Electromagnetic fields at 2.45 GHz trigger changes in heat shock proteins 90 and 70 without altering apoptotic activity in rat thyroid gland

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Biology Open 1, 831–838
doi: 10.1242/bio.20121297
Received 12th March 2012
Accepted 7th June 2012

Summary

Non-ionizing radiation at 2.45 GHz may modify the expression of genes that codify heat shock proteins (HSP) in the thyroid gland. Using the enzyme-linked immunosorbent assay (ELISA) technique, we studied levels of HSP-90 and HSP-70. We also used hematoxilin eosin to look for evidence of lesions in the gland and applied the DAPI technique of fluorescence to search for evidence of chromatin condensation and nuclear fragmentation in the thyroid cells of adult female Sprague-Dawley rats. Fifty-four rats were individually exposed for 30 min to 2.45 GHz radiation in a Gigahertz transverse electromagnetic (GTEM) cell at different levels of non-thermal specific absorption rate (SAR), which was calculated using the finite difference time domain (FDTD) technique. Ninety minutes after radiation, HSP-90 and HSP-70 had decreased significantly ($P<0.01$) after applying a SAR of 0.046±1.10 W/Kg or 0.104±5.10^{-3} W/Kg. Twenty-four hours after radiation, HSP-90 had partially recovered and HSP-70 had recovered completely. There were few indications of lesions in the glandular structure and signs of apoptosis were negative in all radiated animals. The results suggest that acute sub-thermal radiation at 2.45 GHz may alter levels of cellular stress in rat thyroid gland without initially altering their anti-apoptotic capacity.

Key words: Apoptotic activity, Electromagnetic fields, Shock proteins, Thyroid gland

Introduction

Over the last decade, numerous scientific studies have described changes in biological parameters after interaction with electromagnetic fields (EMFs) in living organisms. People are generally and daily exposed, silently and continuously, to numerous sources of non-ionizing radiation. Due to individual susceptibility or work exposure, certain segments of the population may be more susceptible to possible adverse effects.

Numerous epidemiological and experimental studies have been carried out to evaluate the risk of cancer (Wakeford, 2004), effects on the nervous system (Regel and Achermann, 2011), hematological effects (Jin et al., 2011), effects on metabolism or the endocrine system (Karasek and Woldanska-Okonska, 2004) and effects on the general population or groups directly at risk from EMF-related exposure.

Heat shock proteins (HSPs) are found in all living organisms, from bacteria to humans (Morimoto et al., 1992). They participate in cellular-physiological processes of hormonal signaling, cellular cycle control and cellular proliferation and differentiation (Morimoto et al., 1992; Helmbrecht et al., 2000). They also help regulate and control cellular environmental stress processes (Gupta et al., 2010; Trautinger et al., 1996) related to the etiopathogeny of various diseases (Kalmar and Greensmith, 2009). Concern regarding potential risks of human exposure to EMFs has led to the creation of numerous experimental models for laboratory detection of biomarkers that are sensitive to this physical stimulus. Evidence has been found for changes in the expression of HSPs in various models of human cell lines (Lee et al., 2005; Caraglia et al., 2005; Alfieri et al., 2006) or *in vivo* rat tissue (Jorge-Mora et al., 2010) after exposure to experimental radio frequency systems. Other studies report no apparent changes in these biological markers after applying other experimental models in the laboratory (Wang et al., 2006; Chauhan et al., 2006; Valbonesi et al., 2008).

Recently, numerous studies have appeared describing the relationship between exposure to electro-magnetic sources, such as extremely low frequency (ELF) EMFs and radio frequency (RF), and various thyroid gland pathologies such as cancer (Milham and Morgan, 2008), alterations in the production of thyroid hormones (Rajkovic et al., 2003; Koyu et al., 2005; Eşmekaya et al., 2010) and other dysfunctions (Bergamaschi et al., 2004). Cellular levels of HSP-90 and HSP-70 in the thyroid gland are known to be linked to homeostasis (Wallin et al., 1992) and levels of cytotoxicity in certain glandular pathologies (Samadi et al., 2009; Paggi et al., 1995). These proteins may be biomarkers for detecting toxicity or environmental stress that

affects normal thyroid tissue functioning. In this study, we used levels of HSP-90 and 70 to analyze cellular stress induced by radiation (Jarosz and Lindquist, 2010), and how anti-apoptotic activity and integrity (Joly et al., 2010) are affected in female rat thyroid tissue exposed to 2.45 GHz radio frequency in an experimental GTEM system. We also used rectal temperature probes to measure body stress in animals, in order to determine if there was any interaction between variations in the post-radiation temperature of the animals and cellular stress.

Materials and Methods

Animals

All experiments were carried out according to European regulations on animal protection (Directive 86/609), the Declaration of Helsinki and/or the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (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 were used in the study. The rats weighed 230–250 g, were housed in individual cages with free access to food and water and were maintained at ±22℃ under a 12:12 h light/dark regimen. There is already evidence that estrogen may act directly in female rat thyroid cells to modulate their proliferation and functioning (Santin and Furlanetto, 2011).

