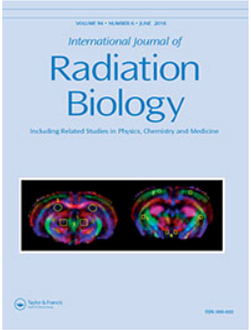

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ORIGINAL ARTICLE

Exposure to radiation from single or combined radio frequencies provokes macrophage dysfunction in the RAW 264.7 cell line

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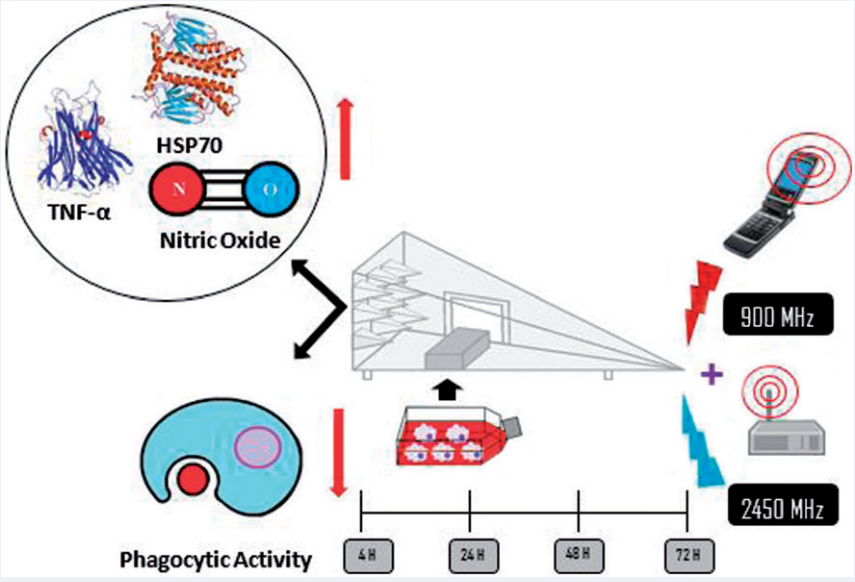
ABSTRACT

Purpose: The aim of this study was to determine whether exposure to radiation from single or multiple radio-frequency (RF) signals at 900 and 2450 MHz would induce effects in the RAW 264.7 cell line.

Materials and methods: Cell cultures were exposed to single or combined RF for 4, 24, 48, or 72 h in a GTEM electromagnetic test chamber. At the end of the radiation exposure time, viability and cell growth were analyzed by flow cytometry, nitric oxide (NO) production was measured by colorimetry, the expression of HSP70 and TNF-α was ascertained by qPCR, and the phagocytic activity was observed by microscopy.

Results: NO production increased after 48 h exposure at 2450 MHz, compared with controls. The group subjected to the combined interaction of two RFs showed an increase of HSP70 after 48 h exposure and a significant increase of NO and TNF-α after 72 h. The phagocytic activity of macrophages decreased in all groups as exposure time increased.

Conclusions: Our results indicated a decrease in phagocytic activity and an increase in inflammatory, cytoprotective, and cytotoxic responses in macrophages after continuous and combined exposure of multiple RF signals. Multiple RF interact in everyday life, the immune response in humans is unknown.



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KEYWORDS
Combined radiofrequencies; macrophage; RAW 264.7

1. Introduction

Exposure of the general population to radiofrequency (RF) fields from mobile phones, wireless networks, television, radio broadcasting, and other communication technologies has become universal and continuous in recent years. Humans are also simultaneously exposed to multi-signal RF and there is very little information about the biological effects of this. Legal limits on the specific absorption rate (SAR) associated with exposure to multiple signals (REAL

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DECRETO 1066/2001) have not been established based on experimental evidence, and there is accordingly significant uncertainty regarding the effectiveness of these limits. Only a handful of epidemiological (Thomas et al. 2008a, 2008b), in vitro (Kang et al. 2014), or in vivo (Lee et al. 2011, 2012; López-Furelos et al. 2012, 2016) studies have been done for exposure to combined RF.

Macrophages are fundamental components of the immune system and play a crucial role in the activation of innate and acquired immunity (Regueiro-González et al. 2011) through detoxification and the generation of free radicals when a harmful agent acts (Korc et al. 1995). The immune system protects mammals from external or internal aggressions; electromagnetic fields (EMF) could alter the correct functioning of systemic repair mechanisms (Helga et al. 2006; Guerriero and Ricevuti 2016). More specifically, EMF may influence the innate immune system, as induced extracellular neutrophil (EN) formation is dependent on the NADPH oxidase pathway and production of reactive oxygen species (Lieke et al. 2015). Results from experiments with RF-field exposure at 2.45 GHz SAR at 10 W/kg have shown very little or no effects on either chemotaxis or phagocytosis in neutrophil-like human HL-60 cells (Koyama et al. 2015). However, exposure of macrophagic immune cells to RF in vivo (Mayers and Habeshaw 1973; Zafra et al. 1988) and in vitro (Gamaley et al. 1995) may change cellular function. The RAW 264.7 macrophage cell line has proven optimal for exploring immune responses to the application of electromagnetic fields in a GTEM chamber (Lin and Lin 2011).

Nitrogen-reactive species are a family of nitric oxide (NO) molecules produced by the enzymatic activity of NO synthase (NOS). NOS is not constitutive in macrophages but induced in situations where these cells are activated by cytosines, particularly interferon-gamma (IFN-γ) (San-Miguel et al. 2006). NO produced by macrophages acts as a cytotoxic free radical (Albina and Reichner 1998). Uncontrolled production can cause cellular damage that influences the activation of apoptosis pathways and various defense responses against oxidative stress (Hirvonen et al. 1996).

Electromagnetic fields can regulate certain genes involved in the acceleration of aging processes by inducing cellular stress and provoking the synthesis of cytoprotective heat shock proteins (HSP) (Pérez-Bruzón et al. 2011; Kim et al. 2012). Increased HSP in tissue is often associated with a resistance to or decrease in apoptosis (programmed cellular death) (Hsin-Yi and Yu-Jen 2011). In addition, the expression of the tumor necrosis factor-alpha (TNF-α) is crucial in the development of the protective response to infections and the immunological activity that destroys tumor cells (Hirvonen et al. 1996; Petrini et al. 1997). Induction of heat shock protein HSP70 and TNF-α in response to stress from an electromagnetic field may suggest that these agents shift cellular equilibrium towards survival or initiate alterations in cell physiology. Observing the expression of TNF-α or HSP after radiation makes it possible to evaluate macrophage function and viability (Grunwald et al. 2014).

