated animals ((I) and (II)), both groups fixed at 90 minutes post-irradiation. Calibration bars**: (a, c)** = 500 µm (**b, d)** = 50 µm.

- At the level of the **somatosensorial cortex (parietal cortex)** there were significant differences as regards the radiation factor between the HSP-90 positive cells in layer 3 constituted by pyramidal neurons) corresponding the different levels of radiation and non irradiated animals ($p < 0.007$) (see Figures 4(a) and 4(b)). Equally, the duration of the post-irradiation period had a positive effect on establishing significant differences between the groups ($p < 0.05$) in

this area of the brain.

The cell counts corresponding to exposure SAR value 0.27 ± 21.10^{-3} W/kg reached high levels and there were significant differences between the groups of rats maintained for 90 min and 24 h post irradiation ($p < 0.001$) (see Table 3).

Table 3. Summary of the mean values ± standard errors of the counts of HSP-90 positive cells in the different brain tissues, taking into the factors Power x Time post-irradiation) 1, 2, 3, 4 indicate significant different in relation to the radiation power (1 = 0 W, 2 = 1.5 W, 3 = 3 W and 4 = 12 W) during acute (30 min) exposure. * Indicates significant differences between the groups 90 min and 24 h post-irradiation for the different powers.

Counts of HSP-90 positive cells								
Anatomical Areas	**Time post-irradiation**							
	90min				**24 h**			
	0 W	**1.5 W**	**3 W**	**12 W**	**0 W**	**1.5 W**	**3 W**	**12 W**
Cortex								
Parietal cortex	41±4^{2,3,4}	65±2*^{1,4}	71±2*^{1}	76±3*^{1,2}	40±3^{2,3,4}	75±2*^{1,4}	76±2*^{1}	82±3*^{1,2}
Entorhinal cortex	68± 5*^{2,3,4}	80±5^{4}	82± 5^{4}	89± 6*^{1,2,3}	97±4*^{2,3,4}	81±4^{1,4}	80± 4^{1,4}	47± 6*^{1,2,3}
Hippocampus								
DG	44±3*	45±3*	53 ±3	52±3*	68±3*^{3,4}	69±3*^{3,4}	56±3^{1,4}	24±3*^{1,2,3}
CA1	57±4	64±4*^{4}	67±4*^{4}	52±4*^{3}	51±4	51±4*	44±4*	35±5*
Hypothalamus								
Pa	33±8*^{2,3,4}	24±7*^{4}	23±6*^{4}	63±5*^{1,2,3}	83±6*^{2,3,4}	98± 5*^{4}	100±5*^{4}	128±7*^{1,2,3}
Arc	39±5*^{2,3,4}	62±4*^{1,4}	64±3*^{1,4}	86±3*^{1,2,3,}	85±3*^{4}	85±3*^{4}	80±3*^{4}	99 ±3*^{1,2,3}
LH	50±4^{2,3,4}	70± 3^{1,4}	65±3*^{1,4}	89±3*^{1,2,3,}	56±4^{2,3,4}	68± 4^{1,4}	77±5*^{1}	85± 3*^{1,2,3,}

In this area of the cortex, there were significant differences in the numbers of HSP-90 positive cells between all irradiated groups — maintained for 90 min and 24 h post-irradiation — except for comparisons between SAR values 0.069 ± 5.10^{-3} and 0.034 ± 3.10^{-3} W/kg or 0.27 ± 21.10^{-3} W/kg and 0.069 ± 5.10^{-3} ($p = 0.093$ or $p = 1$ and $p = 0.202$ or $p = 0.076$) (see Table 3).

- In the **limbic cortex (entorhinal cortex)** the numbers

of HSP-90 positive cells in rats exposed to SAR values 0.27 ± 21.10^{-3} W/kg (and maintained for 90 min post-irradiation) were significantly different from the numbers of HSP-cells in rats exposed to the other levels of radiation ($p < 0.001$) (see Table 3). There were no significant differences in the number of these cells between non irradiated rats and those exposed SAR values 0.034 ± 3.10^{-3} W/kg and 0.069 ± 5.10^{-3} W/kg ($p = 0.275$ and $p = 0.643$) (see Figure 5(a)). There were significant differences in the number of these cells between those exposed and non irradiated rats SAR value 0.27 ± 21.10^{-3} W/kg ($p < 0.001$) (see Figures 5(a) and 5(b)).