Experimental design

A total of 96 female Sprague-Dawley rats were used and distributed equally into the following groups:

Group I (n=48): The rats were divided into 4 subgroups (n=6); each rat was exposed to 30 min* of microwave radiation at different levels: 0 (control), 1.5, 3.0 and 12 W**. The rats were kept alive for 90 min***, then slaughtered and perfused with fixative.

Group II (n=48): The rats were divided into 4 subgroups (n=6); each rat was exposed to 30 min of microwave radiation at different levels: 0 (control), 1.5, 3.0 and 12 W**. The rats were kept alive for 24 h***, then slaughtered and perfused with fixative.

*The minimum time exposure was tested previously.

**Power levels of 1.5, 3 and 12 W were used in this experiment, as they are multiples of the initial frequency (×2 or ×8); and 12 W is the maximum power of the amplifier.

***90 min post-radiation was chosen as the moment of slaughter for Group I animals because peak expression of the HSP-90 and 70 proteins occurs at that time. Group II animals were examined 24 h after exposure to radiation, which is the generally-recognized recovery interval.

Experimental radiation system protocol and description of the numerical simulations by FDTD SAR estimation

The power was delivered by a controlled signal during the radiation procedure. The amplifier (AMP) output was connected to the directional coupler (DC) that fed the Gigahertz Transverse Electromagnetic cell (Schaffner GTEM 250, 1.25 m×0.65 m×0.45 m). The rat (R) was placed inside the cell and positioned in the region of maximum field uniformity.

The DC makes it possible to measure the input power (PIN) and reflected power (PREF) values. The PIN was read by a power meter that also monitored the purity of the input signal; while the PREF value was obtained directly by means of a spectrum analyzer. All the measuring instruments were certified and calibrated by Agilent Technologies. This exposure setup provides good field uniformity and the behavior of a traveler wave can be simulated. The system components and operation were described in detail by Jorge-Mora et al. (Jorge-Mora et al., 2010).

The specific absorption rate (SAR) values were estimated with the aid of SEMCAD X (Schmid & Partner Engineering AG, Reference manual for the SEMCAD simulation platform for electromagnetic compatibility, antenna design and dosimetry (2009), available from http://www.semcad.com) and a finite difference time domain (FDTD)-based software tool. A Sprague-Dawley model R8 numerical (voxel) phantom rat (Schmid & Partner Engineering AG, 2009 reference manual) was used, weighing 198.3 g and composed of 60 different tissues assembled in 1.15 mm thick slices (tissue morphologies obtained by magnetic resonance images). The numerical phantom was radiated by a plane wave impinging its left side, with the magnetic field H parallel to its main axis (Jorge-Mora et al., 2010). The electrical field value E was determined by Eqn 1:

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

where h is the septum height in the exposure zone (position of the MH), P_{TR} ζ is the input power of the GTEM cell, $P_{TR}=P_{IN}-P_{REF}$, $Z_0=50'\Omega$ is the input impedance of the cell, and ζ is the coefficient that depends on the ripple field in the position MH, which is considered to have a value of 2 (Schaffner Electrotest Gmbh GTEM Test Cells, Datasheet 2005).

The SARs were estimated by applying 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:

$$SAR_E = SAR_S \times W_S/W_E \quad (2)$$

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

Stress levels and changes in rectal temperature after radiation

Using a Eutech digital thermometer, animal temperatures were measured before placement in the radiation chamber, and again at 0, 30, 60, 90 min and 24 h after radiation. Monitoring the rectal temperature of radiated and non-irradiated rats made it possible to determine temporal variation in levels of body stress (Dallmann et al., 2006), as well as differences in responses among the animals in the experiment.

Tissue extraction and preparation of cell extracts

A total of 24 animals were used for the ELISA technique. Once the 90 min and 24 h time periods had elapsed after radiation, the animals were thoroughly anesthetized with ethyl ether and the thyroid gland tissue was extracted under a microscope with a Nikon Eclipse CFI60 optical system. The animals were subsequently slaughtered. The extracted thyroid glands were stored at −30℃ for use.

Enzyme-linked immunosorbent assay (ELISA)

The glands were removed from cold storage and tissue lysis was carried out using the ProteoJet Mammalian Cell Lysis Reagent kit (Fermentas), following manufacturer's instructions. Then, the concentration of the protein in the extracted tissue of each sample was quantified using the Bio-Rad Protein Assay kit (BioRad Laboratories), with bovine seroalbumin (BSA) as the standard protein.

To each ELISA polystyrene sample plate well (Iwaki), 1 µg of each sample was added and then incubated overnight at 4℃ in 100 µl/well of coupling buffer (Na_2CO_3 0.015 M, $HNaCO_3$ 0.035 M, pH 9.6). Then, the plates were blocked for 2 h at room temperature using Tris buffer saline (TBS: Tris 50 mM, NaCl 0.15 M, pH 7.2) containing 0.2% Tween 20 and 5% skim milk. After washing with PBS we added a 1/200 diluted solution of anti-HSP-90 and anti-HSP-70 monoclonal antibody (Santa Cruz Biotechnology) and this was incubated for 1 h at 37℃. After several TBS rinses, the samples were placed in a 1/2000 diluted solution of rat anti-IgG polyclonal rabbit antibody (Dakopatts) and incubated for 1 h at 37℃. Finally, the plates were washed with TBS and then a 0.04% ortofenilendiamin (Sigma) substrate was added in phosphate-citrate buffer (Na_2HPO_4 0.2 M, citric acid 0.1 M; pH 5.0) and 0.001% H_2O_2. The reaction was stopped after 20 min incubation and H_2SO_4 3 N was added. The optical densities (OD) were then read in an ELISA microplate reader at 492 nm. A qualitative relationship was established in which absorbance was proportional to the concentration of protein detected in 1 µg/µl.