Phagocytosis is the most specific immune function of macrophages; alteration of their properties by RF may compromise the integrity of the immune response and the proper functioning of biochemical processes related to the metabolism of unwanted substances (Kolomytseva et al. 2002; He et al. 2014).

Recently, we described the acute cellular stress response of Sprague–Dawley rat brain tissue to the combined action of two radiofrequencies. The evidence that the effect of combined frequencies is different from the effect of a single frequency might indicate that a different mechanism is involved in the interaction of multiple radio frequencies with respect to single frequencies (López-Furelos et al. 2016). Different macrophage immune responses resulting from exposure to electromagnetic sources have also been described (Seaman et al. 2002; Maresuke et al. 2016), but there is no experimentally based description of what happens when the interaction of more than one radiofrequency signal occurs in said immune cell. Thus, the cytoprotective, cytotoxic, anti-inflammatory, or phagocytic functions of the immune cells could be affected differently by single frequencies or the interaction of multiple frequencies.

To compare macrophage immune responses, we applied one or more frequencies to the RAW 264.7 macrophage cell line for 4, 24, 48, or 72 h in a GTEM chamber. Studies were then performed to evaluate macrophage immune functions, specifically: cell viability, oxidative stress by quantification of NO production, cytoprotective, and inflammatory function using HSP70 and TNF-α, and macrophage phagocytic activity.

2. Material and methods

2.1. Experimental radiation system

Figure 1(A) depicts the experimental system. There are two Vector Signal Generators, VSG1 Frec #1, Agilent E8267D (250 kHz–20 GHz) and VSG2 Frec #2, Agilent E 4438C (250 kHz–4 GHz) (Agilent Technologies, Palo Alto, CA) which generate pure sinusoidal signals of 900 MHz and 2450 MHz, respectively, and are regulated to the required power during exposure. Both generators are connected to an Agilent 11636A Power Divider (PD), which is used as a power combiner, and the signal passes to the 1551G3 Amplifier (AMP) (0.8–3 GHz). Once the signal has been amplified, it is introduced into a NARDA 3282B-30 Directional Coupler (DC) and passes directly to the Shaffner 250 GTEM Radiation Chamber (DC-20 GHz), where the Element Under Test (EUT) is placed in the area of maximum field uniformity. The DC makes it possible to measure the incident power values P_{IN} using an Agilent E4418B Power Meter (PM), and thereby establish the desired entry power for the system. The reflected power value, P_{REF}, can also be measured and monitored using the Agilent E4407B Spectrum Analyzer (SA) (9 kHz–26.5 GHz).

We can observe that the field impinges on B in the direction k, with the E and H vectors positioned perpendicularly to the propagation direction. T Wavecontrol EF Cube (30–3000 MHz) Isotripic Probe (IP) was used to ascertain peak value. This measurement was taken without placing the culture flask inside the chamber, using the desired entry signal values. In this way, we can more precisely describe the behavior of the chamber in the measurement zone.

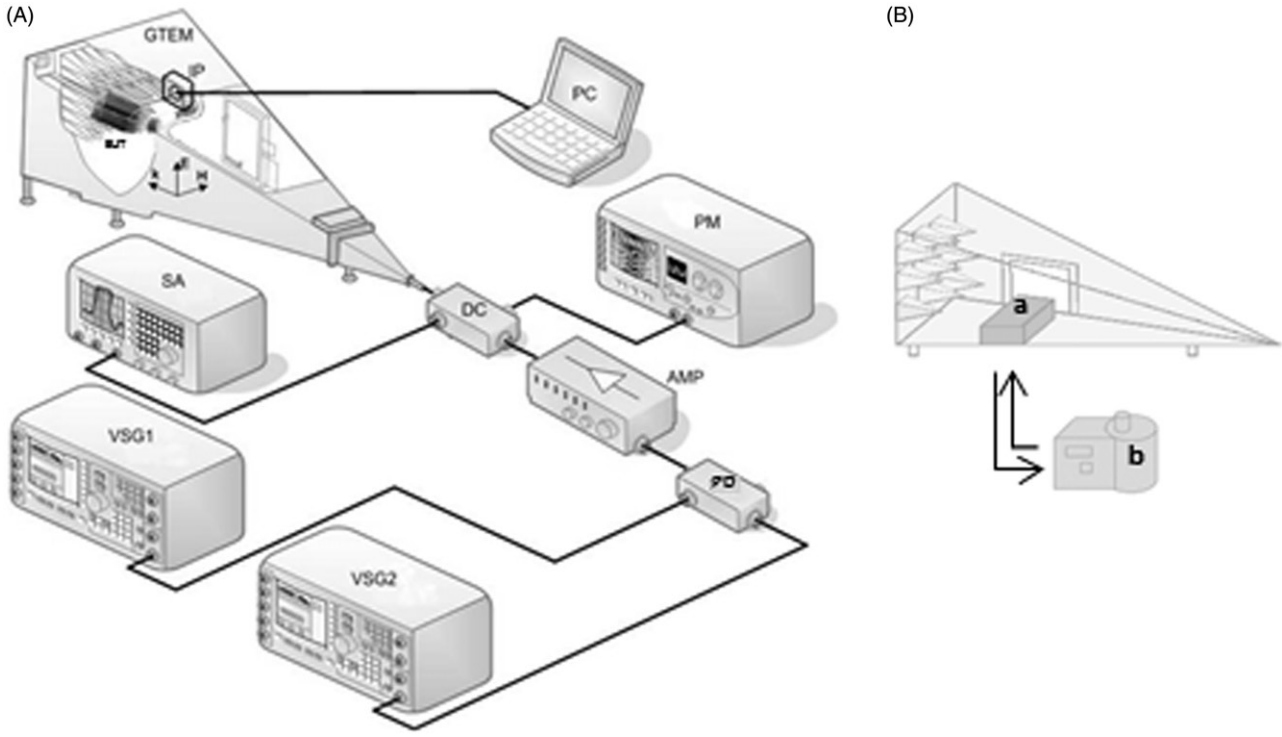

Figure 1. (A) Diagram of experimental system. GTEM: Schaffner 250 GTEM: Chamber (DC-20GHz); VSG Frec #1: Agilent E4438C Vector Signal Generator (250 kHz–4 GHz) operating at 2.45 GHz; VSG Frec #2: Agilent E4438C Vector Signal Generator (250 kHz–4 GHz) operating at 900 MHz; PD: Agilent 11636A power Divider (used as a power combiner); AMP: Aethercomm Amplifier 0.8–3.2–10; DC: NARDA 3283B-30 Directional Coupler (800–4000 MHz); SA: Agilent E4407B Spectrum Analyzer (9 kHz–26, 5 GHz); PM: Agilent E4418B Power Meter; IP: (30–300 MHz) EF cube Wave control Isotropic Probe; EUT: element under test. (B) Diagram of thermoregulation system, with heating pedestal inside the radiation Chamber (a) and external heating system (b).