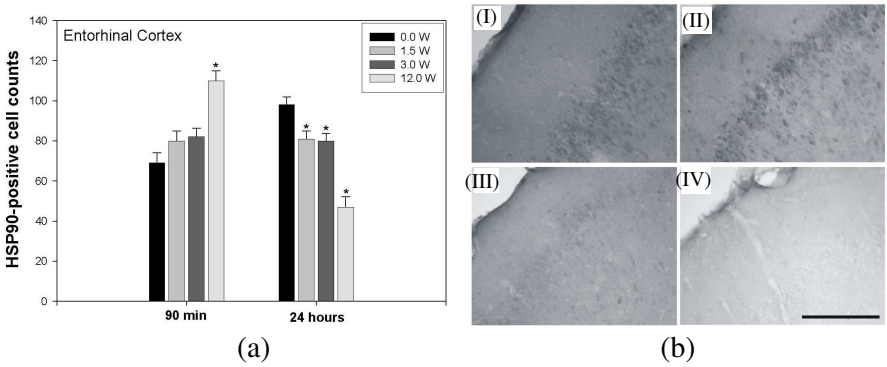


Figure 5. (a) Histogram showing the number of HSP-90 positive cells in the entorhinal cortex. Significant differences (at $p < 0.05$) were established by two-way ANOVA and posterior Bonferroni test for multiple comparisons. Values are cells/mm^2 means ± S.E. * indicate significant differences respect non irradiated animals. (b) Photomicrograph showing the more intense staining for HSP-90 in the cells of the entorhinal cortex of rats exposed to microwave radiation at 0.27 ± 21.10^{-3} W/kg (II) compared with non-irradiated animals, both groups fixed at 90 minutes post-irradiation (I). However, there is a large decrease in the intensity of staining 24 h post irradiation between non-irradiated animals (III) and those exposed to radiation at 0.27 ± 21.10^{-3} W/kg (IV). Calibration bar: 100 µm.

The number of HSP-90 positive cells was significantly different in rats exposed to radiation at SAR values 0.27 ± 21.10^{-3} W/kg then maintained for 24 h post-irradiation than in those maintained for 90 minutes post irradiation ($p < 0.001$) (see Table 3). For the other levels 0.034 ± 3.10^{-3} W/kg and 0.069 ± 5.10^{-3} W/kg the number of cells was not significantly different from those in the groups maintained for 90 min post-irradiation ($p = 0.93$ and $p = 0.693$). However, there were

also significant differences between non irradiated group and the groups exposed to radiation to SAR 0.069 ± 5.10^{-3} W/kg ($p < 0.007$) and 0.034 ± 3.10^{-3} W/kg ($p < 0.011$) and 0.27 ± 21.10^{-3} W/kg ($p < 0.001$) (see Figures 5(a) and 5(b)).

The differences between the cell counts corresponding to rats exposed to radiation at 0.27 ± 21.10^{-3} W/kg and those exposed to the other levels were highly significant ($p < 0.001$ for all comparisons) (see Table 3).

- In the **Hippocampus** (in GD and CA1) and considering groups maintained for both 90 min and 24 h post-irradiation, there were significant differences between those rats exposed to SAR values 0.27 ± 21.10^{-3} W/kg radiation and the other groups ($p < 0.001$) (see Table 3). There were significant differences between the groups maintained for 90 min and 24 h post irradiation for all levels of exposure ($p < 0.05$).

Ninety minutes post-irradiation there were no significant differences in the cell counts between the different groups in either GD or in CA1 (see Table 3).

In the groups irradiated at SAR values 0.27 ± 21.10^{-3} W/kg, the number of HSP-90 positive cells was significantly lower 24 hours post irradiation than 90 minutes post-irradiation ($p < 0.001$) both in the GD and in CA1(see Table 3). Likewise, in the GD, the number of HSP-90 positive cells in the group of rats irradiated at SAR values 0.27 ± 21.10^{-3} W/kg differed from those in the other three groups ($p < 0.001$) (see Table 3, Figures 5(a) and 5(c)); with significant differences also between the group irradiated SAR values 0.069 ± 5.10^{-3} and the group irradiated at 0.034 ± 3.10^{-3} W/kg and the non irradiated group ($p = 0.013$ and $p = 0.046$, respectively) (see Figure 6(a)). However, there were no significant differences among the 4 groups at the level of the CA1 (see Figures 6(b), 6(c) and Table 3).