Two samples of thyroid tissue were extracted from each animal and the technique was replicated.

Tissue extraction and preparation of morphological techniques

After radiation, the rats were left to rest for 90 min or 24 h and then slaughtered for tissue extraction. The slaughter procedure involved an intra-peritoneal injection of sodium pentothal to diminish stress levels, followed by an overdose of ethyl ether just prior to slaughter. The tissues were fixed in paraformaldehyde for 24 h (10% in buffer phosphate), rinsed in alcohols and toluene, placed in a block of paraffin and cut into sections 5–6 microns thick. Three sections of each animal from each group (2) and the four subgroups (6 animals per subgroup) were used for each morphological technique described as follows (H&E and DAPI).

Hematoxylin-eosin (H&E) staining

The sections were then placed in xylol for 15 min, after which tissue was then hydrated alternately in pure 96% and 70% alcohol for 5 min periods, with a final rinse in distilled water. Hematoxilin tincture was then applied for 5 min, followed by a final 3 min rinse in water. Eosin tincture was applied for 1 min and then dehydration was carried out with 70%, 96% and 100% alcohol. Finally, the samples were submerged in xylol and mounted. The samples were examined under a conventional optical microscope to look for signs of lesions in the follicular cells and connective or colloid tissue of the thyroid gland.

DAPI staining of nuclei

These sections were rinsed in PBS wash 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/glycerin. DAPI-stained tissues were visualized by fluorescence microscopy.

Quantification and statistical analysis

An ELISA microplate reader at 492 nm was used to quantify HSP-90 and HSP-70. Signs of apoptosis were quantified using a Zeiss fluorescent microscope at 100× magnification, with 100 cells per field of vision examined for signs of apoptosis in each sample (three slides per animal) of each of the groups studied (48 animals×3 slides each=144 slides).

The results in the text and figures are expressed as averages and standard error of the mean ($P<0.05$). The following statistical tests were applied:

1) Two-way ANOVA was used to evaluate significant differences in stress levels as indicated by temperature, as well as in HSP-90 and HSP-70 concentrations in the thyroid gland, followed by a Holm-Sidak multiple comparison test. The factors taken into consideration were power used during exposure to radiation (0, 1.5, 3 and 12 W) and the time elapsed between radiation and slaughter of the animals (90 min and 24 h).
2) One-way ANOVA was used to study the SAR values (Table 1), followed by a Holm-Sidak test.

Transformations of natural logarithms were applied as needed to obtain normality and homoscedasticity.

Results

SAR

Table 1 shows the mean values of the average SAR in rat thyroid and body, as well as the mean values of the peak SAR per 1 g of thyroid and body, obtained from individual measurements of rats exposed to different powers (P) and electric fields (E). Using a one-way ANOVA test followed by a Holm-Sidak test, statistically significant differences were found among all the SAR values measured for the various powers (1.5, 3 and 12 W) applied to the radiated subgroups and for peak SAR values in 1 g of rat thyroid or body (*$P<0.001$, significant differences $P<0.05$*). The increase in average and maximum SAR values is directly proportional to the initial power (P) and the electric field value (E) for each subgroup (Fig. 1).

Stress levels and changes in rectal temperature after radiation

The effects of the various power levels were not dependent on exposure time. There is no statistically significant interaction between power and time ($P=0.886$).

Comparisons of the power factor revealed that there were no significant temperature differences among the groups (1.5, 3 and 12 W) or with regard to the control group (0 W).

Time comparisons revealed significant differences in average temperatures before and after radiation (at 0, 30, 60 and 90 min $P=0.00038$, $P=0.00020$, $P=0.0029$ and $P=0.015$, respectively). There were no significant differences in mean temperatures after radiation and at 24 h ($P=0.230$). Significant differences ($P=0.003$) only appeared at maximum power (12 W) with respect to the group exposed for 0 min.

Levels of HSP-90 and HSP-70 determined by ELISA

Results for HSP-90

The two factors considered in the Two-Way ANOVA test were power (0, 1.5, 3 and 12 W) and the time elapsed between exposure and slaughter (90 min and 24 h) In the thyroid gland, the difference in the mean values for the different power levels (0, 1.5, 3, 12 W) was statistically significant (*$P<0.001$*), after allowing for effects of time differences of 90 min or 24 h after radiation. The difference in the mean values for the different times elapsed after radiation was not statistically significant (*$P=0.127$*) and not large enough to exclude the possibility that the difference was due to random sampling variability after allowing for the effects of differences in power.