Table 1. Experimental conditions of experimental groups subjected to RF radiation.

	f (MHz)	P_{TR} (W)	E_m (V/m)	P_D (W/m^2)	Mean SAR (W/kg) 24-well plate	Mean SAR (W/kg) Culture flasks
Group 1	900	12	96.8	24.9	0.1477	0.2467
Group 2	2450	12	102.8	28.0	0.1740	0.4060
Group 3	900 + 2450	6 + 6	71.5	13.6	0.0842	0.1834

f: frequency; P_{TR}: transmitted power, E_m: measured r.m.s. electrical field strength; and P_D: incident power density; mean SAR: FDTD-calculated specific absorption rate using E_m.

The values obtained with the IP were found to generally agree with those recommended by the makers of the chamber, based on the following expression:

$$E = \sqrt{Z_0 P_{TR}/(h^2\zeta)} \quad (1)$$

where h is the height of the septum in the area of exposure (EUT position), see Figure 1(A), P_{TR} is the power at entry in the GTEM $(= P_{IN} - P_{REF}), z_0 = 50[\Omega]$ the entry impedance of the GTEM, and ζ is the coefficient that depends on the field ripple within the EUT positioning zone and is considered equal to 2. However, we have opted to use the values measured with the IP for greater precision in this study.

The electromagnetic parameters used in this experimental radiation system and simulation results related to the FDTD-calculated specific absorption rate (SAR) of these cell cultures are described in Table 1. The specific absorption rate (SAR) values were estimated using SEM-CAD X finite-difference time-domain (FDTD)-based software (Schmid and Partner Engineering 2009).

2.1.1. Maintaining the temperature

To maintain a constant temperature of 37 °C, an automatic thermoregulation system was designed. It consists of two pieces: (a) inside the GTEM chamber, a PVC pedestal containing water that circulates via an internal plastic coil that transfers heat to the water and is connected by two tubes; (b) the external heating unit (Figure 1(B)) consisting of: a heating element, a coil connected to an air cooling system, a thermostat to control the temperature, a pump to recirculate the liquid and a water deposit. The system makes it possible to maintain a constant temperature of 37.5 ± 0.5 °C on the surface of the pedestal, where the culture flasks are placed (Figure 1) (López -Furelos et al. URSI 2016).

2.2. The raw 264.7 cell line culture

2.2.1. The RAW 264.7 cell line

The RAW 264.7 macrophage cell line, purchased from the American Type Culture Collection (ATCC, cat. no. TIB-71), was initially generated from a murine leukemia virus-induced tumor in BALB/C. It lacks surface immunoglobulins and has phagocytic activity.

2.2.2. Culture media

IMDM (SIGMA). Iscove's Modified Dulbecco's Medium (IMDM) is a modification of Dulbecco's Basal Eagle Medium that includes selenium, additional amino acids, and vitamins. IMDM does not contain growth factors and thus requires supplementation with 10% fetal bovine serum (FBS) (PAN

Biotech, Dorset, UK). IMDM uses a system of sodium bicarbonate buffers (3.024 g/l), which requires an environment with 5–10% CO2 to maintain physiological pH.

LEIBOWITZ-L15 (SIGMA). The Liebowitz L-15 salt mixture is enriched with much larger amounts of amino acids than other commercial solutions. It also has vitamins and other essential components for cell growth. It does not require use of a CO_2 stove, because its formulation does not contain bicarbonate as buffer.

2.2.3. Subculture

In the sub-confluent cultures (70–90%), 90% of the old medium was removed. The cells were mechanically re-suspended by scraping and transferred to new flasks at a concentration ranging from 1:3 to 1:6, depending on the initial concentration of the cells obtained. A new IMDM medium was added at 5% CO_2 and 37 °C. The cells are semi-adherent, so some grow in suspension, some are vaguely attached to the surface and others are flattened and attached to the flask. The cells should not be allowed to grow and become confluent, because this can cause them to lose their adherent qualities.

2.3. Experimental design

The experimental groups were divided according to the following experimental design:

- Group I: Cell cultures irradiated at 900 MHz and 12 W.
- Group II: Cell cultures irradiated at 2450 MHz and 12 W.
- Group III: Cell cultures irradiated at 900 MHz and 2450 MHz at 6 W power, respectively, and simultaneously.
- Group IV: Non-irradiated control cultures.

These four groups were also subdivided according to exposure time (4, 24, 48, or 72 h).

2.4. Radiation protocol

2.4.1. Culture flasks

Cells were introduced into 25 cm^2 culture flasks (Thermo Fisher Scientific, Waltham, MA) and the culture medium was replaced with L-15 supplemented with 10% inactivated fetal bovine serum (PAN Biotech, Dorset, UK). Then, the 32 independent cultures of the RAW 264.7 macrophage cell line were individually exposed to radiation in the GTEM chamber (two cell cultures per group and time) at the point of maximum field uniformity with the culture flask placed horizontally and the flask cup oriented towards the source of radiation.

2.4.2. Sterile 24-well plates

To determine phagocytic activity, four sterile 24-well plates (Corning Life Sciences, Corning, NY) (one for each group) with a square glass deposited on the bottom of each well were radiated. Then, 2 ml of IMDM medium with a RAW 264.7 cell concentration of 1×10 cells/ml were added to each well and incubated 24 h. The IMDM medium was subsequently removed and replaced with 3 ml of L-15 medium. The radiation protocol was then applied and 6 crystals with cells attached to their surfaces were extracted at 4, 24, 48 and 72 h. The 24-well plates were radiated at the point of maximum field uniformity with the plate placed horizontally and the longer axis perpendicular to the source of radiation.

2.5. Biological studies

2.5.1. Viability study and growth curve

To perform the viability and growth curve study, Cell Analyzer Muse equipment was used according to the manufacturer's recommended protocol, using fluorochrome propidium iodide (SIGMA, St. Louis, MO) and analyzing the number of cells/ml for each time interval.

Cell growth data were obtained by comparing the slopes of the equations of the line resulting from the graphical representation of growth (concentration/time) for each of the cultures exposed to radiation.