- In the **Hypothalamus**, ninety minutes and twenty-four hours post-irradiation the cell counts in the paraventricular nucleus (Par) revealed maximal increases in expression of HSP-90 in those animals exposed to SAR values 0.27 ± 21.10^{-3} radiation, and were significantly higher than in non irradiated rats ($p < 0.012$ or < 0.001) and those exposed to radiation at SAR values 0.034 ± 3.10^{-3} W/kg and 0.069 ± 5.10^{-3} W/kg ($p < 0.001$ or < 0.003 or < 0.007). The HSP-90 positive cell counts were also significantly different for the different SAR values (0.034 ± 3.10^{-3}, 0.069 ± 5.10^{-3}, 0.27 ± 21.10^{-3} W/kg) compared for 90 min and 24 h post-irradiation ($p < 0.001$) with important increases for the paraventricular nucleus 24 h post-irradiation (see Table 3 and Figures 7(a) and 7(d)).

In the arcuate nucleus, the HSP-90 positive cells were maximal at

SAR values 0.27 ± 21.10^{-3} W/kg. For all SARs, there were significant differences between cell corresponding to post-irradiation periods of 90min and 24 h ($p < 0.003$ in all cases). For ninenty minutes post-

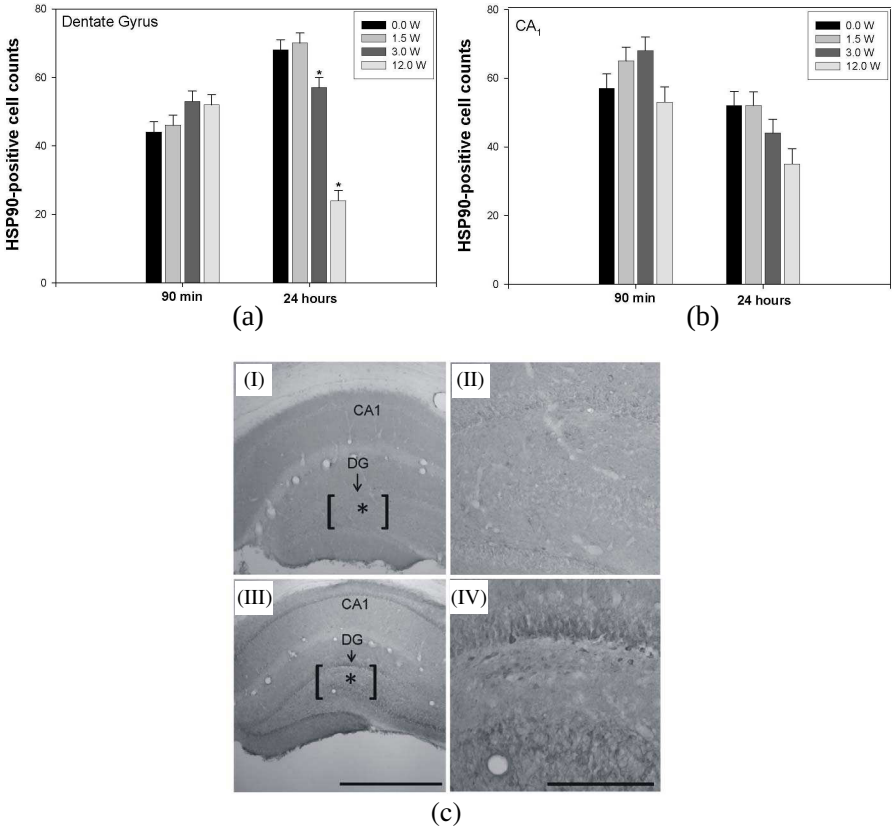


Figure 6. (a) and (b) Histogram showing the number of HSP-90 positive cells in the hippocampus (a) Gyrus dentate and (b) CA1. Significant differences (at $p < 0.05$) were established by two-way ANOVA and posterior Bonferroni test for multiple comparisons. Values are cells/mm^2 means ± S.E. * indicate significant differences respect non irradiated animals. (c) Photomicrograph showing low intensity of staining for HSP-90, in the gyrus dentate (GD) and CA1 ((I) and (III)) in rats exposed to microwave radiation at 0.27 ± 21.10^{-3} W/kg, in comparison with non-irradiated animals, detail of GD in rats exposed to microwave radiation at 0.27 ± 21.10^{-3} W/kg (II) and non-irradiated (IV) 24 h post-irradiation. Calibration bars: **(a, c)**= 500 µm (**b, d)** = 100 µm.