The effect of different power levels was found to depend on the time elapsed after radiation. There was a statistically significant interaction between power and time elapsed after radiation (*$P=0.018$*).

Comparisons of the power factor showed significant differences between the control subgroup (0 W) and groups exposed to 1.5 W (*$P=0.00008$*) or 3 W (*$P=0.0003$*), but not between the control and the group exposed to 12 W (*$P=0.0905$*).

The subgroup irradiated at minimum power (1.5 W) showed significant differences in HSP-90 levels at 90 min after irradiation, compared to levels at 24 h after irradiation (*$P=0.001$*) (Fig. 2A).

Group I (rats slaughtered and fixed 90 min after irradiation): The amount of HSP-90 expressed in the thyroid gland of rats radiated at 1.5 W and 3 W was significantly different from that found in non-irradiated rats (*$P<0.0001$, $P<0.022$*, respectively). Protein levels in rats irradiated at minimum power (1.5 W) also showed significant differences with respect to rats irradiated at 3 W or 12 W (*$P<0.009$, $P<0.0001$*). Protein levels in rats irradiated at maximum power (12 W) were not significantly different from those in the non-irradiated group (*$P<0.283$*) (Fig. 2A).

Group II (rats slaughtered and fixed 24 h after irradiation): The amount of HSP-90 expressed in the thyroid gland of rats exposed to radiation at 3 W was significantly different from that found in non-irradiated rats (*$P<0.003$*). However, there were no significant differences in HSP-90 levels in rats irradiated at 1.5 W or 12 W when compared to non-irradiated-rats (Fig. 2A).

Results for HSP-70

The difference in the mean values for each power level was statistically significant (*$P=0.037$*) in the thyroid gland, after allowing for the effects of time differences after radiation. The difference in the mean values for the times elapsed after radiation was not statistically significant (*$P=0.924$*).

The effect of different power levels was not dependent on the time elapsed after radiation. There was not a statistically

Table 1. SAR values in thyroids and bodies of the experimental rats, calculated from the power (P) and electrical field (E). The SAR values were compared by One-Way ANOVA differences between power (P) or electric field (E) and an *a posteriori* Holm-Sidak test. For multiple comparisons, differences were considered significant at $P<0.05$. *Indicates significant differences between [1]($P=1.5$) [2] ($P=3$) [3]($P=12$).

	Experimental measurement of specific absorption rate by FDTD			
	Mean SAR in thyroid (W/kg)	Peak SAR in 1 g thyroid	Mean SAR in body (W/kg)	Peak SAR in 1 g body
P=1.5 W **E**=28.48 V/m	$0.046\pm1.10^{-3(2,3*)}$	$0.041\pm2.10^{-3(2,3*)}$	$0.0169\pm7.10^{-4(2,3*)}$	$0.089\pm9.10^{-3(2,3*)}$
P=3 W **E**=40.28 V/m	$0.104\pm5.10^{-3(1,3*)}$	$0.076\pm4.10^{-3(1,3*)}$	$0.0364\pm19.10^{-3(1,3*)}$	$0.180\pm9.10^{-3(1,3*)}$
P=12 W **E**=80.56 V/m	$0.482\pm12.10^{-3(1,3*)}$	$0.340\pm10.10^{-3(1,3*)}$	$0.161\pm4.10^{-3(1,3*)}$	$0.795\pm2.10^{-3(1,3*)}$