2.5.2. NO production

To assess NO production, we made a standard straight line using sodium nitrite, then applied Gries reagent (5% phosphoric acid (Merck, Kenilworth, NJ), 0.5% sulfanilamide (Merck, Kenilworth, NJ) and 0.05% naphthylethylenediamide (Sigma, St. Louis, MO)) to our sample at a ratio of 1:1 (v/v). The reaction required incubation for 10 min and the result was read using an absorbance reader (ELx808, Biotek, Winooski, VT) at 530 nm.

2.5.3. Expression detection

mRNA extraction. After irradiation, the cells were centrifuged at 2500 rpm for 5 min. The L-15 culture medium was removed and the pellets re-suspended in 1 ml of TriPure reagent (Sigma-Aldrich, St. Louis, MO). Then, 0.2 ml of chloroform (Merck) was added and the culture was incubated for 10 minutes then centrifuged at 1200 *g* and 4 °C for 15 min. The phase containing the DNA, proteins and cell debris was discarded and the fraction containing the RNA was kept. To remove the TriPure reagent residues, the cultures were rinsed with isopropyl alcohol (Merck, Kenilworth, NJ) followed by 70% ethanol (Merck), and centrifuged after each wash. The result was a whitish dehydrated pellet, which was stored at −70 °C.

DNase treatment. The pellet was hydrated in H_2O with diethylpyrocarbonate (Sigma-Aldrich, St. Louis, MO) and allowed to incubate for 15 min at 60 °C. Subsequently, the RNA was quantified using NanoDrop ND-1000 equipment and 4 μg of nucleic acids was added. For DNase treatment, a commercial kit (Thermo Fisher Scientific, Waltham, MA) consisting of DNase I and a reaction buffer with 2.5 mM $MgCl_2$ was used. After incubation for 30 min at 37 °C, the reaction was stopped with 50 mM EDTA from the kit.

rtPCR. We combined 200 ng of the pure RNA sample with 1.25 μM random hexamer primers (Roche, Basel, Switzerland) and 250 μM deoxynucleosidostyphosphate (dNTPs) then allowed it to incubate at 65 °C for 5 min. Reagents were then added from the GeneAmp® RNA PCR kit (Thermo Fisher Scientific, Waltham, MA) consisting of 200 U of MMLV (Moloneymurineleukemia virus) reverse transcriptase (Promega, Madison, WI) in a reaction buffer of 30 mM Tris and 20 mM KCl (pH 8.3) and ribonuclease inhibitor. These were then introduced into a thermocycler that was programmed as follows: 25 °C for 5 min, 37 °C for 50 min and 70 °C for 15 min. As a result, we obtained the cDNA of the total mRNA.

Q-PCR. Once the cDNA had been synthesized, we performed a quantitative PCR analysis to study tumor necrosis factor-alpha (TNF-α) gene expression: forward/reverse primer 5′-AGCCCCCAGTCTGTATCCTT-3′/5′-CTCCCTTTGCAGAACTCAGG-3′, and the HSP70 forward/reverse primer pair 5′-CATCATC AATGAGCCCACAG-3′/5′-TCTTGTGTTTGCGCTTGAAC-3 using in each case the forward/reverse β-actin (ACT) primer pair 5′-AGCCATGTACGTAGCCATCC-3′/5′-CTCTCAGCTGTGGTGGTGAA-3′. ACT was used to normalize the data, which were expressed in relative arbitrary units. Each sample was introduced in triplicate onto a multi-well plate; the first forward and reverse of the gene of interest were introduced into each well for each case. The cDNA sample and the cromogen kapasybrfast (Kapa Biosystems, Wilmington, MA) were also placed on the multi-well plate. Eco™ equipment (Illumina, San Diego, CA) was used for amplification.

2.5.4. Phagocytic activity

After irradiation, the crystals were deposited on a new plate with 750 μl of medium containing 1.5 mg/ml of lyophilized *Saccharomyces cerevise* and incubated for 2 h. Three PBS washes were then performed to remove excess yeast, and the plate was incubated for 5 min in 100% methanol and allowed to dry at 20 °C. The next step consisted of staining the cells and yeasts with Giemsa dye (Harleco, Gibbstown, NJ) at 100% concentration for 2 min and at 50% concentration for 1 min. The samples were washed three times with tap water and the preparation was assembled with Entellan (Merck, Kenilworth, NJ).

Measurement of phagocytic activity. To measure phagocytic activity, a Nikon ECLIPSE E2000 microscope (Nikon, Tokyo, Japan) was used at 100× resolution. Nine-hundred cells were examined and measured in each of the groups (I, II, III, and IV) and for each radiation time (4, 24, 48 and 72 h). From the macrophage count, we determined the percentage of macrophages that had internalized yeasts with respect to those that had not for each group and exposure time.

2.5.5. Quantification and statistical analysis

The results shown in the text and figures are expressed as means ± SEM; significant differences ($p < .05$).

a. One-way ANOVA was used to evaluate changes in cell growth in relation to radiation exposure. The factors considered were the interaction of one or more frequencies compared to the non-radiated group.
b. Two-way ANOVA was used to evaluate changes in viability in relation to radiation exposure. The factors considered were the interaction of one or more frequencies and the exposure times. The Holm–Sidak test for multiple comparisons was subsequently applied.
c. The results obtained from the NO absorbance test were analyzed using two-way ANOVA. The factors considered were the interaction of one or more frequencies and exposure times of 48 and 72 h (Figure 2). The Holm–Sidak test for multiple comparisons was subsequently applied.
d. The PCR levels (Figure 3(A,B)) for the expression of HSP70 and the TNF-α line were determined by two-way ANOVA, using the quantification in the amplification of the mRNA in the macrophage cells and the interaction of one or more frequencies. The Holm–Sidak test for multiple comparisons was subsequently applied. Natural logarithm transformations were also applied to the data as needed, to obtain normality and homoscedasticity.
e. Phagocytic activity rate results were analyzed with two-way ANOVA, using radiofrequencies (non-radiated, one or both RF) and exposure time. The Holm–Sidak test for multiple comparisons was subsequently applied (Figures 4–7).