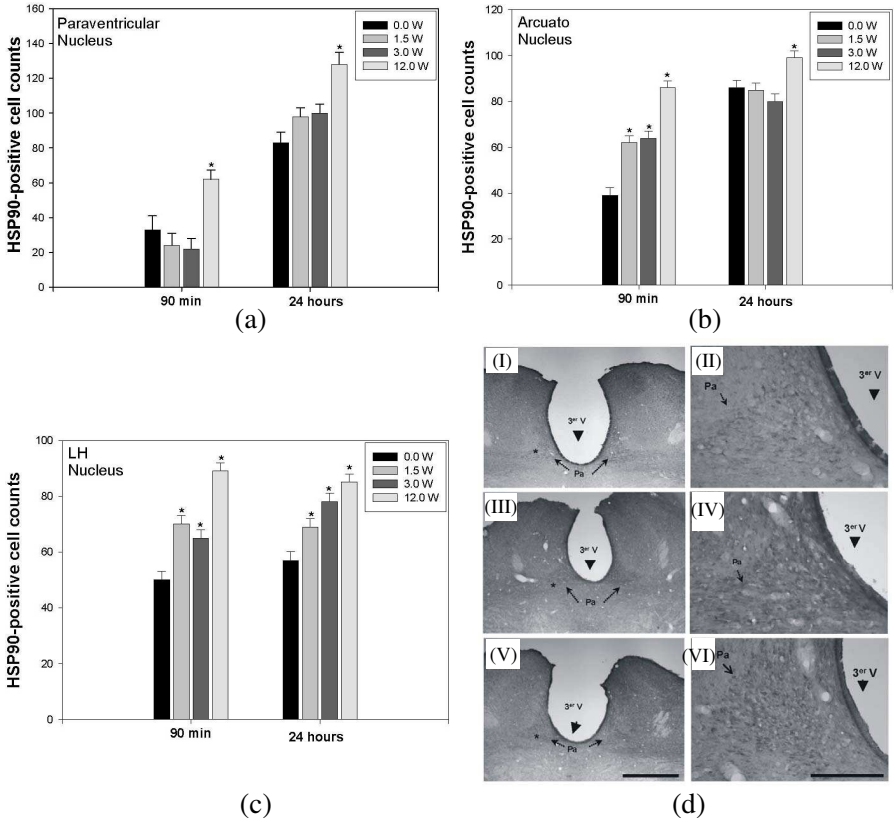


Figure 7. (a) Histogram showing HSP-90 positive cells in the paraventricular nucleus (Pa), (b) the arcuate nucleus (Ar), (c) the Lateral Hypothalamic nucleus (LH). Significant differences (at $p < 0.05$) were established by two-way ANOVA and posterior Bonferroni test for multiple comparisons. Values are cells/mm^2 means $\pm$ S.E. * indicate significant differences respect non irradiated animals. (d) Photomicrograph showing the different intensities of staining for HSP-90, in the Pa nucleus of non-irradiated rats ((I), (II)), and rats exposed to microwave radiation at 0.034 ± 3.10^{-3} W/kg ((III), (IV)) and 0.27 ± 21.10^{-3} W/kg ((V), (VI)), and fixed 90minutes post-irradiation. Calibration bars: **(a, c, e)** = 500 µm (**b, d, f)** = 100 µm.

irradiation there were significant differences in cell counts for the different SARs and the non irradiated rats ($p < 0.001$). However, twenty-four hours post-irradiation, there were significant differences alone in cell counts at SAR values 0.27 ± 21.10^{-3} W/kg and non

irradiated rats (see Figure 7(b)). In addition, there were significant differences between HSP-90 positive cells for the different levels of radiation ($p < 0.001$), except for SARs values 0.034 ± 3.10^{-3} and 0.069 ± 5.10^{-3} W/kg, for which there were no significant differences ($p = 1$) (see Table 3).