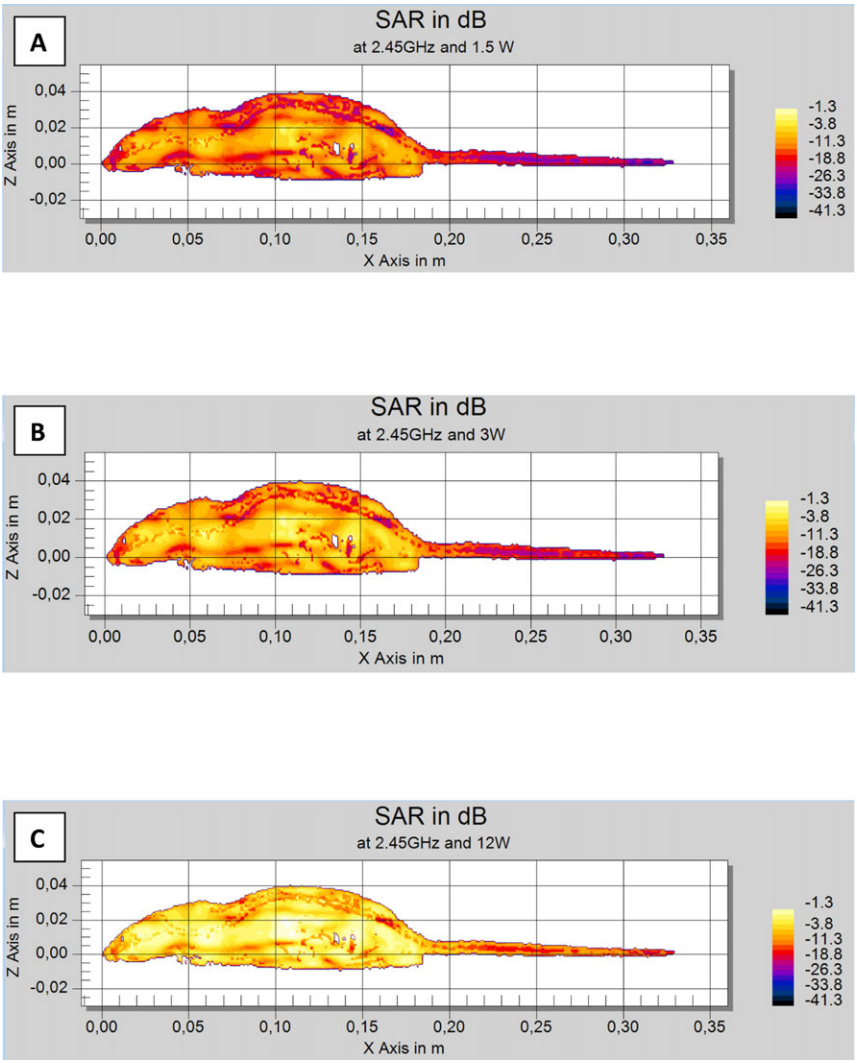


Fig. 1. Local SAR distribution in the phantom rat 'exposed' to (**A**) 1.5 W, (**B**) 3 W and (**C**) 12 W of 2.45 GHz, in the X=0.33 m plane.

significant interaction between power and time after radiation ($P=0.556$).

Comparisons of the power factor showed significant differences between the control subgroup (0 W) and groups that received 1.5 W or 3 W ($P=0.0098$, $P=0.0149$), but there were no significant differences with respect to the group exposed to 12 W ($P=0.142$).

Group I: The amount of HSP-70 expressed in the thyroid of rats exposed to radiation at 1.5 W was significantly different from that observed in non-irradiated rats ($P<0.010$). However, the HSP-70 levels in the thyroid of rats exposed to radiation at 3 W or 12 W were not significantly different from levels observed in non-irradiated rats ($P=0.060$, $P=0.156$) (Fig. 2B).

Group II: Expression of HSP-70 in the thyroid of rats exposed to radiation at 1.5 W, 3 W and 12 W was not significantly different from levels observed in non-irradiated rats ($P=0.065$, $P=0.371$ and $P=0.50$). See Fig. 2B.

H&E analysis

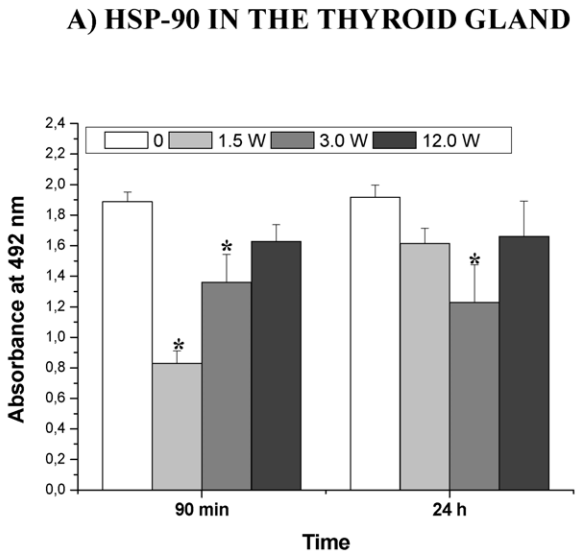
At 90 min and 24 h after radiation, examination of the sections tinctured with H&E under an optical microscope at 40× and 100× magnification did not show lesion alterations in the thyroid tissue structure that would affect the follicular cells, connective tissue or colloid tissue in the animals exposed to 1.5 W and 3 W when compared to the thyroid tissue of non-irradiated animals (Fig. 3A–D). At 90 min and 24 h after exposure to non-ionizing radiation exposure to 2.45 GHz, only a slight loss of cell cohesion was observed in some follicles (3E,F).

Results of DAPI staining

Fluorescence microscopy with a DAPI staining was used to examine the nuclear morphology of different cells for signs of apoptosis. No morphological changes, such as chromatin condensation or nucleus fragmentation, were observed in DAPI-stained sections from irradiated (all groups and different SARs) or non-irradiated animals. However, when the samples were compared to positive controls subjected to gamma radiation, we found positive signs of chromatin condensation 6 h after radiation (Fig. 4).