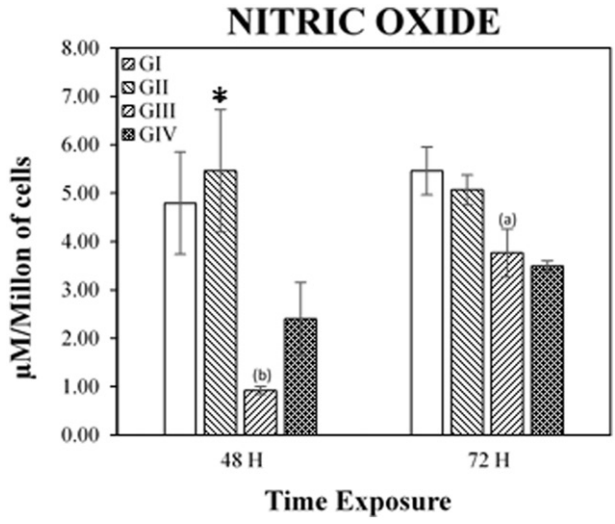

Figure 2. Histograms showing the concentration of nitric oxide in relation to time in GI (900 MHz), GII (2450 MHz), GIII (900 MHz + 2450 MHz), and GIV (non-radiated) groups. Values show the means (seven samples for each group/time) of radiated groups (GI, GII, and GIII) and the non-radiated group (GIV). Error bars indicate the standard error of mean (SEM) within each group (radiated or non-radiated). *Significant differences in relation to the non-radiated group ($p < .05$). (a and b) Indicate significant differences for each radiated group in relation to time: 48 h (a), 72 h (b) ($p < .05$).

3. Results

3.1. Viability study and growth curve

Viability and cell growth of the radiated groups were measured by flow cytometry, using six samples per group/time. The results showed that RAW 264.7 cell line viability was not

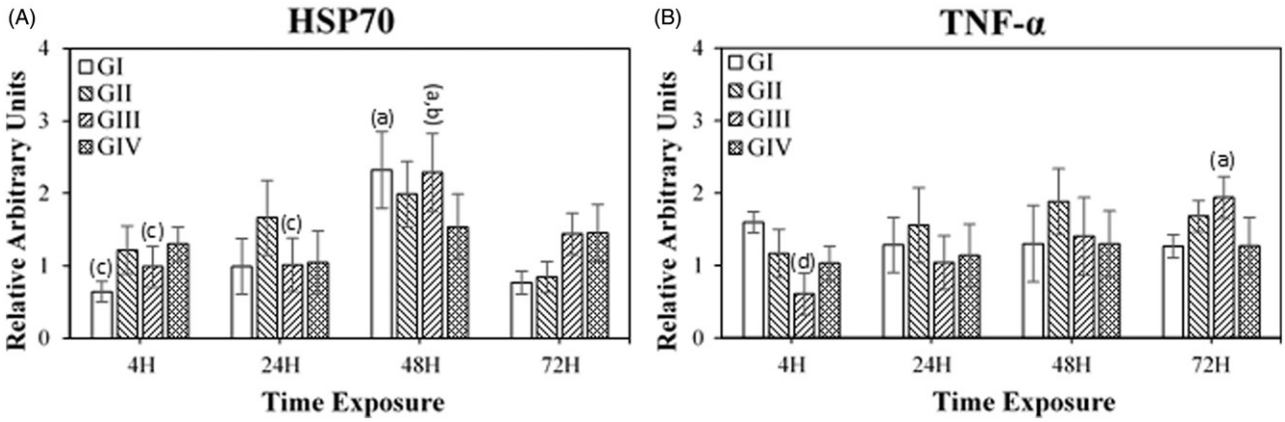

Figure 3. Histograms of mRNA expression of HSP70 (A) and TNF-α (B) proteins in relation to time in GI (900 MHz), GII (2450 MHz), GIII (900 MHz + 2450 MHz), and GIV (radiated). Values show the means (12 samples in total) of the radiated groups (GI, GII, and GIII) and the non-radiated group (GIV). Error bars indicate the standard error of the mean (SEM) within each group (radiated or non-radiated). (a,b,c,d) Indicate significant differences of each radiated group in relation to time: 4 h (a), 24 h (b), 48 h (c), and 72 h (d) ($p < .05$).

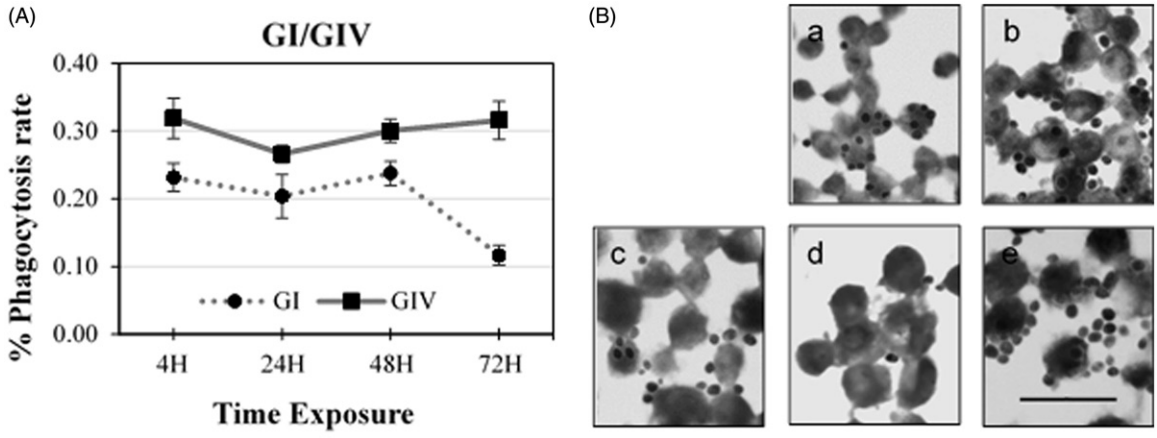

Figure 4. (A) Graph showing the evolution (from 4 to 72 h) of phagocytic activity in group I (900 MHz) compared with group IV (non-radiated). (B) Microphotographs show the internalization of yeasts in the macrophages of group I at 4 h (a), 24 h (b), 48 h (c), and 72 h (d), compared with control (e). Photographs taken at 100× magnification. six samples for each group/time were used.