In LH the maximal numbers of HSP-90 positive cells also corresponded to SAR values of 0.27 ± 21.10^{-3} W/kg. For 90 min post-irradiation, the cell counts for all groups exposed to radiation were significantly different from those corresponding to non irradiated rats ($p < 0.001$) (see Figure 7(c)), and there were also significant differences between the groups exposed to the different levels ($p < 0.001$) except when comparing SARs of 0.034 ± 3.10^{-3} and 0.069 ± 5.10^{-3} W/kg, which were not significantly different ($p = 1$). The number of HSP-90 positive cells in rats maintained for 24 h post-irradiation were significantly different from those maintained for 90 min only in those groups irradiated at SAR values 0.069 ± 5.10^{-3} W/kg ($p < 0.003$). Twenty four hours post-irradiation, the cell counts corresponding to all SARs were significantly different from those corresponding to the non irradiated animals ($p < 0.05$ in all cases), although significant differences between the irradiated animals were only apparent between the groups exposed to the maximum and minimum levels ($p < 0.001$) of radiation ($p < 0.001$) (see Figure 7(c) and Table 3).

3.4. Histopathology

The histopathological examinations revealed scattered dark neurons, which were shrunken, dark stained, homogeneous and with loss of discernible internal structures. Some dark neurons were observed in all locations when the animals were exposed at maximal SAR (0.27 ± 21.10^{-3} W/kg). Specifically, we studied the somatosensorial, limbic cortex, hippocampus and hypothalamus regions, which displayed a moderate occurrence of dark neurons. Non-parametric test for the groups revealed significant differences between the numbers of dark neurons in animals 0W and 12 W, 90 m and 24 hours post exposure ($p < 0.05$). The results regarding SAR radiation and evaluation of dark neurons expressed in number of animals per group are described in Table 4 (see Figure 8).

3.5. DAPI Staining of Nuclei

The nuclear morphology of cells was examined by fluorescence microscopy for signs of apoptosis. No morphological changes, such as chromatin condensation or nucleus fragmentation, were observed under fluorescence microscopy after DAPI staining of sections from irradiated (in any groups and SAR) or non irradiated animals.

Table 4. Distribution of histopathological scores for the occurrence of dark neurons as a function of SAR exposure to electromagnetic fields (EMF).

SAR (W/kg)	Grade 0	Grade I	Grade II
0.0	11	1	-
0.034	10	2	-
0.069	7	5	-
0.27	4	7	1

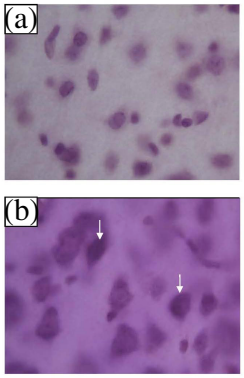


Figure 8. The photomicrograph shows pyramidal neurons in the somatosensorial cortex. (a) Grade 0. (b) Grade I, histopathological appearance of parietal cortex. *Struken nerve cells are dark neurons, indicated with arrows. Calibration bar: 25 μm.

4. DISCUSSION

Although various authors have associated changes in levels of expression of heat-shock proteins with exposure to non ionizing radiation [3, 9, 37], other authors of in vitro studies with embryos [38] and of in vivo studies attribute such changes to thermal stimuli. To our knowledge no in vivo studies have described changes in the levels and distribution of the heat shock protein HSP-90 in several rat brain regions analyzed after acute controlled exposure of the animals to 2.45 GHz at non thermal SAR in a GTEM cell (see Tables 2, 3).

In this study, we found that at 90 minutes post-irradiation, there was an increase in cellular HSP-90 and the same regional pattern of distribution was observed as in the cerebral cortex of non-

irradiated rats [12] as also observed with other stimuli such as hyperthermia [39, 40] anoxia [41] and alcohol [42]. As the cortex is the uppermost part of the brain structure, it may initially be the most strongly affected [43] in relation to the energy of non-ionizing radiation that the tissue absorbs — expressed as the specific absorption rate (SAR). The amounts of HSP-90 in the cerebral cortex increase in parallel to the SARs, although they do not reach thermal levels, which are $< 0.4\,\mathrm{W/kg}$ for the whole body [44] and $< 2\,\mathrm{W/kg}$ for brain [45] (see Table 2). Therefore, it cannot be stated categorically that the mechanisms that cause effects on the heat shock protein in the cerebral cortex are thermal [46], non-thermal [8, 47, 48], or that both mechanisms act together [49].