Discussion

We are not aware of any prior studies defining stress levels in thyroid gland cells that quantify HSP indices in live animals exposed to electromagnetic sources. The thyroid gland regulates immune functions and various hormones, so the possibility of non-ionizing radiation provoking changes in the machinery of the



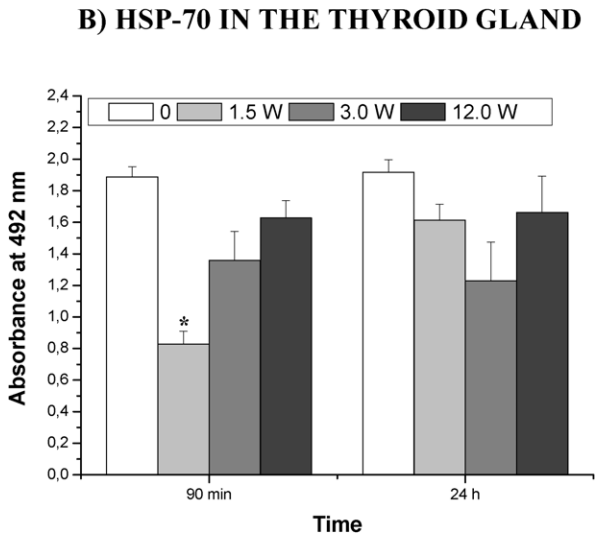


Fig. 2. The histograms represent levels of HSP-90 (**A**) and HSP-70 (**B**) detected by ELISA in the thyroid gland 90 min and 24 h after exposure to 0, 1.5, 3 and 12 W. Each bar represents average values ± standard error. Asterisks indicate statistically significant differences ($P<0.05$) found using a two-way ANOVA followed by a Holm-Sidak test for multiple comparisons.

chaperone cells of the gland constitutes a very relevant matter. Our results indicate important changes in HSP-90 and HSP-70 levels after exposure to 2.45 GHz electromagnetic fields. A significant decrease 90 min after exposure, when maximum values were reached in the control animals, occurs in HSP-90 and HSP-70 when the minimum SAR is applied (0.046 ± 1.10^{-3} and/or 0.104 ± 5.10^{-3}). Furthermore, the thyroid tissue was found to be most resistant to recovery at radiation levels of 3 W. Twenty-four hours after radiation, the basal protein levels of exposed animals had still not returned to the levels of the control or non-exposed animals.

The 2.45 GHz frequency was used in this experiment based on biological effects described by other authors (Malyapa et al., 1998; Paulraj and Behari, 2006) and our own previous studies of

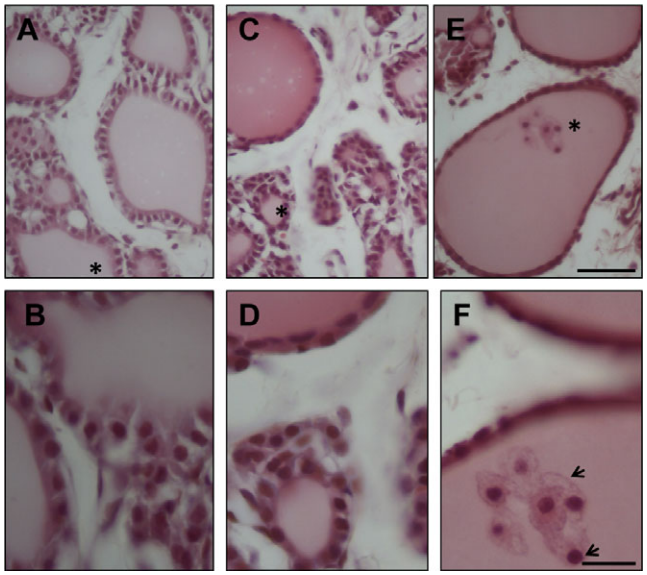


Fig. 3. The photographs of rat thyroid tissue stained with Hematoxylin-Eosin (H&E) show no signs of cellular lesions in rats exposed to 1.5 W (**A**) and 3 W (**C**) and a slight loss of cohesion in the follicular cells at 12 W (**E**) at 40× magnification. Photos (**B**,**D**,**F**) show the areas marked by * at 100× magnification. Arrows indicate follicular cells. Calibration bars: (A,C,E)=50 µm, (B,D,F)=20 µm.

HSP-90 in several regions of the brain (Jorge-Mora et al., 2010). This frequency is commonly used in telecommunications and for therapeutic purposes. At sub-thermal levels, *in vitro* human cell models showed significant changes in the expression of HSP-90 but not HSP-70 (Lee et al., 2005; Wang et al., 2006; Alfieri et al., 2006; Perez et al., 2008). However, other authors report changes in HSP-70 only when very high, thermal levels of 20 W/kg power were applied (Tian et al., 2002). The results of the present experiment indicate that low SAR levels (peak SAR

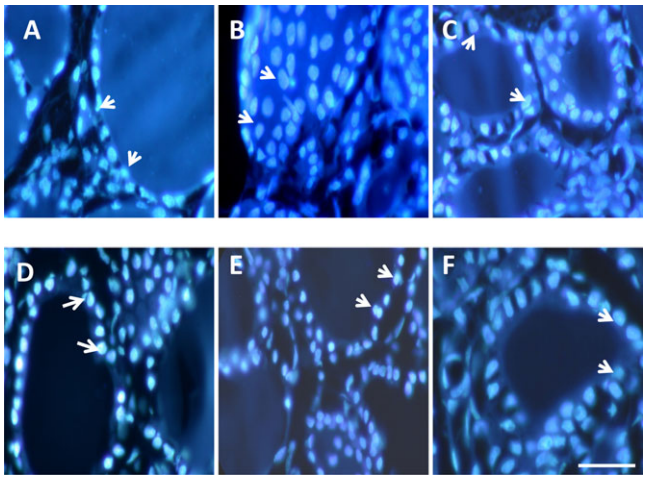


Fig. 4. The photographs of rat thyroid tissue with fluorescent DAPI staining show no evidence of nuclear fragmentation of the nucleus or chromatin condensation in the epithelial cells of the thyroid follicles in any of the experimental groups. Photos (**A**) (**B**) (**C**): animals slaughtered 24 h after exposure to 0 W (control), 1.5 W or 12 W, respectively; (**D**) (**E**) (**F**): animals slaughtered 90 min after exposure to 0 W (control), 1.5 W or 12 W. Arrows indicate follicular cells with no signs of apoptosis. 100× magnification, calibration bar 20 µm.