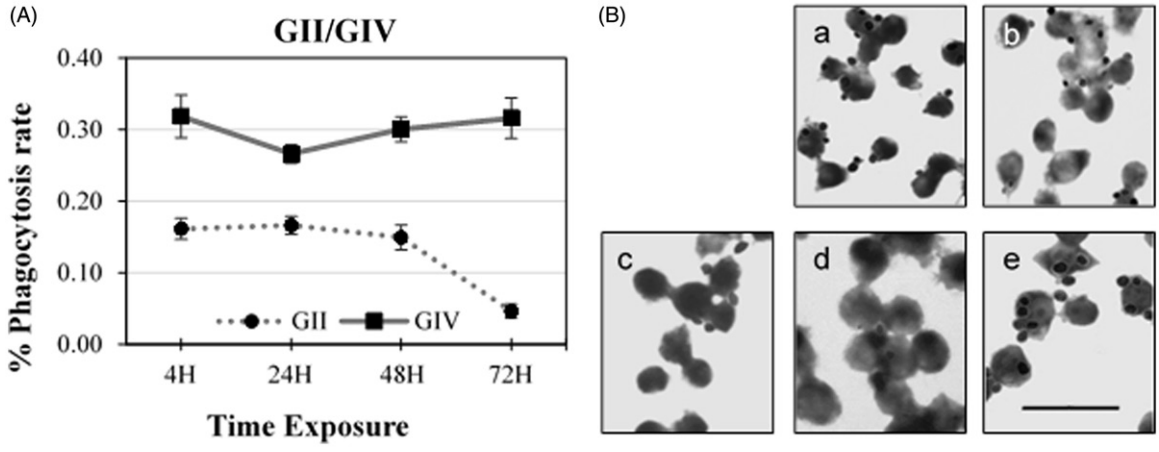

Figure 5. (A) Graph showing the evolution (from 4 to 72 h) of phagocytic activity in group II (2450 MHz) compared with group IV (non-radiated). (B) Microphotographs show the internalization of yeasts in the macrophages of group II at 4 h (a), 24 h (b), 48 h (c), and 72 h (d), compared with control (e). Photographs taken at 100× magnification. six samples for each group/time were used.

affected in radiated groups I (900 MHz), II (2450 MHz), or III (900 + 2450 MHz).

There were no significant differences between groups exposed to radiation (I, II, and III) and the non-radiated group (IV). Similarly, there were no significant differences in cell viability resulting from the different radiation exposure times.

As for growth, there were no significant differences in the growth curve slopes from 4 to 72 h for groups exposed to

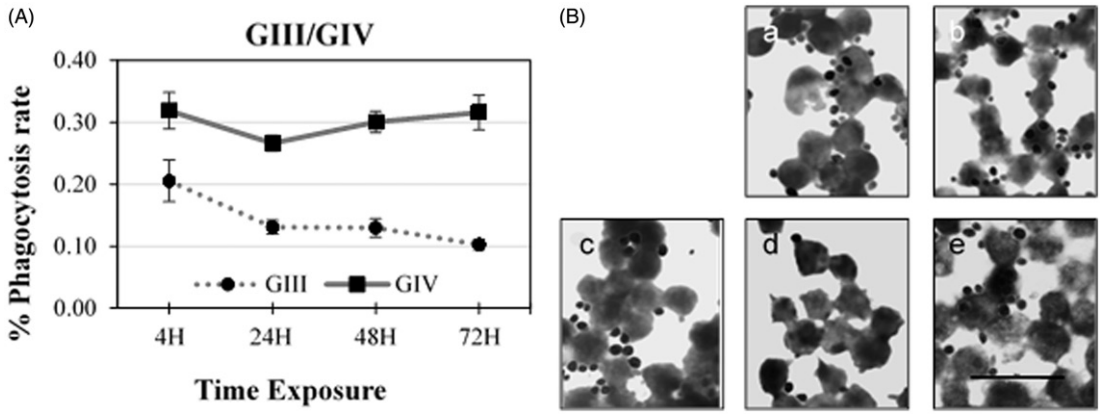

Figure 6. (A) Graph showing the evolution (from 4 to 72 h) of phagocytic activity in group III (900 MHz + 2450 MHz) compared with group IV (non-radiated). (B) Microphotographs show the internalization of yeasts in the macrophages of group III at 4 h (a), 24 h (b), 48 h (c), and 72 h (d), compared with the control (e). Photographs taken at 100× magnification. Six samples for each group/time were used.

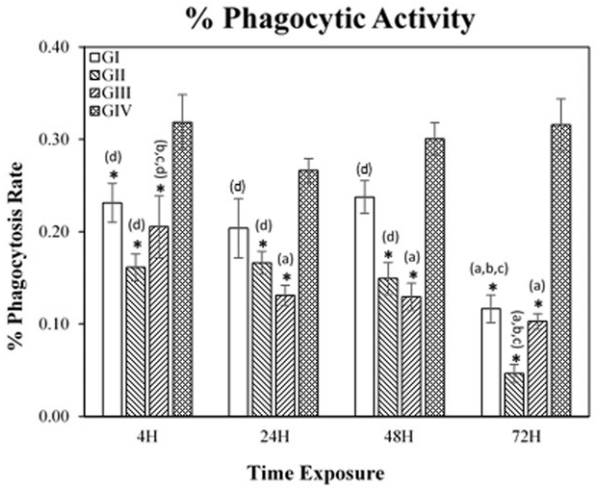

Figure 7. Histogram showing the percentage of phagocytic activity in relation to time in GI (900 MHz), GII (2450 MHz), GIII (900 MHz + 2450 MHz), and GIV (non-radiated). Values show means (nine samples of 100 cells each) of radiated groups (GI, GII, and GIII) and non-radiated group (GIV). Error bars indicate the standard error of the mean (SEM) within each group (radiated or non-radiated). Ninety-six samples were used, six for each group/time. * Indicates significant differences in relation to the non-radiated group ($p < .05$). (a,b,c,d) Indicate significant differences of each radiated group in relation to time, 4 h (a), 24 h (b), 48 h (c), and 72 h (d) ($p < .05$).

radiation (I, II, and III) in relation to the non-radiated (IV) group.

3.2. NO production

The difference in mean values (seven samples per group/time) among the levels in the groups (GI, GII, GIII, and GIV) was statistically significant ($p \leq .001$) and greater than would be expected by chance, after allowing for effects of differences in time exposure. We used a multiple comparison procedure to isolate the groups that differed from others.

The difference in mean values among the different time exposure levels was statistically significant ($p = .023$) and greater than would be expected by chance, after allowing for the effects of differences in the groups. We used a multiple comparison procedure to isolate the groups that differed from others.

The effect of different irradiation levels did not depend on time exposure. There was no statistically significant interaction between groups and time exposure ($p = .074$).

Forty-eight hours after radiation exposure, RAW 264.7 cultures showed a significant increase in NO values in group II (2450 MHz) compared with the non-irradiated group IV ($p = .013$). Significantly lower levels of NO were also found in group III compared with group I ($p = .010$) and group II ($p = .009$).

A significant increase in NO values was observed in macrophage cultures subjected to combined irradiation in group III (900 + 2450 MHz) when the exposure time increased from 48 to 72 h ($p = .050$) (Figure 2).

3.3. Expression detection

3.3.1. HSP70

The difference in mean values among the different levels in the groups (GI, GII, GIII, and GIV) was not statistically significant ($p = .588$) or great enough to exclude the possibility that the difference was due to random sampling variability after allowing for the effects of differences in time exposure.