In the present study, the subcortical levels of the intracytoplasmic protein HSP-90 were higher 90 min and 24 h post-irradiation in the hypothalamus nucleus for all SARs than the corresponding non irradiated rats; however similar levels of HSP-90 were observed in the hippocampus of rats 90 post irradiation and non-irradiated rats, and lower levels in the hippocampus of rats 24 h post-irradiation than in the corresponding non irradiated rats. This difference in the levels of the chaperone protein in the hippocampus and hypothalamus may be related to other factors such as circadian rhythms and levels of cortisol [19]. We therefore consider that the response may occur later in the hippocampus and be directly associated with the hypothalamus-hypophysis-suprarenal feedback [50] although the hypothalamus may respond to stimulus of other more general functions of under homeostatic control [51]. However, studies of the levels of corticoids following exposure to electromagnetic fields do not provide clear data, as high levels of glucocorticoids in the hippocampus [32], increased sensitivity of glucocorticoid receptors [52], as well as stabilization of the levels of ACTH and glucocorticoids [53] and a decrease and posterior recovery — four weeks after exposure [54] have been reported in response to exposure to microwave radiation. Therefore we cannot deduce if there is a direct or causal relationship between the levels of HSP-90 and the levels of stress associated with cortisol.

In recent in vivo studies at other frequencies than those here, an important degree of neuronal cortical and subcortical activation c-fos has been observed [47, 55, 56]; changes in expression of different genes, including a decrease in the expression of HSP-90, have been observed in vitro studies involving acute exposure to 2.45 GHz radiation [6], with important changes, at non-thermal SARs. Furthermore, there is a relationship between exposure to electromagnetic fields that may induce inactivation of this protein and activation of proteases

that trigger apoptosis [9], with a demonstrated decrease in the cytoprotective mechanisms [37]. In this sense, the decrease in the levels of HSP-90, 24 h after exposure to radiation at most SARs in the hippocampus and entorhinal cortex suggest that the effects of non ionizing radiation may be related to a decrease in the levels of protection provided by this protein. However we did not find any signs of apoptosis such as chromatin condensation and nucleus fragmentation in any sections of brain region at any of the SAR levels applied in this experiment. The appearance of some dark neurons throughout the nervous tissues revealed that there may be some other non enzymatic injury mechanism (via apoptosis) that provokes signs of cellular death, as already described by other authors [57]. It is likely that several parallel active programmed and passive mechanisms of cell death may be initiated by microwave radiation [58]. In this experiment the anti-apoptotic function of HSP-90 activated by microwaves radiation is sufficient for it not to cause cell death by activation of enzymatic pathways. It is known that microwaves may trigger injury in the cellular cytoplasm [59] and that HSP-90, an intracellular protein ensures the stability of different proteins [27], although the role of this chaperone is unknown in relation to necrosis.

Expression of HSP-90 depends on the stimulus [60], which leads us to suggest the possibility that the biological effects of radiation may also depend on the anatomical area under study. On one hand, the protein may activate neuroprotective mechanisms [24], which in the present study may be related to high levels of HSP-90 in the parietal cortex and the hypothalamic nucleus, and on the other hand, the decrease in levels of the chaperone protein in limbic cortex and hippocampus may indicate suppression of the cell defence function of the protein.

High levels of cytoprotection in the somatosensorial cortex and hypothalamus and an acute decrease in protection in the limbic cortex and hippocampus indicate a high degree of complexity in the cell defence mechanisms, as well as variation in the response of cell populations to microwave radiation.

5. CONCLUSIONS

A wide response in the induction of the chaperone protein HSP-90 in diverse neuronal populations studied in the rat brain indicates different degrees of sensitivity to the biological effect of non ionizing radiation. The observed decrease in the levels of the chaperone protein in the limbic regions of the cortex and hippocampus places in question the real function of this protein and the effects that it

may have during prolonged exposure. The physiological study of this biomarker of brain damage may help us to understand the implications, particularly as regards brain pathologies, of chronic and daily exposure to electromagnetic to which humans are subjected nowadays.

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