0.089 ± 9.10^{-3} or 0.180 ± 9.10^{-3} in the body and 0.041 ± 2.10^{-3} or 0.076 ± 4.10^{-3} in the thyroid), cause decreases in levels of heat shock proteins in thyroid cells. There is a wide variety of environmental stimuli that can cause cellular stress and modify the synthesis of HSP-90 and/or HSP-HSP-70 in mammal tissue, significantly affecting the maintenance of cellular homeostasis (Morimoto, 1993; Kregel, 2002; Jarosz and Lindquist, 2010), anti-apoptotic processes and cellular differentiation (Schmitt et al., 2007; Lanneau et al., 2007). The most well-known inductors are temperature (Kregel, 2002), toxic agents (Gupta et al., 2010) oxidative stress (Kalmar and Greensmith, 2009) and ultraviolet radiation (Trautinger et al., 1996) or ionizing radiation (Calini et al., 2003; Kang et al., 2002).

Although some authors describe what seems to be a cellular stress response, one would expect it to take the form of an increase in HSP expression rather than a decrease. De Pomerai et al., for example, later rectified their study, saying that what they had taken to be a 'non-thermal' effect (de Pomerai et al., 2000; de Pomerai et al., 2003) was in fact a thermal one (de Pomerai et al., 2006). Our experience, which has been corroborated by other studies, suggests that the interaction of RF in the tissues might activate thermal and non-thermal mechanisms simultaneously (Jorge-Mora et al., 2010; Jorge-Mora et al., 2011; López-Martín et al., 2006; López-Martín et al., 2009; Curcio et al., 2005). In some cases one mechanism is more apparent than the other; in this experiment, rectal temperature was slightly higher in animals with a higher SAR. However, this did not result in a significant increase in the expression of HSP-70 and 90, which is probably compensated by the thermoregulatory mechanisms of the animals (Table 2).

The decrease in heat shock proteins seems to indicate the existence of non-thermal physical stimuli acting by unidentified mechanisms via low-intensity electrical fields, with no direct relationship between power and magnitude of effect. Because the animals received non-ionizing radiation to the entire body through the skin, the system is subject to multiple physical stimuli with biological repercussions that are difficult to identify precisely. Tissue stress can be added to other mechanisms induced by the simultaneous action of different functional circuits (Jorge-Mora et al., 2011).

The literature also describes decreases in HSP-70 and 90 twelve hours after a state of anoxia (Ramaglia and Buck, 2004) and during cerebral ischemia (Yang et al., 2005). These data indicate that increased HSP expression is not critical in early adaptation. However, late regulation involving the increase of heat shock proteins suggests that stress proteins play a role in promoting long-term tolerance.

The decreased levels of HSP-90 in the thyroid gland after radiation are much lower than those required for normal cellular functions. In a stressful environment this protein can destabilize and alter the relationship between genotype and phenotype (Jarosz and Lindquist, 2010). HSP-90 expression in the thyroid gland varies significantly; in normal tissue the protein levels are much higher than in the hyperplasic gland or in papillary cancer of the thyroid (Wallin et al., 1992). Various authors relate this to cellular differentiation, or a physiological process that has been altered. In some cases the levels of this or other heat shock proteins in the thyroid gland may indicate normal (Ginsberg et al., 2006) or pathological (Medeiros-Neto et al., 1996; Boltze et al., 2003; Baryshev et al., 2004) thyroid functioning. There is even an important relationship between the activity of HSP-90 and the degree of hypophysary stimulation of the thyroid cells (Ginsberg et al., 2006).

After exposing the rat thyroid gland to radiation, HSP-70 levels also decreased, with a rapid recovery that is probably related to the reparative or cytoprotective effects of this protein (Rajdev and Sharp, 2000). In any case, the HSP-90 and HSP-70 levels accompanying thyroid cells are present under normal physiological conditions and in pathological changes. Thus, human thyroid cell experimental models are optimal for studying how HSP-90 and HSP-70 expression responds to stress from thermal or chemical stimuli in auto-immune diseases of the thyroid gland (Youde et al., 1998).