The difference in mean values among the different levels of time exposure was statistically significant ($p \leq .001$) and greater than would be expected by chance, after allowing for effects of differences in the groups. We used a multiple comparison procedure to isolate the groups that differed from others.

The effect of different levels in the groups did not depend on time exposure. There was no statistically significant interaction between groups and time exposure ($p = .605$).

The cytoprotective action of HSP70 increased temporarily after 48 h combined radiation in group III (900 + 2450 MHz), compared with the groups radiated for 24 ($p = .010$) and 4 h ($p = .009$). HSP70 also increased in group I (900 MHz) after 48 h exposure, with respect to cells radiated for 4 h ($p = .009$) (Figure 3(A)).

3.3.2. TNF-α

The difference in mean values among the different levels in all groups was not statistically significant ($p = .612$) or great enough to exclude the possibility that the difference was due to random sampling variability after allowing for the effects of differences in time exposure.

The difference in mean values among the different levels of time exposure was not statistically significant ($p = .471$) or great enough to exclude the possibility that the difference was due to random sampling variability after allowing for the effects of differences in the groups.

The effect of different levels in the groups did not depend on time exposure. There was no statistically significant interaction between the groups and time exposure ($p = .874$).

Inflammatory activity, measured by quantification of TNF-α expression, increased significantly in the RAW 264.7 cell line exposed to combined radiation in group III (900 + 2450 MHz) after 72 h exposure, compared with the same group after 4 h exposure ($p = .009$) (Figure 3(B)).

3.4. Phagocytic activity

The difference in mean values among the different levels in the groups (GI, GII, GIII, and GIV) was statistically significant ($p \leq .001$) and greater than would be expected by chance after allowing for effects of time exposure. A multiple comparison procedure was used to isolate groups that differed from the others.

The difference in the mean values among time exposures was statistically significant ($p \leq .001$) and greater than would be expected by chance after allowing for effects of time exposure. A multiple comparison procedure was used to isolate groups that differed from the others.

The effect of different levels in the groups depended on time exposure. There was a statistically significant interaction between the groups and the time exposure ($p = .001$).

Phagocytic activity in the RAW 264.7 macrophage cell line decreased significantly in group II (2450 MHz) ($p \leq .010$) (Figures 5 and 7) and group III (900 + 2450 MHz) ($p \leq .010$) compared with the non-radiated group (GIV) for all radiation exposure times. However, in group I, a significant decrease in phagocytic activity compared with the non-radiated group only appeared at 4 h ($p = .013$) and 72 h ($p = .013$) (Figures 4 and 7).

Phagocytic activity decreased significantly in relation to time exposure. We observed a significant decrease in the group III (900 + 2450 MHz) phagocytic activity values of radiated cultures exposed for 24 h ($p = .013$), 48 h ($p = .010$), and 72 h ($p = .009$), compared with the values after exposure for 4 h (Figures 6 and 7).

Finally, in groups I (900 MHz) and II (2450 MHz), which were exposed to a single radiation frequency, we found a significant decrease in phagocytic activity after exposure for 72 h compared with the groups exposed for 4 h ($p = .010$), 24 h ($p \leq .013$), or 48 h ($p \leq .013$) (Figures 4, 5 and 7).

4. Discussion

We are not aware of any experimental studies describing changes in immune activity due to the interaction of single or combined radiofrequencies in macrophages of the RAW 264.7 cell line. The scarce research available on magnetic fields, low frequencies, and ultra-wideband pulses offers contradictory findings (Seaman et al. 2002; Glushkova et al. 2015; Maresuke et al. 2016). In this work, we found that the interaction of one or more frequencies caused a significant decrease in macrophage phagocytic activity. Also, anti-inflammatory and cytoprotective responses were especially dependent on exposure time when the two combined RF signals interacted with the macrophages.

In the present work, no changes became evident in the viability of the RAW 264.7 cell line when it was exposed to radiation at multiple frequencies for different lengths of time. These observations corroborate other in vivo studies in which no changes in tumor growth were detected after exposure at a frequency near 900 MHz (Higashikubo et al. 1999; Heikkinen et al. 2001; Bartsch et al. 2002). Similarly, in a different study, no effects were observed in three generations of rats after exposure to 2140 MHz for 20 h/d (Tomoyuki et al. 2014). In studies on cell lines exposed to low frequency, no significant differences in viability due to radiation were found for the U937 macrophage cell line (Karolina et al. 2014). In vitro studies on rat glioma cells exposed to 836.55 MHz showed no evidence of increase or decrease in cellular proliferation when the cells were exposed for 24 h in a TEM chamber at SAR levels ranging from 0.15 to 15 mW/kg (Stagg et al. 1997). In another study using 900 MHz RF, no alterations were detected in the viability of the U937 cell line or the SK-N-SH neuroblastoma cell line (Ebru et al. 2006). When human U251 and A172 glioblastoma cell lines and the human SH-SY5Y neuroblastoma cell line were irradiated at 1800 MHz and 4.0 W/kg SAR for a maximum of 24 h, there was no evidence that radiation influenced viability (Liling et al. 2017). Again, when the A172 line was subjected to RF exposure at 2450 MHz, no important decreases in viability were detected so long as the SAR remained between 5 and 100 W/kg, although a 70% decrease in viability was observed at 200 W/kg SAR (Wang et al. 2006). All these studies support our findings that there were no changes in macrophage viability in the RAW 264.7 cell line under the conditions applied in the present work.

Some data seem to indicate that NO levels increased when organs such as the lungs, liver, testicles and heart, or breast tissue or nasal mucus were radiated at 900 MHz (Yariktas et al. 2005; Meric et al. 2011). Exposure time was also crucial: at 900 MHz for short periods (30 min/d for 7 d), NO production levels were not altered, but levels of this biomarker increased with 12 h exposure/day for 30 d (Irmak et al. 2002). In our study, exposure of the RAW 264.7 macrophage cell line to 900 MHz radiation did not appear to increase stress levels enough to register a significant difference in NO levels ($p = .17$). Other authors likewise found no differences in NO production after radiation of the RAW 264.7 cell line at 50 Hz (Maresuke et al. 2016). Production of NO after exposure for 30 min in a GTEM ultra-wideband pulse

chamber for 1 ns or repeated exposure at 600 Hz and approximately 0.106 W/kg SAR did not significantly alter the RAW 264.7 cell line (Seaman et al. 2002). The results for 2450 MHz RF are supported by prior studies in which the early response to radiation was an increase in NO production and a decrease in time until normal levels were reached in rat brain radiated at 2100 MHz (Hidisoglu et al. 2016). In contrast, the results of our experiment (Figure 2) showed that macrophages in the group exposed to combined frequencies (group III) suffered attenuation of the initial response at 48 h followed by a drastic increment in NO production, measured at 72 h exposure. This may indicate a delay in NO production that would normalize at 72 h or even increase with greater exposure times.