In spite of the fact that various thyroid pathologies involve increases in HSP-90 and HSP-70 (Medeiros-Neto et al., 1996; Boltze et al., 2003; Baryshev et al., 2004), there is also evidence of situations where conditions such as hyperthermia, aging or illness can diminish the response of heat shock proteins in various tissues (Gutsmann-Conrad et al., 1998; Pardue et al., 2007). This suggests that an increase in heat shock protein synthesis may be necessary in some cellular responses, but not all (Carper et al., 1987). The decrease in the chaperone levels may indicate that the magnitude of heat shock protein responses in various types of cells may affect the survival of these cells after stress (Pardue et al., 2007). Great diversity of vulnerability and response to stress by induction of heat shock proteins has been observed in nervous system tissue studies of different animals (Jorge-Mora et al., 2010; Gutsmann-Conrad et al., 1998).

The similar levels observed for Heat Shock Proteins 90 and 70 indicate a common, non-discriminatory functioning mechanism, as was observed in some cases of continuous and planned therapeutic stimulation of Heat Shock Factor-1 (HSF-1) (Powers and Workman, 2007). This induces the increase in both HSP-90 and 70 chaperones (Kieran et al., 2004). Is it possible that the radiation from a non-thermal mechanism could inhibit (the effects of) HSF1 in some organs or tissues? This hypothesis will require investigation in the future.

However, the two chaperones have different functions in mammals, which are manifest in their different responses to the same physical stimulus (Parcellier et al., 2003; Lanneau et al., 2007). Thus, HSP-90 thyroid cells are more sensitive to 3 W

Table 2. Experimental measurement of rectal temperature; *indicates significant differences before and after radiation.

	Experimental measurement of rectal temperature					
	Time					
Power	Before	0 minutes	30 minutes	60 minutes	90 minutes	24 hours
0 W	36.9 ± 14.10^{-2}	36.9 ± 14.10^{-2}	37.3 ± 20.10^{-2}	37.1 ± 20.10^{-2}	37.1 ± 20.10^{-2}	37.1 ± 20.10^{-2}
1.5 W	37.0 ± 4.10^{-2}	37.5 ± 18.10^{-2}	37.4 ± 13.10^{-2}	37.2 ± 26.10^{-2}	37.3 ± 10.10^{-2}	37.0 ± 14.10^{-2}
3 W	37.0 ± 13.10^{-2}	37.5 ± 10.10^{-2}	37.6 ± 24.10^{-2}	37.2 ± 25.10^{-2}	37.4 ± 40.10^{-2}	37.1 ± 17.10^{-2}
12 W	36.8 ± 19.10^{-2}	$37.7 \pm 12.10^{*-2}$	37.5 ± 11.10^{-2}	37.5 ± 11.10^{-2}	37.4 ± 15.10^{-2}	37.2 ± 1.10^{-2}

power. This also appears in repeated exposures of nervous tissue (Jorge-Mora et al., 2011), but is not the case with HSP-70.

In spite of the decrease in stress protein response levels, we did not find evidence of lesions or apoptosis, contrary to the findings of other authors that associate high levels of HSP-90 and HSP-70 with an increase in the cytoprotective and anti-apoptotic functions of these chaperones (Parcellier et al., 2003; Kang et al., 2002). Exposure to EMFs at 900 MHz has been reported to cause pathological changes associated with an increase in the caspase pathway, which is dependent on apoptosis, in the thyroid gland structure (Eşmekaya et al., 2010).

Although some studies do not establish a causal relationship between EMFs and thyroid cancer (Lope et al., 2006; Kang et al., 1998), there is much research describing the functional and histological effects of EMFs on the thyroid gland. Exposure to LF-EMF or RF at 900 MHz causes changes in hormonal secretion of T3 and T4 (Rajkovic et al., 2003; Koyu et al., 2005; Bergamaschi et al., 2004) and morphological alterations that affect the epithelium, connective tissue, follicular and inter-follicular cells and mast cells (Rajkovic et al., 2003; Rajkovic et al., 2005; Rajkovic et al., 2006). Such changes in the thyroid gland after exposure to non-ionizing sources of radiation can be seen to recover morphologically, but not functionally (Rajkovic et al., 2003).

There is evidence to support the hypothesis that estrogen has a direct role in thyroid follicular cells and influences thyroid growth and function in normal and abnormal conditions (Santin and Furlanetto, 2011). However, there are no indications that RF can interfere with the estrogenic activity of female rats (Yamashita et al., 2010) to provoke an indirect effect on thyroid function.

This leads us to consider that in our study the lack of visible alterations or signs of apoptosis in the glandular structure might be due to the very short time of exposure to non-ionizing radiation.

Conclusions

The results of this study have demonstrated that: 1) the thyroid gland is sensitive to 2.45 GHz RF; 2) non-thermal mechanisms cause a transitory decrease in the values of HSP-90 and HSP-70, which determine cellular stress levels in the thyroid gland; 3) recovery of HSP-90 basal levels is slower at the 3 W power setting; 4) there are no signs of lesion or apoptosis in the glandular structure that would indicate the beginning of damage caused by non-ionizing radiation.

Acknowledgements

The authors wish to thank the *Dirección General de Investigación y Desarrollo* of the *Xunta de Galicia* for financing part of this research through project 09TIC006206PR. We greatly appreciate the help received from the technical assistance of Rafael Fuentes, Alberto López, and Isabel Tarrio.

Competing Interests

The authors have no competing interests to declare.

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