Normally, an increase in NO production would lead to the activation of HSP70 expression, which serves to protect the cells from thermal and oxidative stress and helps avoid the loss of protein structure (Tavaria et al. 1996). Cellular growth in an oxidative environment induces the expression of TNF-α, which is crucial in developing both a protective response against infections and immunological activity that can destroying tumor cells (Hirvonen et al. 1996; Petrini et al. 1997).

In the present study, there were no significant changes in HSP70 expression after single or combined RF exposure (900 MHz and 2450 MHz). At 2450 MHz RF, our results were supported by previous studies in which no significant differences in HSP70 expression appeared in rat thyroid tissue 90 min or 24 h after exposure to radiation at 12 W power (Misa Agustiño et al. 2012). Similarly, no signs of changes in HSP70 concentration were found in rat brains exposed to 900 MHz RF and 7.5 W/kg SAR (Fritze et al. 1997), or to 900 MHz or 1800 MHz and SAR less than or equal to 5 W/kg for 2 h per day or for 12 weeks (Sandrine et al. 2008). Again, exposure of the human Mono Mac 6 cell line to 1800 MHz RF–EMF did not change the levels of HSP70 expression (Simkó et al. 2006).

In this work, the increased mRNA expression of HSP70 in all groups studied after 48 h exposure can be explained by a mechanism similar to what has been observed in exposures at low frequencies. Some authors observed that the mRNA expression of HSP72 increased after 15 min of exposure at 1 mT, although the concentration of HSP72 did not. They argued that activation of the HSP72 gene was independent of protein synthesis (Gottwald et al. 2007). Another explanation may be that combined exposure from various sources of stress and exposure to electromagnetic radiation generated a decrease in the activity of some enzymes, provoking a temporary inhibition of the RNases and mRNA stabilization of HSP70 (Roberta et al. 2006).

As with the expression of HSP70, the following studies seem to demonstrate that the effects of radiation on the expression of TNF-α are heavily influenced by the type of tissue radiated and the exposure frequency. In hippocampus extracts from rat brain, an increase in TNF-α levels was observed at 900, 1800, and 2450 MHz RF and SAR = 0.59 mW/kg in a GTEM chamber, when the tissue was exposed for 2 h/d (5 d/week) for 60 d (Megha et al. 2015). In nerve tissue, increased TNF-α production was observed in the N9 microglial cell line exposed to 1800 MHz and 2450 MHz, but no change in production was found for the C8-D1A astroglial cell line (Yang et al. 2010; Lu et al. 2014). In contrast to these studies and in support of what occurred in the astroglia, RF exposure of primary microglial cell cultures from neonate rats to 1950 MHz at 0.2, 0.8, and 2.0 W/kg SAR did not provoke changes in TNF-α production (Hirose et al. 2010). In other tissues, no effects were detected from exposure of patients with atopic dermatitis to 900 MHz RF and 1 W/kg SAR for 30 min, proceeding from a telephone antenna (Johansson et al. 2008). Likewise, there were no changes in TNF-α production in the testicular tissue of rats exposed to 2450 MHz and 3.21 W/kg SAR for 60 min/d for 28 d (Saygin et al. 2011).

In applying electromagnetic fields at low frequency, we obtained contradictory results indicating that TNF-α expression increased in intraperitoneal macrophages (Makar et al. 2006), while the TNF-α produced by mononuclear phagocytes decreased significantly (Ignacio et al. 2011). Similarly, at 50 and 60 Hz, TNF-α production and immune functions did not change in human peripheral blood mononuclear cells (Ikeda et al. 2003).

In consonance with our results, Helga et al. (2006) found that in human immune cells extracted from blood donors and pulse-radiated at 1950 MHz and 1 W/kg SAR for 8 h, there were no differences in TNF-α synthesis compared with control monocytes. From this, we assume that we are correct in affirming that the various frequencies and exposure times in our study did not provoke changes in TNF-α production. However, one especially significant finding in our research was the important increase in TNF-α levels after 72 h continuous exposure of the RAW 264.7 macrophage line to the combined interaction of both RF (group III) (Figure 3(B)). From this, we deduce that the macrophage inflammatory response increases with continuous and combined exposure to multiple RF signals. This could point to important repercussions on mammalian immune systems in a world where multiple radiofrequencies interact constantly.

Phagocytosis can be considered as a means of defense against invading microorganisms and a way of eliminating (or even recycling) dead tissue. Thus, modification of this trait in macrophages would endanger the integrity of the immune response and the proper functioning of biochemical processes related to the metabolization of unwanted substances (Regueiro-González et al. 2011). Our results show a decrease in phagocytic activity that is most precocious in group III and increases after 72 h continuous exposure (Figure 7). Studies by other authors on peripheral rat blood report that the phagocytic activity of neutrophils was suppressed by approximately 50% after 2–3 h exposure at high frequency and low intensity (42.0 GHz, 0.15 mW/cm^2, 20 min daily). The effect lasted 1 d and fully disappeared after 3 d (Kolomytseva et al. 2002). Another study reported a reduction in microglial phagocytosis after exposure for 20 min to pulsed waves at 2.45 GHz and 6 W/kg SAR (He et al. 2014). We also found results, similar to our own, indicating diminished phagocytosis in murine macrophages for RF exposure at 2.45 GHz (Mayers and Habeshaw 1973).

Other studies of murine peritoneal macrophage lines exposed to non-ionizing radiation have described increased phagocytic activity (Zafra et al. 1988). However, exposure of the HL-60 cell line to 2.45 GHz and 2 or 10 W/kg SAR for 4 and 24 h resulted in very little or no effect on phagocytosis at 2 W/kg SAR, which is the maximum value recommended by the ICNIRP (International Commission for Non-Ionizing Radiation Protection), or at 10 W/kg SAR (Koyama et al. 2015).

5. Conclusions

The results of exposure of the RAW 264.7 macrophage cell line to the single or combined frequencies included in this experiment indicate that

1. Significant cytoprotective, cytotoxic and anti-inflammatory actions appear in the cells with the interaction of multiple frequencies and are dependent on exposure time.
2. Phagocytic activity diminished with the interaction of one or more frequencies.

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